Studies on glucose tolerant β-glucosidases from a novel *Byssochlamys fulva* and their applications in biomass to ethanol conversion

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12 December 2012

DECLARATION

I hereby declare that the work presented in this thesis entitled "Studies on glucose tolerant β-glucosidases from a novel *Byssochlamys fulva* and their applications in biomass to ethanol conversion" is based on the original work done by Mr. Abraham Mathew (Reg # 3171), under my guidance and supervision, at the National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, India. I also declare that this work or no part of this work has been submitted for the award of any degree, diploma, associateship or any other title or recognition.

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Thiruvananthapuram

12 December 2012

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Studies on glucose tolerant β-glucosidases from a novel *Byssochlamys fulva* and their applications in biomass to ethanol conversion"** is based on the original work done by me under the guidance of Dr. Rajeev Kumar Sukumaran, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, India and the thesis or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associateship or any other Title or Recognition.

Abraham Mathew

Dedicated to my parents and teachers

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Review

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1. Introduction

1.1. Bioethanol from biomass

Renewable resources are the only reliable sources of energy for the future. With the declining fossil reserves, the transportation industry faces serious problems in the form of non availability of transportation fuel and the ever increasing oil prices. The use of biofuels is therefore considered as the most promising alternative. Bioethanol from biomass is proposed as one of the most important alternative fuels. With an octane rating of 112 - 114 compared to that of 85 - 94 for fossil petrol, engines fired on ethanol can run at a much higher compression ratio without octane boosting additives. This prevents premature ignition of fuels which otherwise cause stress on the engine and there by damaging the engine. Thus bioethanol can replace lead as an octane enhancer in petrol. Ethanol burns more effectively as it has higher oxygen content thereby reducing CO emission to nearly 90 % compared to engines run on gasoline (Jeuland et al., 2004; Szulczyk, 2010).There are three primary ways by which ethanol can be used as transportation fuel- as a 5 % or 10 % blend with petrol, as a fuel with 15 % gasoline (E-85), or as a component of reformulated gasoline [ethyl tertiary butyl ether] (Srivastava et al., 2010). In 2008, Government of India announced the National Policy on Biofuels, mandating a phase wise implementation of ethanol blending in gasoline. Five percent ethanol blend with petrol was made mandatory from October 2008, and projected a target of 20 % blend by 2017. However, the program could not be implemented fully due to shortage of ethanol. In 2006-09, a shortage of 60 % and 2009-2010, a shortage of 85 % was recorded (Corporate Planning and Economic studies, 2011).

Bioethanol can be produced by the fermentation of hexose or pentose by selected microbes. Starch rich cereals like corn or sucrose rich plants like sugar cane and sugar beet can be a good source of hexose. In North America, bioethanol is produced from

maize while in Europe wheat, rye or barley are used (Jeuland et al., 2004). However the extensive use of food resources will result in food scarcity. In India, currently ethanol is produced from sugar cane. With a mandatory 5 % blend, total ethanol production including potable and alcohol based industry requires 545 million tonnes of sugarcane. But the total sugarcane production in India during 2007-08 was only 340 million tonnes. Moreover, the price and availability of sugar fluctuates with yield (Ray et al., 2012). Thus the use of less economical materials as a source for hexose is gaining momentum and hence several research efforts are directed to meet this criterion.

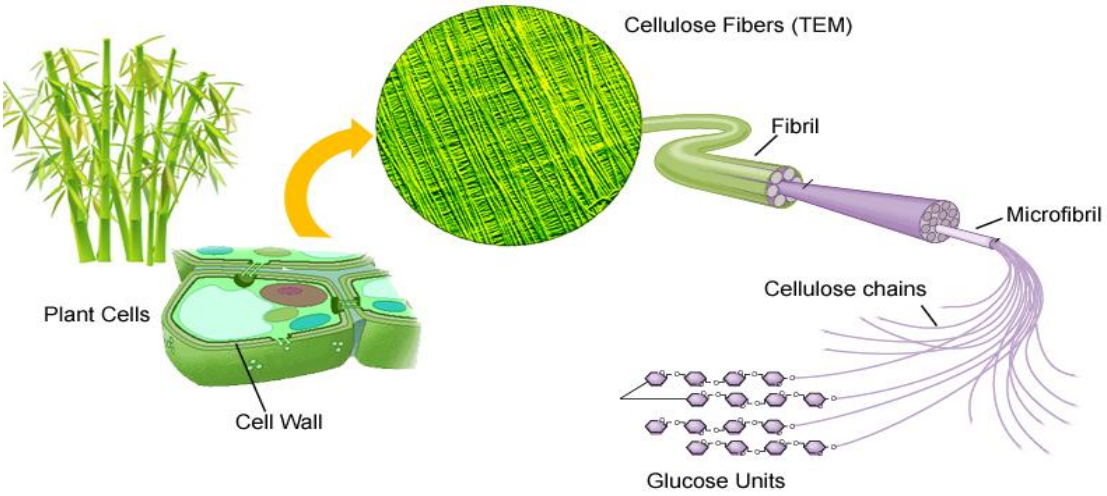
Lignocellulose can be a good alternative for food commodities as their hydrolysis also provide plenty of hexose. They form the major part of plant biomass and consist of cellulose, hemicellulose and lignin. The proportion of each constituent depends on the biomass; the concentration of cellulose is usually 30-50 %. A country like India with agriculture as the chief source of livelihood produces several types of agro residues. The huge amount of agro residues generated can be potentially employed for producing value added products like biofuels (Chapla et al., 2010). The production of ethanol from lignocelluloses involves four major steps – pretreatment, saccharification, fermentation and ethanol recovery. Pretreatment removes recalcitrant residues like lignin, making cellulose more amenable to hydrolysis. Hydrolysis of pretreated biomass can be carried out either using dilute acids or by means of enzymes. Acid hydrolysis generates unwanted by-products making sugar recovery difficult. These by-products are also potential inhibitors for subsequent fermentation. Moreover, acid forms a hazardous waste. Enzymatic method is more specific with no by-product formation thus minimizing the need for technological developments to remove inhibitors developed during hydrolysis. The major hindrance for the commercialization of enzymatic hydrolysis is the cost of enzyme.

1.2. Cellulose

Cellulose is a linear bio polymer of glucopyranose molecules connected by β-1,4 glycosidic linkages (Aslam et al., 2010). In plant cell wall, cellulose occur as microfibrils with 2-10 nm diameter, cross linked by other cell wall components like

xyloglucans. Each microfibril is unbranched and consists of 30-36 glucan chains aggregated laterally by means of hydrogen bonds and van der Waals force to form crystalline structures (Figure 1.1). These structures are highly resistant to enzymatic attack (Arantes and Saddler, 2010; Eriksson et al., 2002). In addition to this, cellulose contains non-crystalline regions called amorphous regions within the microfibrils. The ratio of crystalline and amorphous region depends on the type of biomass (Vries and Visser, 2001).

Figure 1.1: Distribution of cellulose in plant cell wall



1.3. Cellulase

Enzymatic saccharification of cellulose is a heterogeneous reaction system in which cellulases in aqueous environment react with insoluble macroscopic and structured cellulose (Arantes and Saddler, 2010). Cellulase refers to a family of enzymes that synergistically act to hydrolyze cellulose. Cellulases come under glycosyl hydrolase family 3 and thus are hydrolytic in function. The three major enzyme classes that constitute cellulase are

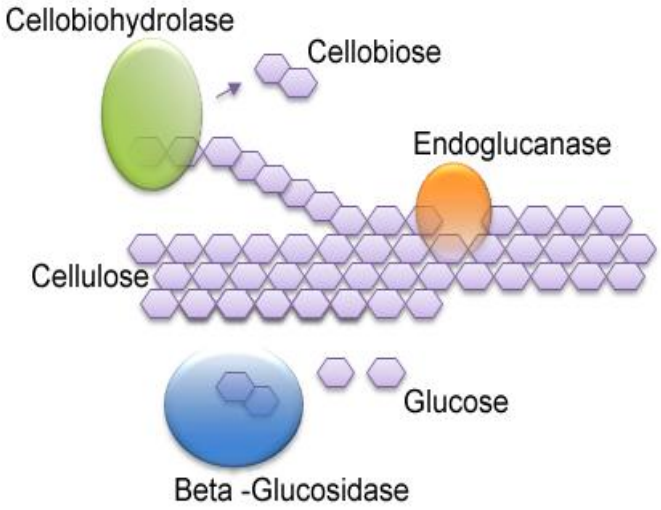
a) Endoglucanases (ENG) – EC 3.2.1.4

b) Exoglucanases (EXG) – EC 3.2.1.91

c) Beta-glucosidases (BGL) – EC 3.2.1.21

Endoglucanse (4-β-D-glucan 4-glucanohydrolase) also known as CMCase is a de-polymerizing enzyme. It hydrolyzes accessible intramolecular β-1,4 glucosidic bonds of cellulose randomly creating shorter chains with new chain ends (Zhang et al., 2006). ENGs also act on cellodextrins with decreased action towards smaller chains. Exoglucanase (4-β-D-glucan cellobiohydrolase) acts on non-reducing ends of cellulose releasing cellobiose. Thus it progressively decreases the chain length of cellulose. Beta-glucosidase (β-D-glucoside glucohydrolase) catalyzes hydrolysis of cellobiose into glucose. The enzymes cleave β-1,4 linkages between two glucose residues (cellobiose) to release beta-glucose (Figure 1.2). β-glucosidase can also act on cellodextrins, the activity decrease with increase in chain length (Bisaria and Mishra, 1989).

Figure 1.2: Schematic diagram showing enzymatic hydrolysis of cellulose



Cellulase from *Trichoderma reesei* have been the focus of research for over 50 years and it has been widely used in laboratory and pilot scale bioethanol production (Gray et al., 2006). Cellulase from *Trichoderma viride* and *Aspergillus niger* are also used commercially for biomass hydrolysis (Barbagallo et al., 2004; Gottschalk et al., 2010; Passos et al., 2009). Many of the commercially available enzyme preparations are actually a cocktail consisting of cellulase from *Trichoderma* or *Aspergillus* supplemented with β-glucosidase from other sources.

1.4. Beta-glucosidase – the rate limiting enzyme

Among cellulases, β-glucosidase forms the centre of interest because of its peculiar features. In cellulose hydrolysis, β-glucosidase forms the rate limiting factor. Wood rotting fungi, both ascomycetes and basidiomycetes are good sources of cellulase. However, many of these fungi produce only small amounts of β-glucosidase. Even hyper cellulase producing mutants of *Trichoderma reesei* are found to be deficient in β-glucosidase (Duff et al., 1986). Most β-glucosidases reported so far are subjected to product inhibition. That is, when the concentration of glucose increases in the medium, the activity of enzyme decreases. This decrease in activity may be sudden with small increase in glucose or gradual. Thus β-glucosidases vary in their levels of tolerance to glucose. The decrease in β-glucosidase activity results in accumulation of cellobiose which in turn inhibit endoglucanase and exoglucanase (Harhangi et al., 2002; Kaur et al., 2007; Riou et al., 1998). Many reports indicate substrate inhibition of β-glucosidase with high concentration of cellobiose (Bravo et al., 2000; Krogh et al., 2010; Woodward and Wiseman, 1982). β-glucosidase from *Pyrococcus furiosus* was found to be un-competitively inhibited by cellobiose (Bruins et al., 2003). The mechanism of inhibition may be explained by transglycosylation. When cellobiose concentration in the reaction mixture reaches higher than a certain threshold, transglycosylation becomes dominant. This results in the formation of cellotriose instead of hydrolysis of cellobiose, resulting in substrate level inhibition to hydrolysis (Bohlin et al., 2010).

The cellulases from *Trichoderma* are often supplemented with β-glucosidase from other sources for enhanced cellulose hydrolysis. The cellulolytic system of *Penicillium funiculosum* is found to efficiently complement the β-glucosidase deficient cellulolytic pool of *Trichoderma reesei* (Castro et al., 2010). Similarly the purified β-glucosidase from *Penicillium decumbens* could enhance saccharifying ability of cellulase from *Trichoderma reesei* QM 9414 (Chen et al., 2010). Addition of purified β-glucosidase from *Volvariella volvacea* (0.08 U/ml) to cellulase of *Aspergillus niger* (0.22 U/ml) resulted in a 9.7 % enhancement of saccharification of microcrystalline cellulose (Cai et al., 1998).

1.5. Multiplicity of β-glucosidases

Isoforms are enzymes with different amino acid sequences but catalyze the same chemical reactions. They are essential for fine-tuning of metabolism based on the specific environment to which the cell or organism is exposed. If the isoforms are produced by different genes, they are called isozymes while isoforms produced by different alleles of a gene are termed allozymes. The isoforms vary in their kinetic properties and hence can have much importance in metabolism. Hence they are preserved over evolution. Thus the same species may have strains that vary in the number and type of isoforms.

Isoforms are of common occurrence in β-glucosidases. *Botrytis cinerea* shows three isoforms, all being intracellular in location (Gueguen et al., 1995). Four isoforms were reported in *Aspergillus terreus*, *Aspergillus caespitosus*; three isoforms in *Humicola insolens*, *Humicola fuscoatra*, *Chaetomium thermophilum*; two in *Absidia corymbifera*, *Penicillium lagena*, *Emericella nidulans* var. *lata* (Sonia et al., 2008). In *Aspergillus tubingensis*, four isoforms of β-glucosidase were reported and were found to exhibit high diversity with respect to pH and temperature optima, temperature stability, substrate specificity and glucose tolerance. This high diversity of kinetic properties, help the fungus to utilize variety of substrates by making use of the synergistic activity of different enzymes (Decker et al., 2001). The multiplicity in BGL production may be due to differential expression under the influence of culture conditions or carbon source. In *Aspergillus terreus*, use simple substrates like fructose, glucose or cellobiose resulted in the production of a single isoform while up to three isoforms were noticed with crude media like corn cob (Nazir et al., 2009). In *Aspergillus niger* and *Aspergillus carbonarius*, thin layer counter current distribution of culture filtrate revealed the presence of three isoforms (Brumbauer et al., 2000). In *Aspergillus kawachii*, three isoforms were noted, two extracellular and one cell wall bound. All the three isoforms were produced from the same gene *bgl*A, as disruption of the gene resulted in the absence of all the three isoforms. It is not clear whether the same mRNA is transcribed in to the three isoforms. Also reports indicate rapid production of β-glucosidase from the same strain in late phase of culture and at lower temperatures. This all indicate the

possibilities of numerous factors in the regulation of gene expression and multiplicity of BGL (Iwashita et al., 1999).

1.6. Glucose tolerant β-glucosidases

Beta-glucosidases are hydrolyzing enzymes subject to product inhibition. That is when the glucose level in the medium reaches to a certain threshold, β-glucosidase activity is inhibited. β-glucosidase show wide variation in this threshold value and in the nature of inhibition shown towards glucose levels. Some enzymes show inhibition even under low concentration of glucose and this inhibition progressively increases with glucose concentration. While in certain enzymes, glucose inhibition starts only when glucose level reaches to a certain threshold level. This threshold level varies with enzymes and hence β-glucosidase from different sources shows different levels of tolerance to glucose. Glucose at higher concentrations may either block the active site preventing the entry of substrate or may prevent the release of hydrolyzed substrate. The exact mechanism is difficult to understand. As low cellobiose concentration only can ensure constant reaction rates, hydrolysis of this can produce only small amount of glucose. For inhibition studies, addition of high concentration of glucose will be required; and to detect the minor amount of glucose produced by cellobiose hydrolysis, high analytical precision is required (Krogh et al., 2010). Enzyme from *Aspergillus oryzae* had shown high glucose tolerance with a *Ki* value of 1.36 M (Riou et al., 1998) while Gűnata and Vallier (1999), reported a β-glucosidase with *Ki* value of 953 mM from *Aspergillus oryzae*. One of the highest tolerance to glucose by a β-glucosidase (*Ki* = 1.4 M) was reported from *Candida peltata* (Saha and Bothast, 1996).

1.7. Sources of β-glucosidases

β-glucosidases are ubiquitous enzymes present in all forms of life ranging from Archaea to most advanced plants and animals. The properties and nature of the enzyme varies with life forms to suit their physiological needs.

1.7.1. Plant sources

β-glucosidases are reported in a number of plant species (Table 1.1). They are generally associated with defense mechanism in plants, 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), beta-glucan synthesis during cell wall development, pigment metabolism, fruit ripening (Khan and Akhtar, 2010) etc. β-glucosidase help in the release of active cytokinins from kinetin and cytokinin glucosides (Brzobohaty et al., 1993). Mace (1972), had reported the role of β-glucosidase in converting the monoglucoside of 2,4, dihydroxy 7 methoxy 2 H-1,4 benzoxazin -3-one to glucose and the aglycone which had a mycotoxic effect in maize plants. Flower scent compounds like 2-phenyl ethanol, geraniol, benzyl alcohol exist as glycoconjugates and β-glucosidases are involved in the hydrolysis and thereby the release of these volatile compounds producing the characteristic fragrance to flowers and plant parts (Sakai et al., 2008).

Table 1.1: β-glucosidases from plants

Enzyme	Plant source	Substrate	Activity	Reference
Torvosidase	*Solanum torvum*	Torvoside	Defense mechanism	(Arthan et al., 2006)
β-glucosidase	*Costus speciosus*	Furostanol gylcosides	Defense mechanism	(Inoue and Ebizuka, 1996)
Linamarase	*Trifolium repens*, *Manihot esculenta*	Linamarin Lotaustralin	Release of HCN	(Barrett et al., 1995; Liddle et al., 1998)
Myrosinase	*Brassica*	Glucosinolates	Release of thiocyanates	(Fenwick et al., 1983)
Avenacosidase	*Avena sativa*	Avenacoside	Defense mechanism	(Gus-Mayer et al., 1994)
Dhurrinase	*Sorghum*	Dhurrin	Release of HCN	(Cicek and Esen, 1998)

1.7.2 Animal sources

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. They are acid β-glucosidases that show an optimum activity at acidic pH (Burrow and Grabowski, 2011). The deficiency of this enzyme can cause Gaucher's disease (Khan and Akhtar, 2010).The disease occurs by the accumulation of glucocerebroside in spleen, liver, white blood cells and can cause liver and skeletal malfunctioning, neurological disorders, anemia etc.

1.7.3. Bacterial sources

Bacteria utilize β-glucosidase as a catabolic enzyme to break down complex cellooligosaccharides to obtain glucose. Plant pathogenic bacteria that cause soft rot, utilizes these hydrolytic enzymes for virulence. Over expression of *bgl* gene in *Dickeya dadantii* during plant infection indicate the role of β-glucosidase in pathogenesis (Antunez-Lamas et al., 2009). Strains of *Agrobacterium tumefaciens* with high β-glucosidase activity have higher virulence. Coniferin secreted by plants is hydrolyzed by bacterial β-glucosidase to produce coniferyl alcohol which seems to be a potent inducer of virulence gene (Castle et al., 1992). The endotoxin secreted by *Bacillus thuringiensis* is found to show β-glucosidase enzyme activity (Papalazaridou et al., 2003). Bacterial sources of BGL and their properties are listed in table 1.2.

Table 1.2: β-glucosidases from bacteria

Bacteria	Localization	Mol. Weight	Optimum Temp	Optimum pH	*Km* value	Reference
Leuconostoc mesenteroides	In	360 kDa (native)	50 °C	6.0	0.07 mM pNPG 3.7 mM laminarin	(Gueguen et al., 1997a)
Lactobacillus plantarum	Ex	40 kDa (native)	45 °C	5.0	1.82 mM pNPG	(Sestelo et al., 2004)
Acetobacter xylinum	Ex	81.2 kDa (SDS)	40 °C	5.5	3.7 mM cellotriose	(Tahara et al., 1998)
Bacillus circulans	In	51.3 kDa (SDS)	55 °C	6.0-9.0	-	(Paavilainen et al., 1993)
Lactobacillus brevis	In	330 kDa (native)	45 °C	5.5	0.22 mM pNPG	(Michlmayr et al., 2010)
Cellulomonas biazotea	Ex	109 kDa (SDS)	70 °C	4.8	0.025 mM pNPG	(Lau and Wong, 2001)
Clavibacter michiganense	In	65 kDa (SDS)	45 °C	7.5	-	(Nakano et al., 1998)
Sulfolobus shibatae	In	-	98 °C	5.5	-	(Wolosowska and Synowiecki, 2004)

1.7.4. Fungal sources

Fungi are mostly decomposers of plant biomass and hence require complete cellulolytic machinery for nutrition. Filamentous fungi are widely used in fermentation industry as a principal source of enzymes and metabolites with low cost and high volume (Wanga et al., 2004). Compared to bacteria, fungal strains are found to be efficient producers of cellulase (Amouri and Gargouri, 2006). For cellulose hydrolysis on a commercial scale, cellulases secreted by *Trichoderma reesei* are used. Since the fungus produces only low amounts of β-glucosidases, enzyme cocktails are created by supplementing the cellulase with β-glucosidase from other fungal sources. Fungal β-glucosidases show considerable variations in molecular mass and kinetic properties (Table 1.3), several being commercially used for cellulose hydrolysis.

Table 1.3: β-glucosidases from fungi

Organism	Localization	Mol. Weight (native)	Mol. Weight (SDS)	Temp. Optimum	pH Optimum	*Km* value	*Ki* for glucose	References
Debaryomyces pseudopolymorphus	Ex	100 kDa	-	40 °C	4.0	11.9 mM cellobiose	-	(Villena et al., 2006)
Aspergillus sp.	Ex	44 kDa	-	60 °C	4.0	-	-	(Elyas et al.,
	Ex	30 kDa		60 °C	3.0	-	-	2010)
Talaromyces emersonii	Ex	260 kDa	130 kDa (dimer)	71.5 °C	4.02	0.13 mM pNPG	0.25 mM	(Murray et al., 2004)
Candida peltata	Ex	43 kDa	43 kDa	50 °C	5.0	2.3 mM pNPG, 66.0 mM cellobiose	1.4 M	(Saha and Bothast, 1996)
Humicola grisea var. *thermoidea*	Ex	57 kDa	-	50 °C	6.0	0.12 mM pNPG 0.27 mM cellobiose	-	(Nascimento et al., 2010)
Monascus purpureus	Ex	>100 kDa	-	50 °C	5.5	0.39 mM pNPG 2.86 mM cellobiose	-	(Daroit et al., 2008)
Scytalidium thermophilum	My	40 kDa	-	60 °C	6.5	0.29 mM pNPG 1.61mM cellobiose	-	(Zanoelo et al., 2004)
Paecilomyces thermophila	Ex	197 kDa	116 kDa	75 °C	6.2	0.25 mM pNPG 0.65 mM pNPG	73 mM	(Yang et al., 2008)
Pichia etchellsii	Ce	186 kDa	-	50 °C	6.0	0.33 mM pNPG	12 mM	(Wallecha and Mishra, 2003)
	Ce	340 kDa		50 °C	6.0	0.33 mM pNPG		
			-			24.0 mM cellobiose	25 mM	
Saccharomyces cerevisiae	In	-	-	20 °C	4.0	2.55 mM pNPG	-	(Spagna et al., 2002)
Aspergillus oryzae	Ex	43 kDa	43 kDa	50 °C	5.0	0.55 mM pNPG	1.36 M	(Riou et al., 1998)
Aspergillus pulverulentus	Ex	240 kDa	118 kDa (dimer)	60 °C	4.0	-	-	(Mase et al., 2004)
Aspergillus terreus	In	275 kDa	-	50 °C	4.8	0.4 mM pNPG 0.78 mM cellobiose	3.75 mM	(Workman and Day, 1982)
							-	

Organism	Localization	Mol. Weight (native)	Mol. Weight (SDS)	Temp. Optimum	pH Optimum	*Km* value	*Ki* for glucose	References
Candida wickerhamii	In	180 kDa	94 kDa (dimer)	35 °C	6.0	0.28 mM pNPG		(Skory et al., 1996)
Chaetomium thermophilum	Ex	40 kDa	43 kDa	65 °C	5.5	0.76 mM pNPG 3.13 mM cellobiose	-	(Venturi et al., 2002)
Fusarium oxysporum	Ex	110 kDa	110 kDa	60 °C	5	0.093 mM pNPG 1.8 mM cellobiose	2.05 mM	(Christakopoulos et al., 1994)
Thermoascus aurantiacus	Ex	350 kDa	120 kDa (trimer)	80 °C	4.5	0.11 mM pNPG	0.29 mM	(Parry et al., 2001)
Talaromyces thermophilus	In	49 kDa	50 kDa	50 °C	5.5	1.2 mM pNPG 5.7 mM cellobiose	66.0 mM	(Nakkharat and Haltrich, 2006)
Sclerotinia sclerotiorum	Ex	196 kDa	96.5 kDa (dimer)	60 °C	5.0	0.1 mM pNPG 1.9 mM cellobiose	-	(Issam et al., 2003)
	Ex	76.5 kDa	76.5 kDa	60 °C	5.0	2.8 mM pNPG 8.0 mM cellobiose		
Penicillium decumbens	In	50 kDa	28 kDa (dimer)	65 °C	7.0	0.007 mM pNPG	0.24 mM	(Mamma et al., 2004)
	In	460 kDa	115 kDa (tetramer)	75 °C	7.0	0.013 mM pNPG	0.29 mM	
Paecilomyces sp.	Ex	305 kDa	102 kDa (trimer)	55 °C	3.5	0.11 mM pNPG	-	(Yan et al., 2008)
Penicillium decumbens	Ex	-	120 kDa	65-70 °C	4.5-5.0	0.0064 mM pNPG 0.0188 mM salicin	-	(Chen et al., 2010)
Penicillium oxalicum	In	-	133.5 kDa	55 °C	5.5	0.37 mM pNPG	-	(Copa-Patiño et al., 1990)
Penicillium purpurogenum	In	-	-	50 °C	5.5	-	-	(Dhake and Patil, 2005)
Botrytis cinerea	In	350 kDa	88 kDa (tetramer)	50 °C	6.5-7.0	0.06 mM pNPG	-	(Gueguen et al., 1995)
Penicillium purpurogenum	Ex	-	110 kDa	65 °C	5.0	5.1 mM pNPG	21.5 mM	(Jeya et al., 2010)
Melanocarpus sp.	Ex	102 kDa	92 kDa	60 °C	6.0	3.3 mM pNPG	-	(Kaur et al., 2007)
Aspergillus sojae	Ex	250 kDa	118 kDa	60 °C	5.0	0.14 mM pNPG	-	(Kimura et al., 1999)

Organism	Localization	Mol. Weight (native)	Mol. Weight (SDS)	Temp. Optimum	pH Optimum	*Km* value	*Ki* for glucose	References
Penicillium brasilianum	Ex	-	115 kDa	70 °C	4.8	0.09 mM pNPG 1.58 mM cellobiose	2.3 mM	(Krogh et al., 2010)
Neocallimastix frontalis	Ex	-	125.5 kDa	65 °C	5.5-7.0	2.5 mM pNPG	10.5 mM	(Li and Calza, 1991)
Trichoderma koningii	Ex	-	69.1 kDa	50 °C	5.0	2.67 mM pNPG	-	(Lin et al., 2010)
Humicola grisea	My	94 kDa	55 kDa (dimer)	60 °C	6-6.5	-	-	(Souza et al., 2009)
Acremonium persicinum	Ex	-	128 kDa	-	5.5	0.30 mM pNPG 0.91 mM cellobiose	0.35 mM	(Pitson et al., 1997)
Cladosporium fulvum	Ex	180 kDa	-	45 °C	5.5	0.19 mM pNPG	-	(Zhao et al., 2009)
Fusarium proliferatum	Ex	78.7 kDa	39.1 kDa (dimer)	50 °C	5.0	-	-	(Su et al., 2009)
Humicola insolens	My	94 kDa	55 kDa (dimer)	60 °C	6.0-6.5	-	-	(Souza et al., 2009)
Stachybotrys sp.	Ex	-	170 kDa (dimer)	50 °C	5.0	0.27 mM pNPG 2.22 mM pNPG	-	(Amouri and Gargouri, 2006)
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	0.35 mM pNPG	1.3 mM	
	Ex	-	54 kDa	60 °C	5.0	3.2 mM pNPG	470 mM	
	Ex	-	54 kDa	60 °C	5.0	6.2 mM pNPG	600 mM	
Aureobasidium sp.	In	331 kDa	-	80 °C	4.0	8.85 mM cellobiose	-	(Hayashi et al., 1999)
Aspergillus terreus	Ex	-	29 kDa	60 °C	2.0-8.0	14.2 mM pNPG	-	(Nazir et al., 2009)
	Ex	-	43 kDa	60 °C	5.0	4.37 mM pNPG		
	Ex	-	98 kDa	70 °C	5.0	11.1 mM pNPG		
Volvariella volvacea	My	158 kDa	-	55-60 °C	7.0	0.09 mM pNPG	-	(Cai et al., 1998)
	My	256 kDa	-	55-60 °C	6.2	0.50 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	
Termitomyces clypeatus	My	-	116 kDa	45 °C	-	0.148 mM pNPG	-	(Pal et al., 2010)
Candida cacaoi	In	220 kDa	-	60 °C	4.0-5.5	0.44 mM pNPG	-	(Drider et al., 1993)
Ceriporiopsis subvermispora	Ex	-	110 kDa	60 °C	3.5	3.29 mM pNPG 2.63 mM cellobiose	-	(Magalhães et al., 2006)
	Ex	-	53 kDa	-	-	-	-	

1.8. Mode of action

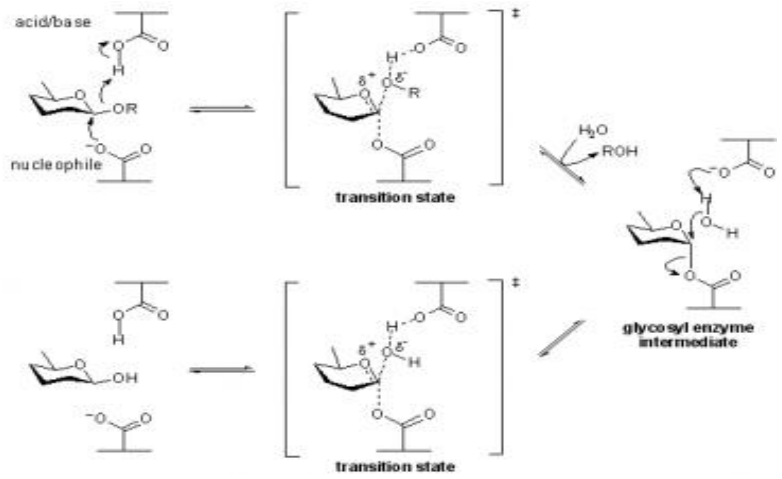
Though β-glucosidases are basically hydrolytic enzymes, they also perform reverse hydrolysis and transglycosylation reactions. The catalytic activity is thought to be provided by specific amino acids in the active site. The catalysis involves general acid catalysis in which two amino acids are thought be involved (Henrissat, 1991). Hydrophobic amino acids like tryptophan is thought to involve in the catalysis with the aromatic ring helping in substrate binding, orienting the glutamic acid nucleophile and stabilizing the deprotonated state (*Clostridium papyrosolvens*, *Agrobacterium* sp., *Aspergillus niger*). In most of the β-glucosidases, the -COOH group in the side chain of acidic amino acids like glutamic acid and aspartic acid acts as nucleophile and participates in catalytic action by forming covalent glycosyl-enzyme intermediate. They also modulate the ionization state of acid/base catalytic residues, form hydrogen bond to the sugar 2-OH group and stabilize the oxocarbenium ion like transition state (*Agrobacterium faecalis*, *Aspergillus wentii*, *Pichia etchellsii*, *Schizophyllum commune*). In some β-glucosidase (*Trichoderma reesei*), histidine is thought to be the proton donor while cysteine residues are found to involve in substrate binding (Bhatia et al., 2002; Li et al., 2001; Seidle et al., 2005).

1.8.1. Hydrolysis

Beta-glucosidase catalyzes the hydrolysis of glycosidic linkages formed between hemiacetal -OH group (OH group of anomeric carbon atom) and the OH group of other compounds like sugar, amino alcohol, alkyl or aryl alcohol. Two active site carboxylic acids are involved in catalysis, one act as a catalytic nucleophile and the other as an acid/base catalyst. During hydrolysis, the enzymic nucleophile attacks the anomeric carbon atom resulting in a covalent linkage with the anomeric carbon atom. The beta configuration of anomeric carbon atom is changed so as to form α-D-glycosyl-enzyme intermediate. Another active residue of the enzyme act as a base catalyst and donates H^+ ion to the glycosidic oxygen thereby assisting in the departure of the aglycone (or the other glycone) group. The glycosyl-enzyme intermediate is then hydrolyzed by a general

base catalyzed attack at the anomeric carbon atom by water (Figure 1.3). This trans-addition of -OH group results in the conversion of α in to β anomer and thus β-glucose is released as the product (Bhatia et al., 2002; Chir et al., 2002).

Figure 1.3: Schematic representation of hydrolysis reaction by β-glucosidase (www.cazypedia.org)



1.8.2. Reverse hydrolysis

In reverse hydrolysis, β-glucosidase catalyzes the synthesis of oligosaccharides. The substrate can be a disaccharide or a monosaccharide. When a disaccharide is the substrate, an enzyme substrate complex formed may be attacked by water or by another substrate. In an aqueous solution, lower substrate concentration result in hydrolytic activity of the enzyme and monosaccharides are produced. As the substrate concentration increases, reverse hydrolysis is favored where enzyme substrate complex is attacked by another substrate molecule to form oligosaccharides (Bruins et al., 2003). When substrate is a monosaccharide, condensation reaction can result in the production of oligosaccharides. In this reaction, aldohexose (glucose) binds with the enzyme to form enzyme-glycosyl intermediate. This is intercepted by another sugar or sugar derivative. Glycosidic bond is created between the anomeric OH group of beta-glucose and -OH group of 4^{th} carbon atom of the second sugar resulting in the formation of glycoside and water. The reaction is thermodynamically controlled and occurs through equilibrium displacement (Bhatia et al., 2002; Hancock et al., 2005).

1.8.3. Transglycosylation

In transglycosylation, the substrate is a glycoside (disaccharide or aryl linked glucoside). The enzyme-substrate intermediate makes the substrate an activated donor and the complex may be trapped by a nucleophile like alkyl, aryl alcohol, monosaccharide or disaccharide rather than water. This results in the formation of a new glycoside. The efficiency of product formation depends up on the competition of water and acceptor alcohol for the enzyme-glycosyl intermediate. Transglycosylation is kinetically controlled and can be enhanced by lowering thermodynamic activity of water by using high temperatures and organic solvents (Bhatia et al., 2002; Hancock et al., 2005). β-glucosidases with transglycosylation activity have been reported by a number of workers (Table 1.4).

Table 1.4: Transglycosylation activity of β-glucosidase from fungi

Organism	Donor molecule	Acceptor molecule	Product formed	Reference
Trichoderma reesei	cellobiose	cellobiose	cellotriose, cellotetraose, sophorose	(Saloheimo et al., 2002)
Thermotoga neapolitana	cellobiose	arbutin	β-D-gluco-pyranosyl arbutin	(Park et al., 2005)
Phanerochaete chrysosporium	laminaribose	laminaribose	glucosyl laminaribose	(Kawai et al., 2004)
Pyrococcus furiosus	lactose cellobiose	lactose cellobiose	tri and tetrasaccharides	(Bruins et al., 2003)
Piromyces sp.	cellopentaose	cellopentaose	celloheptaose. cellooctaose	(Harhangi et al., 2002)
Paecilomyces thermophila	cellobiose	cellobiose	cellotriose, cellotetraose, cellopentaose	(Yang et al., 2008)
Paecilomyces thermophila	cellotriose	cellotriose	cellotetraose, cellopentaose	(Yang et al., 2008)

1.9. Classification of β-glucosidase

Based on substrate specificity, β-glucosidase can be classified in to aryl β-glucosidase that act on aryl substrates, cellobiases that acts on cellobiose and broad substrate specificity enzymes that can act on both alkyl and aryl substrates (Riou et al., 1998; Yang et al., 2008). Most of the β-glucosidases so far characterized act both on alkyl and aryl substrates (Bhatia et al., 2002).

Based on nucleotide sequence identity (NSI) and folding similarities, glycosyl hydrolases are grouped in to different families (Henrissat and Bairoch, 1996) and at present 128 families are identified, of which β-glucosidase fall in family 1, family 3 and family 5 (www.cazy.org).

Glycoside Hydrolase Family 1 (GH1).

Family GH1 enzymes are found across a large spectrum of life forms. They are more common in bacteria and plants and have eight β/α barrel motif with active site placed in a wide cavity along the axis of barrel (Figure 1.4). The acid/base catalyst is located at the end of β strand 4 and catalytic nucleophile near the end of β strand 7. Glutamic acid acts as acid/base catalyst and catalytic nucleophile. Enzymes generally have β-glucosidase and β-galactosidase activities.

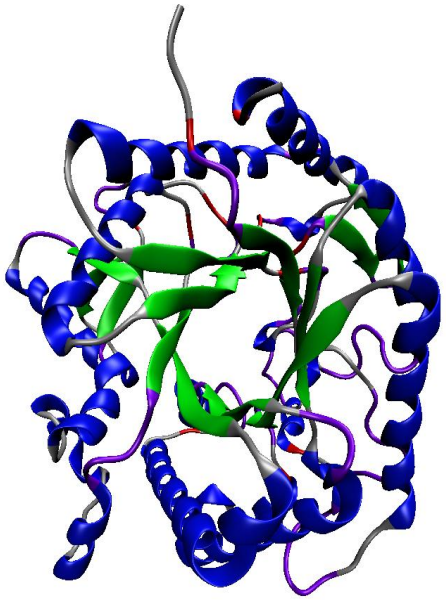
Glycoside Hydrolase Family 3 (GH3).

Most of the enzymes are microbial in origin. In many cases, the enzymes have broad substrate specificities. The catalytic nucleophile is aspartic acid while acid/base catalyst is glutamic acid. They are generally globular monomeric proteins of molecular mass around 60-70 kDa. Enzyme has two domains (barley β-D-glucan glucohydrolase), one with eight barrel and other with six barrel interconnected by 16 amino acid helix linker (Figure 1.4).

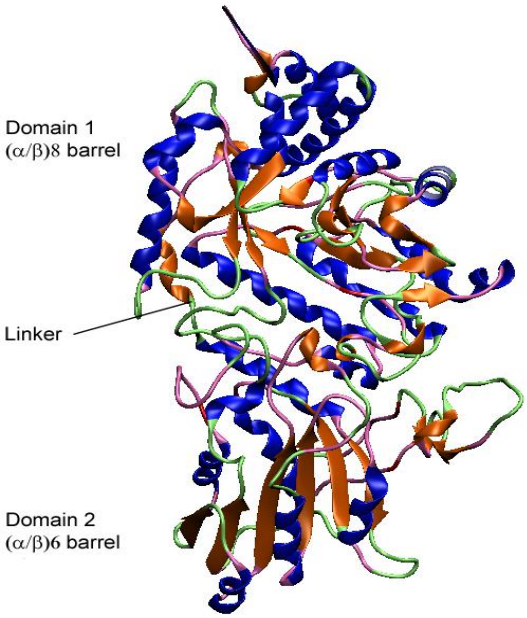
Glycoside Hydrolase Family 5 (GH5).

Found widely across different life forms but more common in fungi and plants. Enzymes are similar in structure with that of GH1 with eight β/α barrel motif. Glutamic acid acts as acid/base catalyst and catalytic nucleophile.

Fig 1.4: Representative structure of GH1 and GH3 β-glucosidase.



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



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

1.10. Applications of β-glucosidase

Beta-glucosidases are ubiquitous in nature and vary in kinetic parameters and substrate specificity. They can perform hydrolytic, reverse hydrolytic and transglycosylation reactions and hence have many potential applications.

1.10.1. Application based on synthetic activity

Biotransformation using β-glucosidase is favored over chemical synthesis owing to higher regio and stereo specificity (Choi et al., 2008). The synthesis ability, both

transglycosylation and reverse hydrolysis are employed for product formation. Glycosidases are inexpensive compared to transglycosidases and do not need complex and expensive substrates like sugar nucleotides (Bruins et al., 2003). The most important application is the synthesis of alkyl glycosides which are biodegradable non-ionic surfactants with good emulsifying and antimicrobial properties. Hexyl, heptyl and octyl glucosides are used as drug carriers and as solubilizing agents of biological membranes (Kiwada et al., 1985; Shinoyama et al., 1991). As liquid crystal generators, gemini surfactants are highly valued and β-glucoside, forms the precursor for the surfactant (Castro et al., 1997). Natural compounds like aryl glucoside with anti-feedant and repellant properties are used in pest control (Trincone and Pagnotta, 1995). Drugs like n-alkyl glucoside ester formed by the reaction of β-glucosidase-lipase on butyl glucoside in presence of phenyl butryic acid are effective in the treatment of fever and rheumatism (Otto et al., 1998). Non digestible oligosaccharides produced by transglycosylation can have positive effect on the growth of essential microbes in human gut flora (Bruins et al., 2003).

1.10.2. Application based on hydrolytic activity

Many of the natural flavor compounds such as monoterpenols, C-13 norisoprenoid and shikimate-derived compounds exist as flavorless glycosides (Winterhalter and Skouroumonis, 1997). The enzymatic cleavage of glycosidic bonds results in the release of flavor. Beta-glucosidases are the key enzymes that release aromatic compounds from their glycoside precursors (Gueguen et al., 1997b). During wine making process, β-glucosidase activity of *Saccharomyces* strains contributes to aroma formation. Along with rhamnosidase and arabinosidase, β-glucosidase play a key role in the release of aglycones, mostly terpenes (Barbagallo et al., 2004). To enhance aroma, commercial enzyme preparations of *Aspergillus* sp. are used (Villena et al., 2007). Microbial β-glucosidases are used to remove bitterness in citrus fruit juice caused by the glycoside naringin. Also cyanogenic glycosides can be degraded by applying β-glucosidases (Harhangi et al., 2002; Lei et al., 1999). Commercial β-glucosidase enzyme

from almonds is used to release vanillin from the natural parent compound glucovanillin during curing of vanilla (Havkin-Frenkel et al., 2004).

Use of glufosfamide drug in cancer treatment relies upon β-glucosidase activity. The glycoconjugate drug is preferentially absorbed by cancerous cells and the cystosolic and lysosomal β-glucosidase cleaves glufosfamide to release the aglycon moiety. This aglycon molecule has cytotoxic effect upon human cancerous cells (Arafa, 2009). Nystatin is toxic to both fungi and mammalian cell and hence intravenous injection of nystatin is not used for controlling pathogenic fungal infection in humans. However inactive nystatin can be produced by creating glucopyranoside conjugates. This gets decomposed only in the presence of fungal β-glucosidase releasing nystatin in a controlled manner affecting the fungus but not the mammalian cells (Bílková et al., 2010). Gaucher's disease, the most common lysosomal storage disease is caused by deficiency of active acid β-glucosidase. This results in the accumulation of glucosyl ceramide. The treatment involves enzyme replacement therapy in which recombinant β-glucosidase (Cerezyme) is administered to patients as intravenous injections (Brumshtein et al., 2009).

1.10.3. Beta-glucosidase in bioethanol industry

Ethanol is an important alternative energy source used in many countries to curb the dependency of fossil fuels. Recent biotechnological developments aims at use of lignocelluloses as a feedstock for bioethanol production (Buaban et al., 2010). Saccharification of cellulose is effected by cellulase enzyme consisting of endoglucanase, exoglucanase and β-glucosidase. In the bioethanol industry, *Trichoderma* and *Aspergillus* form the sources of cellulase (Pandey et al., 2000). However β-glucosidase of *Trichoderma reesei* and several *Aspergillus* spp. are subjected to glucose inhibition. Thus, as the concentration of glucose reaches to a threshold value, activity of β-glucosidases decreases considerably. This in turn results in accumulation of cellobiose that cause decrease in endo and exoglucanase activity. Thus cellulase from *Trichoderma* or *Aspergillus* alone cannot bring out complete hydrolysis of cellulose.

However, cocktails of cellulase can be made with β-glucosidase from other sources to enhance cellulose hydrolysis. The common commercially available cellulase preparations are given in Table 1.5 (Genencor, 2012; Novozymes, 2012; Nieves et al., 1998).

Table 1.5: Source and properties of commercially available cellulase

Product name	Company	Source	Optimum pH	Optimum temperature
Cellic CTEC3	Novozymes	*Mixed*	4.75 -5.25	50-55 °C
Accelerase Trio	Genencor	*Mixed*	4.30-4.50	50 °C
Biocellulase TRI	Quest Intl.	*T. reesei*	4.0-5.0	50 °C
Biocellulase A	Quest Intl.	*A. niger*	5.0	55 °C
Celluclast 1.5L	Novo Nordisk	*T. reesei*	5.0	50 °C
Cellulase TAP	Amano Enzyme	*T. viride*	5.0	50 °C
Cellulase AP30K	Amano Enzyme	*A. niger*	4.5	60 °C
Cellulase TRL	Solvay Enzymes	*T. reesei*	4.5	50 °C
Econase CE	Alko EDC	*T. reesei*	5.0	50 °C
Multifect CL	Genencor Intl	*T. reesei*	4.5	50 °C
Multifect GC	Genencor Intl	*T. reesei*	4.0	50 °C
Spenzyme#1	Genencor Intl	*T. reesei*	4.0	50 °C
Spenzyme#2	Genencor Intl	*T. reesei*	4.0	50 °C
Spenzyme#3	Genencor Intl	*T. reesei*	4.0	50 °C

1.11. Objectives of the Current Study

Beta-glucosidases are critical enzymes in biomass hydrolysis process and is important in creating highly efficient enzyme cocktails for the bio-ethanol industry. Among the two strategies proposed for overcoming the glucose inhibition of commercial cellulases, one is to use heavy dose of BGL in the enzyme blends and the second is to do simultaneous saccharification and fermentation where glucose is converted to alcohol as

soon as it is being generated. While the former needs extremely high quantities of enzyme, the latter is inefficient since the conditions for hydrolysis and fermentation are different. This makes the process technically challenging and also in this case, the alcohol generation is lesser, making its recovery difficult. A third option is to use glucose tolerant β-glucosidases which can work at elevated glucose concentrations. However, there are very few reports on such enzymes from microbial sources especially filamentous fungi which can be cultivated on cheap biomass as raw material. There has been very less number of studies directed at this, though there is every possibility that filamentous fungi that are efficient degraders of biomass may harbor such enzymes. The study therefore aimed at isolating a fungus capable of secreting glucose tolerant β-glucosidase enzyme. Production, characterization of β-glucosidases and application of BGL for bioethanol production were attempted. The objectives of the present study included

1. Screening of filamentous fungi as source of glucose tolerant BGL (GTBGL).

2. Production optimization of BGL in submerged and solid state fermentation using statistical experiment design and design of a solid state fermenter.

3. Studies on BGL induction using different carbon sources.

4. Purification of BGL isoforms and characterization of isoforms.

5. Confirmation of BGL multiplicity by partial sequencing of *bgl* gene.

6. Immobilization of purified BGL on to magnetic nanoparticle and its application.

7. Anti β-glucosidase antibody production and its application.

8. Bioethanol production by selected fungal strains from hexoses and pentoses.

9. Enzyme cocktail with purified BGL in bioethanol production.

Chapter 2

Materials and Methods

Chapter 2
Materials and Methods

2.1. Microorganisms and Preparation of inoculum

The fungal cultures used in the present study were isolated from soil and screened for β-glucosidase activity. The strain AM 130 was identified as *Byssochlamys fulva* and is deposited in the NII culture collection with accession number NII 0930. *Saccharomyces cerevisiae* NCIM 3288 was used for ethanol production and was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India.

Fungal cultures were inoculated on Potato Dextrose Agar (PDA) slants or Czapek-Dox agar slants and were incubated at 30 $^{\circ}$C. The fully sporulated slants obtained after eight days of incubation were stored at 4 $^{\circ}$C for short term preservation. For preparing the spore inoculum, sterile distilled water containing 0.05 % (w/v) tween-80 was added to the eight day old sporulated slants and the spores were dislodged into it by gentle pipetting under aseptic conditions. The suspension was appropriately diluted with sterile distilled water containing 0.05 % tween-80 to obtain the required spore count. Spore counts were done under a phase contrast microscope using a hemocytometer. One milliliter of this spore suspension was used to inoculate the medium.

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

2.2. Medium preparation

2.2.1. Czapek-Dox medium

The semi-synthetic medium used for fungal isolation was the Czapek-Dox agar medium. It had the following composition in g/L: Sucrose – 30.0, $NaNO_3$ – 3.0, K_2HPO_4

– 1.0, $MgSO_4.7H_2O$ - 0.5, KCl - 0.5, $FeSO_4.7H_2O$ - 0.01. Agar was added at 2 % level for solidification. The pH of the media was adjusted to 7.3 ± 0.2.

2.2.2. Mandel and Weber medium

The basal mineral salt 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, $FeSO_4.7H_2O$ - 0.005, $MnSO_4.7H_2O$ - 0.001, $ZnSO_4.7H_2O$ - 0.001, $CoCl_2$ - 0.001 (Mandel and Weber, 1969). The pH of the media was adjusted with 1N HCl or 1N NaOH wherever required.

2.2.3. Submerged Fermentation

Mandel and Weber minimal medium with 0.1% cellulose as carbon source was used for the production of β-glucosidase during screening experiments. Wheat bran was used as the carbon source during production of enzyme by *B. fulva*. Fifty milliliters of medium was taken in 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121 °C, 15 lbs pressure for 15 min.

2.2.4. 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. Ten gram (dry weight) of the substrate was weighed into 500 ml Erlenmeyer flasks and was moistened with a specific amount of Mandel and Weber mineral salt medium. The pH of the media was adjusted with 1N HCl or 1N NaOH wherever required. Distilled water was added in addition to the medium to attain the appropriate initial moisture content wherever applicable. The moistened bran was mixed well and was sterilized by autoclaving at 121 °C for 15 min at 15 lbs pressure.

2.3. Enzyme Production and Extraction

2.3.1. Solid State Fermentation

Medium prepared for SSF was inoculated with 1.0 ml of fungal spore suspension containing the desired number of spores. The contents were mixed thoroughly and were incubated under controlled conditions of temperature and relative humidity. The contents were mixed by mild shaking every 24 h. Incubation was continued for the duration indicated in the experimental designs 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. The filtered solution containing enzyme was centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was used as the crude enzyme preparation.

2.3.2. Submerged Fermentation and extraction of extracellular enzymes

Mandel and Weber medium with the carbon source as specified in the experiment was inoculated with the specified amount of spores. The flasks were incubated at 30 °C on an incubated shaker at 150 rpm agitation to the desired incubation period. At the end of fermentation, biomass was separated by centrifugation at 10000 rpm for 10 min at 4 °C and the supernatant was used as the crude enzyme preparation.

2.3.3. Submerged Fermentation and extraction of intracellular enzymes

Mandel and Weber medium with 1 % xylose (xylose-filter sterilized) was inoculated with the specified amount of spores. The flasks were incubated at 30 °C on an incubated shaker at 150 rpm agitation for a period of 5 days. At the end of incubation period, fungal mycelium was filtered out using Whatman No. 1 filter paper. The mycelium was suspended in physiological saline for 30 min and then filtered and

washed with physiological saline. The mycelium was then resuspended in 0.05 M citrate buffer (pH 4.8) containing 0.01 % PMSF (phenylmethanesulphonyl fluoride) and was disrupted using ultrasonicater for 30 minutes. The homogenate was centrifuged at 15,000 rpm for 15 minutes at 4º C; the supernatant was used as the source of intracellular enzymes.

2.4. Acetone precipitation of enzyme

For obtaining crude enzyme precipitate, 4 volume of chilled acetone (-20 °C) was slowly added to cooled extract with constant stirring. The mixture was kept for 12 h at -20 °C, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The pellet was allowed to dry at room temperature to remove residual acetone. The pellets were then dissolved in 10.0 ml 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

Beta-glucosidase activity was assayed using p-nitrophenyl- β-D glucopyranoside (pNPG) as substrate. The reaction mixture consisting of 1.0 ml of citrate buffer (0.05 M, pH 4.8), 0.5 ml of culture extract and 0.5 ml of 10 mM pNPG was incubated at 50 °C for 15 minutes. The reaction was terminated by adding 2.0 ml of 0.2 M Na_2CO_3.The absorbance of p-nitrophenol released was measured at 410 nm (Ghose and Bisaria, 1987) using a UV-VIS spectrophotometer (Shimadzu, Japan). For estimating glucose tolerance, instead of citrate buffer, citrate buffer containing 1.0 M or 2.0 M glucose was used. One unit enzyme activity was defined as the amount of enzyme required to release 1 µM of p-nitrophenol per minute and was expressed as U/ml in case of submerged fermentation or units per gram dry substrate (U/gds), in the case of SSF. Glucose tolerance was estimated as the % activity retention when assayed in presence of 0.5 M or 1.0 M glucose.

2.5.2. Cellulase assay

Total cellulase activity was measured using the filter paper assay according to IUPAC (Ghose, 1987). A rolled Whatman # 1 filter paper strip of dimension 1.0 x 6 cm (~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) and were equilibrated for 10 min at 50 °C in a water bath. Half milliliter of an appropriately diluted enzyme was added to the tube and incubated at 50 °C for 60 minutes. Appropriate controls were also run along with the test. At the end of the incubation period, each tube was removed from the water bath and the reaction was stopped by addition of 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).

2.5.3. Protein assay

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

2.5.4. Reducing sugar estimation

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

2.5.5. Estimation of Ethanol

Ethanol estimation was done by gas chromatography as outlined in NREL Laboratory Analytical protocol # 011(Templeton, 1994). One milliliter fermented broth

was centrifuged at 12000 rpm for 5 minutes at 4 °C and the supernatant was filtered through a 0.45 μm PES membrane (Pall, USA) before injecting into the GC. Ethanol was detected using an FID detector kept at 250 °C. Other conditions of operation were mobile phase – N_2 (30 ml/min), column temperature – 150 °C, injector temperature - 175 °C and injection volume 1.0 μl. Ethanol was detected by its elution time compared against a standard sample of pure ethanol and the concentrations were calculated based on the peak areas of known concentrations of ethanol. Ethanol concentrations were expressed as % v/v.

2.6. Electrophoresis and Zymogram Analysis

Standard protocol for SDS and Native PAGE were employed to prepare gels with 5-10 % strength. 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. Differences in fluorescence intensities of bands were measured by pixel density analyses of the photographs using the software Scion Image ® (Scion Corp, USA). Glucose tolerance of BGL bands were expressed in terms of activity retention which was calculated as the % of fluorescence intensity remaining in the BGL activity band in the gel incubated in MUG in presence of glucose to that in the gel incubated in MUG without glucose.

2.7. Chromosomal DNA Isolation from selected strains

Fungal strains were cultivated in potato dextrose broth (PDB) by inoculating 50 ml sterile PDB taken in 250 ml Erlenmeyer flasks with 10^6 spores and incubating at room temperature (28 °C ± 2 °C) for 72 h. One gram wet weight of the mycelium was frozen in liquid nitrogen and were ground to a fine powder. It was suspended in 1.5 ml of lysis buffer (250 mM NaCl, 25 mM EDTA, 0.5 % w/v SDS and 200 mM Tris–HCl, pH 8.5). The suspension was incubated at 68 °C for 15 min with occasional gentle mixing. After centrifugation at 13,000 rpm for 15 min (4 ºC), the supernatant was transferred to a new tube and polysaccharides and proteins were precipitated by adding 750 µl of cold 4.0 M sodium acetate at pH 5.2. This solution was gently mixed by inversion, placed at -20 ºC for 20 minutes and centrifuged at 13000 rpm for 15 min (4 ºC). Clean supernatant was transferred in to a new tube and chromosomal DNA was precipitated by adding 1 volume of chilled iso-propanol. This was gently mixed by inversion for a few minutes and incubated at -20 ºC for 10 minutes and DNA was recovered by centrifugation at 12000 rpm for 10 min. The DNA precipitate was washed twice with 70 % ethanol, allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0) and stored at -20 ºC (Rodrigues et al., 2007).

2.8. Ethanol production from hydrolysate

Ethanol production was studied using the enzymatic hydrolysate of sugarcane bagasse as the substrate for alcohol fermentation. The sugarcane bagasse hydrolysate generated by enzymatic saccharification was clarified by centrifugation at 10000 rpm for 10 min and was concentrated by evaporation (50 °C) to reducing sugar content of either 3 %, 6 % or 12 % w/v. Ten milliliters of the hydrolysate was sterilized by filtration through a 0.22 µm syringe filter and was inoculated with 10 % v/v of a 12 h old seed culture of *S. cerevisiae*. Incubation was carried out in stoppered 15 ml glass vials at room temperature (28 °C ± 2 °C) without agitation for 48 h. The samples were centrifuged at 13000 rpm for 10 min at 4 °C. The supernatant was filtered using 0.22 µ syringe filter and the ethanol content was analyzed by gas chromatography (Section 2.5.5).

Chapter 3

Screening of filamentous fungi as producers of glucose tolerant β-glucosidases

Chapter 3

Screening of filamentous fungi as producers of glucose tolerant β-glucosidases

3.1. Introduction

The success of any industrial process for enzyme production relies on several factors, the first being the isolation of a potential strain. Many microbes capable of secreting enzymes with unique functions have been discovered by extensive screening and are now used for industrial production of enzymes (Ogawa and Shimizu, 2002). Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation and higher production of extracellular enzymes with great industrial potential (Guimarães et al., 2006). Based on data from molecular methods, it is estimated that about 5.1 million fungal species inhabit the planet (Blackwell, 2011). Fungi are diverse in their habitat and they can be free living, parasitic, mycorrhizal or in other symbiotic associations and occurring in a wide array of ecosystems. Majority of fungi are seen in terrestrial habitat, followed by freshwater and marine ecosystems. There are about 3150 species of saprophytic soil fungi, 3000 species of ascomycetes freshwater fungi and 1500 species of marine fungi (Gams, 2006). Aquatic fungi may be associated with decaying algal and plant tissue including marine and fresh water algae, mangrove vegetations, and cellulosic materials arising from the terrestrial habitats. On the basis of available data, it is likely that only less than 5 % of the fungal diversity is described and maintained in culture (Richards et al., 2012).

Since majority of fungi are decomposers that depends on plant material as carbon source, they can be very potent sources of cellulases and hence β-glucosidases. Also because β-glucosidase has multitudes of function, different fungal strains of the same species can be good sources of BGL with varying kinetic properties. Even a single strain can secrete multiple isoforms of BGL. Due to the wide range of distribution of

filamentous fungi, each with several strains adapted to specific environments, it was speculated that a screening of filamentous fungi from different ecological niches would definitely provide isolates capable of producing glucose tolerant BGL(s). Studies were conducted for isolation of fungal strains capable of secreting GBGL.

3.2. Materials and Methods

3.2.1. Sample collection and isolation of fungi

Sterile petri plates were used as sample containers. Samples of decaying wood and humus rich soil or clay were collected from terrestrial, fresh water and brackish water environment. Fungi were isolated by serial dilution and pour plate method using Czapek-Dox agar as media with composition as outlined under section 2.2.1. For isolation of brackish water fungi, NaCl at 1 % level was incorporated in to Czapek-Dox medium. The isolated colonies were streaked on to nutrient agar plates to check for bacterial contamination. Pure colonies were streaked on to Czapek-Dox agar slants and were kept at 30 °C for 8 days to sporulate. The sporulated cultures were stored at 4 °C and sub cultured every 45 days.

3.2.2. Primary screening of fungi as producers of glucose tolerant β-glucosidase

Mandel and Weber medium with composition as given under section 2.2.2 containing 0.1 % cellulose as carbon source was used as the production media. For brackish water fungi, 1 % NaCl was incorporated in to the production media. Inoculum was prepared as outlined under section 2.1. The fungi were inoculated in 50 ml of sterile media taken in 250 ml Erlenmeyer flasks and incubated at 30 °C, 150 rpm for 4 days. Enzyme extraction was carried out as described under section 2.3.2. Enzyme assays were conducted to determine the activity and glucose tolerance (0.5 M glucose) as indicated in section 2.5.1.

3.2.3. Secondary screening of fungi- Submerged fermentation (SmF)

Sixteen fungi, 13 from terrestrial source and 3 from brackish water habitat with high glucose tolerant β-glucosidase production and 17 fungi, 15 from terrestrial ecosystem, 1 from fresh water and 1 from brackish water environment showing high production of β-glucosidase were further screened. The screening procedure was similar to that stated in section 3.2.2, except for incubation period, which was extended to 7 days.

3.2.4. Secondary screening of fungi- Solid State Fermentation (SSF)

Fungi identified as potential strains for glucose tolerant BGL production under SmF were tested for their ability for GBGL production in Solid State Fermentation (SSF) with 60 % initial moisture. Media preparation, enzyme extraction, enzyme assay and acetone precipitation of enzyme were done as detailed under appropriate sections in the Materials and Methods (Chapter 2).

3.2.5. Native PAGE and Zymogram analysis

The acetone precipitated BGL of selected strains (SSF extract of GBGL producers) were subjected to native PAGE (10 % gel strength). Zymogram analysis with MUG as substrate in presence of 0.5 M glucose and without glucose were carried out as outlined under appropriate sections in the Materials and Methods (Chapter 2).

3.2.6. Identification of fungal strains

Fungal strains secreting high titers of GBGL were identified by morphological features and the identifications were confirmed by molecular analysis.

3.2.6.1. Morphological identification of fungal strains

Vegetative and reproductive features of fungal strains were studied by microscopical observations. For identification of fungal taxa, morphological features

were compared with fungal descriptions stated in standard books of mycology (Ainsworth, 1996; Alexopoulos et al., 1996).

3.2.6.2. Molecular identification of potential strain

Molecular identification was carried out by sequencing and BLAST analysis of amplified regions of 18S rRNA gene.

3.2.6.2.1. 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).

3.2.6.2.2. PCR Amplification of 18S rRNA gene

The primers used for amplification of 18S rRNA gene are given in Table 3.1. PCR reactions contained 0.5 units of *Taq* DNA polymerase, 1x *Taq* buffer, 200 µM of each deoxynucleotide triphosphate, 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, 56 °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.

Table 3.1: Primers used for amplification of 18S rRNA gene

SL #	SEQ CODE	SEQUENCE
		Primers
1	$P_{45}F$	5′-ATG CCC GTC CAG TCG GAC CT -3'
2	$P_{47}R$	5′-CTG AAC GGT ACG CTT GAC GGT CCG TAG -3'

3.2.7. Genetic diversity of Paecilomyces species

To assess the genetic diversity of 8 strains of *Paecilomyces* isolated as GBGL producers, genotyping was carried out using ITS and RAPD methodology.

3.2.7.1. ITS amplification

PCR amplification of Internal Transcribed Spacer region (ITS) of rDNA was carried out using universal eucaryotic ITS primers. The primers used for ITS amplification is given in Table 3.2. 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 to that stated under section 3.2.6.2.2.

Table 3.2: Primers used for amplification of ITS region

SL #	SEQ CODE	SEQUENCE
		Primers
1	IF	5′-GTT CCG TAG GTG AAC CTG C -3'
2	IR	5′-ATA TGC TTA AGT TCA GCG GGT -3'

3.2.7.2. RAPD analysis

Ten base pair long oligonucleotides were used as RAPD primers. The details of primers used is given in Table 3.3. PCR reactions contained 0.5 unit of *Taq* DNA polymerase, 1x *Taq* buffer, 200 µM of each deoxynucleotide triphosphate, 2.0 µM $MgSO_4$ (All from Fermentas, USA), 0.2 µg genomic DNA, and 1.0 µM primer. Reaction conditions for PCR amplification were an initial 94 °C for 3 min, followed by 45 cycles of 94 °C for 30 sec, 30-40 °C (as determined by Tm value of the primers) for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 4 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. Images were acquired and documentation was done using an image analysis system (Syngene-GBox, UK). Dendrogram was constructed by UPGMA cluster analysis using the Gene Directory ® software provided in Syngene-GBox documentation system.

Table 3.3: Primers used for RAPD

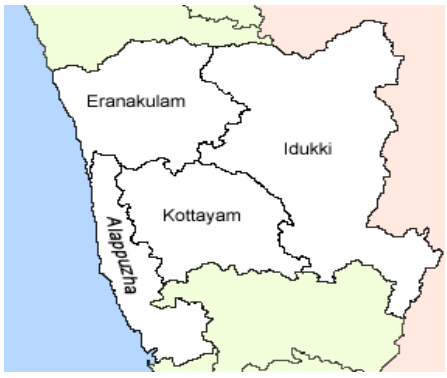
SL #	SEQ CODE	SEQUENCE
		Primers
1	OPJ 06	5′-TCG TTC CGC A -3'
2	OPK 12	5′-TGG CCC TCA C -3'
3	OPX 06	5′-ACG CCA GAG G -3'
4	OPF 10	5′-GGA AGC TTG G -3'
5	OPH 12	5′-ACG CGC ATG T -3'

3.3. Results and Discussion

3.3.1. Sample collection and isolation of fungi

Extensive sampling was carried out in central region of Kerala - Ernakulam, Idukki, Kottayam and Alappuzha districts (Figure 3.1). All together 114 samples from terrestrial environment, 18 from fresh water and 28 from brackish water environment were collected. 455 fungi from terrestrial environment, 103 fungi from brackish water habitat and 50 from fresh water ecosystems were isolated as pure cultures.

Figure 3.1: Schematic representation of sampling



3.3.2. Primary Screening of fungi as producers of glucose tolerant β-glucosidase

Microbial enzymes have several advantage over plant or animal derived enzymes by virtue of their greater variability in catalytic activities, low cost and the regular and abundant supply (Chandrasekaran and Kumar, 2002). Beta-glucosidase production in SmF by pure cultures of fungi is presented in Table 3.4 (BGL production by fungi isolated from terrestrial environment; Table 3.5 (BGL production by fungi isolated from aquatic fresh water environment); Table 3.6 (BGL production by fungi isolated from brackish water environment). Most of the fungal strains collected in the present study from brackish water may not be marine but facultative marine. Facultative marine fungi are from terrestrial or fresh water habitats but may also grow in marine environment (Bugni and Ireland, 2004).

Fungi play an important role in decomposition of plant biomass, both in terrestrial and aquatic habitats. In an earlier study of fungi from composting soil, all the 13 strains isolated were BGL producers; with higher BGL production by thermophilic fungi than by the thermo-tolerant taxa (Sonia et al., 2008). Another study where fungi belonging to deuteromycotina, zygomycotina and ascomycotina were screened, reported that all the 58 species evaluated secreted β-glucosidase (Augustín et al., 1981). In yet another study, among the 13 *Aspergillus* strains isolated from tea garden soil, only 8 were BGL producers (Selvaraj and Sasikala, 2008). In a petri plate based screening method using agar plates with 10 mM MUG, only 20 strains of the 340 strains isolated showed BGL production as evident from fluorescence under UV light (Jeya et al., 2010).

As compared to fungi isolated from terrestrial and fresh water samples, fungi isolated from brackish water environment were the least producers of BGL. Of the 103 fungi isolated from brackish waters, 61.2% showed BGL production and even in the positive cultures, production as well as glucose tolerance was low. A similar result was obtained for alkaline cellulase screening, where among the 54 mangrove fungal strains screened, only three fungal isolates tested positive for enzyme production (Ravindran et al., 2010). In the present study, the low percentage of BGL producers from brackish water may be attributed to the culture conditions used. In screening studies, an acidic pH 5.0 was used in the production media while marine habitats usually have an alkaline pH.

Table 3.4: Screening of fungal strains isolated from terrestrial habitat for GBGL production

Strain	Enzyme activity U/ml		Activity retention %	Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)			(absence of glucose)	(In presence of 0.5 M glucose)	
AM 001	5.27 ± 0.02	0.22 ± 0.01	4.14	AM 047	3.70 ± 0.08	0.16 ± 0.01	4.33
AM 002	4.44 ± 0.03	0.38 ± 0.03	8.49	AM 048	3.57 ± 0.05	0.37 ± 0.06	10.29
AM 003	3.60 ± 0.05	0.22 ± 0.02	6.07	AM 049	8.40 ± 0.03	0.87 ± 0.07	10.37
AM 004	1.22 ± 0.06	0.03 ± 0.00	2.24	AM 050	5.38 ± 0.09	0.51 ± 0.04	9.46
AM 005	2.27 ± 0.02	0.18 ± 0.03	7.76	AM 051	0.38 ± 0.02	0.00 ± 0.00	-
AM 006	1.64 ± 0.03	0.11 ± 0.01	6.77	AM 052	0.30 ± 0.03	0.00 ± 0.00	-
AM 007	1.16 ± 0.01	0.11 ± 0.02	9.09	AM 053	1.89 ± 0.03	0.24 ± 0.05	12.81
AM 008	0.05 ± 0.00	0.00 ± 0.00	-	AM 054	1.69 ± 0.07	0.12 ± 0.02	7.20
AM 009	0.73 ± 0.03	0.06 ± 0.01	7.76	AM 055	0.39 ± 0.03	0.00 ± 0.00	-
AM 010	0.00 ± 0.00	0.00 ± 0.00	-	AM 056	0.18 ± 0.03	0.03 ± 0.01	14.74
AM 011	0.28 ± 0.03	0.00 ± 0.00	-	AM 057	0.29 ± 0.00	0.03 ± 0.00	9.36
AM 012	0.00 ± 0.00	0.00 ± 0.00	-	AM 058	5.83 ± 0.02	0.19 ± 0.01	3.32
AM 013	1.93 ± 0.07	0.20 ± 0.01	10.40	AM 059	2.15 ± 0.04	0.52 ± 0.02	23.99
AM 014	0.48 ± 0.05	0.04 ± 0.01	8.58	AM 060	2.23 ± 0.01	0.06 ± 0.01	2.60
AM 015	2.42 ± 0.01	0.12 ± 0.02	5.01	AM 061	1.81 ± 0.03	0.06 ± 0.00	3.43
AM 016	2.50 ± 0.05	0.17 ± 0.03	6.79	AM 062	1.03 ± 0.01	0.08 ± 0.00	7.53
AM 017	3.21 ± 0.03	0.27 ± 0.01	8.30	AM 063	9.31 ± 0.06	0.30 ± 0.01	3.22
AM 018	0.52 ± 0.02	0.00 ± 0.00	-	AM 064	2.14 ± 0.01	0.49 ± 0.02	22.81
AM 019	2.10 ± 0.08	0.08 ± 0.02	4.04	AM 065	2.91 ± 0.04	0.22 ± 0.01	7.44
AM 020	0.04 ± 0.01	0.00 ± 0.00	-	AM 066	1.44 ± 0.02	0.06 ± 0.00	3.88
AM 021	3.04 ± 0.06	0.88 ± 0.02	29.07	AM 067	1.97 ± 0.02	0.32 ± 0.01	16.31
AM 022	0.00 ± 0.00	0.00 ± 0.00	-	AM 068	5.85 ± 0.08	0.39 ± 0.02	6.74
AM 023	8.25 ± 0.08	0.20 ± 0.01	2.44	AM 069	5.98 ± 0.02	0.41 ± 0.01	6.93
AM 024	0.58 ± 0.02	0.00 ± 0.00	-	AM 070	0.99 ± 0.02	0.14 ± 0.01	14.67
AM 025	1.01 ± 0.02	0.06 ± 0.01	5.82	AM 071	3.95 ± 0.03	0.23 ± 0.01	5.90
AM 026	1.44 ± 0.06	0.47 ± 0.03	32.58	AM 072	0.58 ± 0.01	0.11 ± 0.01	19.41
AM 027	2.48 ± 0.05	0.61 ± 0.02	24.59	AM 073	0.93 ± 0.00	0.53 ± 0.01	57.29
AM 028	1.34 ± 0.05	0.14 ± 0.02	10.22	AM 074	0.47 ± 0.02	0.04 ± 0.01	8.76
AM 029	0.15 ± 0.03	0.00 ± 0.00	-	AM 075	1.56 ± 0.01	0.63 ± 0.01	40.41
AM 030	0.13 ± 0.03	0.00 ± 0.00	-	AM 076	3.21 ± 0.04	0.15 ± 0.00	4.64
AM 031	0.07 ± 0.00	0.00 ± 0.00	-	AM 077	2.72 ± 0.02	0.08 ± 0.00	2.79
AM 032	2.33 ± 0.04	0.29 ± 0.02	12.37	AM 078	8.33 ± 0.08	0.47 ± 0.01	5.61
AM 033	6.65 ± 0.02	0.69 ± 0.02	10.42	AM 079	1.07 ± 0.01	0.45 ± 0.01	41.97
AM 034	1.44 ± 0.01	0.42 ± 0.01	29.11	AM 080	1.52 ± 0.02	0.08 ± 0.00	5.00
AM 035	2.06 ± 0.04	0.37 ± 0.05	17.90	AM 081	0.92 ± 0.08	0.12 ± 0.04	13.46
AM 036	0.65 ± 0.04	0.00 ± 0.00	-	AM 082	2.10 ± 0.01	0.20 ± 0.02	9.73
AM 037	0.68 ± 0.02	0.05 ± 0.01	7.50	AM 083	2.14 ± 0.02	0.13 ± 0.03	6.24
AM 038	0.56 ± 0.01	0.00 ± 0.00	-	AM 084	5.64 ± 0.03	0.65 ± 0.01	11.51
AM 039	9.10 ± 0.02	0.53 ± 0.03	5.84	AM 085	0.00 ± 0.00	0.00 ± 0.00	-
AM 040	2.41 ± 0.02	0.50 ± 0.02	20.59	AM 086	1.69 ± 0.00	0.11 ± 0.00	6.65
AM 041	2.68 ± 0.02	0.20 ± 0.02	7.31	AM 087	5.14 ± 0.07	0.44 ± 0.02	8.64
AM 042	0.87 ± 0.03	0.07 ± 0.00	8.38	AM 088	7.62 ± 0.07	0.33 ± 0.02	4.26
AM 043	1.27 ± 0.02	0.11 ± 0.03	8.43	AM 089	0.00 ± 0.00	0.00 ± 0.00	-
AM 044	2.51 ± 0.02	0.55 ± 0.01	21.98	AM 090	5.00 ± 0.06	0.22 ± 0.00	4.31
AM 045	0.58 ± 0.03	0.00 ± 0.00	-	AM 091	0.74 ± 0.00	0.33 ± 0.02	44.68
AM 046	0.37 ± 0.00	0.00 ± 0.00	-	AM 092	0.15 ± 0.01	0.00 ± 0.00	-

Table 3.4. Continued

Strain	Enzyme activity U/ml		Activity retention %	Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)			(absence of glucose)	(In presence of 0.5 M glucose)	
AM 093	0.17 ± 0.02	0.00 ± 0.00	-	AM 139	0.00 ± 0.00	0.00 ± 0.00	-
AM 094	1.65 ± 0.07	0.14 ± 0.02	8.57	AM 140	2.41 ± 0.12	0.41 ± 0.04	16.98
AM 095	1.65 ± 0.03	0.25 ± 0.01	15.02	AM 141	1.73 ± 0.07	0.61 ± 0.02	35.16
AM 096	0.03 ± 0.00	0.00 ± 0.00	-	AM 142	1.69 ± 0.12	0.07 ± 0.01	4.36
AM 097	1.32 ± 0.04	0.19 ± 0.01	14.47	AM 143	0.35 ± 0.05	0.11 ± 0.00	31.06
AM 098	1.71 ± 0.09	0.94 ± 0.03	54.66	AM 144	2.03 ± 0.14	0.31 ± 0.02	15.48
AM 099	1.78 ± 0.06	0.09 ± 0.03	5.30	AM 145	0.81 ± 0.07	0.26 ± 0.02	31.59
AM 100	15.99 ± 0.03	0.81 ± 0.07	5.05	AM 146	0.07 ± 0.00	0.00 ± 0.00	-
AM 101	1.34 ± 0.05	0.21 ± 0.02	15.58	AM 147	0.65 ± 0.01	0.05 ± 0.00	7.68
AM 102	2.61 ± 0.15	0.47 ± 0.03	17.99	AM 148	0.76 ± 0.04	0.20 ± 0.02	25.88
AM 103	0.00 ± 0.00	0.00 ± 0.00	-	AM 149	0.92 ± 0.01	0.06 ± 0.00	6.92
AM 104	0.00 ± 0.00	0.00 ± 0.00	-	AM 150	1.24 ± 0.03	0.10 ± 0.01	8.14
AM 105	1.21 ± 0.02	0.23 ± 0.02	19.16	AM 151	0.88 ± 0.04	0.14 ± 0.01	15.38
AM 106	2.90 ± 0.19	0.23 ± 0.01	8.09	AM 152	0.03 ± 0.00	0.00 ± 0.00	-
AM 107	6.60 ± 0.36	0.17 ± 0.04	2.64	AM 153	0.00 ± 0.00	0.00 ± 0.00	-
AM 108	3.09 ± 0.06	0.05 ± 0.01	1.65	AM 154	8.96 ± 0.27	0.31 ± 0.06	3.50
AM 109	4.78 ± 0.13	0.24 ± 0.10	5.02	AM 155	0.42 ± 0.08	0.00 ± 0.00	-
AM 110	1.19 ± 0.04	0.14 ± 0.03	11.66	AM 156	0.11 ± 0.02	0.00 ± 0.00	-
AM 111	15.17 ± 0.17	0.98 ± 0.08	6.46	AM 157	0.14 ± 0.01	0.00 ± 0.00	-
AM 112	0.25 ± 0.03	0.00 ± 0.00	-	AM 158	3.11 ± 0.05	0.43 ± 0.02	13.90
AM 113	2.14 ± 0.08	0.34 ± 0.03	15.75	AM 159	1.96 ± 0.04	0.20 ± 0.03	10.33
AM 114	2.98 ± 0.03	0.34 ± 0.02	11.39	AM 160	0.06 ± 0.00	0.00 ± 0.00	-
AM 115	0.70 ± 0.02	0.14 ± 0.02	19.57	AM 161	0.27 ± 0.01	0.04 ± 0.00	15.60
AM 116	0.74 ± 0.04	0.04 ± 0.00	5.24	AM 162	0.11 ± 0.03	0.00 ± 0.00	-
AM 117	0.59 ± 0.06	0.10 ± 0.03	16.84	AM 163	0.00 ± 0.00	0.00 ± 0.00	-
AM 118	0.00 ± 0.00	0.00 ± 0.00	-	AM 164	0.04 ± 0.01	0.00 ± 0.00	-
AM 119	1.14 ± 0.03	0.51 ± 0.03	44.61	AM 165	1.07 ± 0.05	0.06 ± 0.01	5.30
AM 120	0.50 ± 0.05	0.07 ± 0.01	13.24	AM 166	1.14 ± 0.08	0.03 ± 0.00	2.57
AM 121	0.08 ± 0.00	0.03 ± 0.00	38.19	AM 167	1.58 ± 0.05	0.21 ± 0.05	13.36
AM 122	1.68 ± 0.01	0.23 ± 0.02	13.99	AM 168	0.37 ± 0.04	0.00 ± 0.00	-
AM 123	0.11 ± 0.02	0.00 ± 0.00	-	AM 169	0.20 ± 0.03	0.00 ± 0.00	-
AM 124	0.64 ± 0.03	0.07 ± 0.00	11.26	AM 170	0.26 ± 0.01	0.00 ± 0.00	-
AM 125	2.17 ± 0.06	0.33 ± 0.03	15.29	AM 171	0.04 ± 0.01	0.00 ± 0.00	-
AM 126	0.60 ± 0.03	0.03 ± 0.00	4.73	AM 172	0.05 ± 0.00	0.00 ± 0.00	-
AM 127	0.21 ± 0.04	0.00 ± 0.00	-	AM 173	0.75 ± 0.01	0.13 ± 0.00	17.32
AM 128	4.52 ± 0.14	0.27 ± 0.04	7.43	AM 174	0.96 ± 0.02	0.08 ± 0.01	8.64
AM 129	1.13 ± 0.10	0.03 ± 0.00	2.50	AM 175	0.08 ± 0.01	0.00 ± 0.00	
AM 130	3.87 ± 0.17	1.06 ± 0.08	27.26	AM 176	0.32 ± 0.02	0.03 ± 0.01	10.62
AM 131	0.83 ± 0.02	0.05 ± 0.02	5.79	AM 177	3.68 ± 0.11	0.26 ± 0.05	7.16
AM 132	0.64 ± 0.04	0.00 ± 0.00	-	AM 178	1.33 ± 0.08	0.09 ± 0.01	6.76
AM 133	17.72 ± 0.40	0.85 ± 0.16	4.82	AM 179	2.22 ± 0.05	0.42 ± 0.04	18.71
AM 134	1.32 ± 0.11	0.11 ± 0.02	8.30	AM 180	0.11 ± 0.01	0.00 ± 0.00	-
AM 135	6.43 ± 0.24	0.19 ± 0.01	2.98	AM 181	0.22 ± 0.00	0.04 ± 0.00	19.16
AM 136	0.03 ± 0.01	0.00 ± 0.00	-	AM 182	1.04 ± 0.02	0.13 ± 0.03	12.11
AM 137	1.53 ± 0.06	0.34 ± 0.02	22.45	AM 183	1.08 ± 0.03	0.12 ± 0.02	10.24
AM 138	0.41 ± 0.11	0.06 ± 0.01	15.62	AM 184	0.64 ± 0.01	0.03 ± 0.00	5.07

Table 3.4. Continued

Strain	Enzyme activity U/ml		Activity retention %	Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)			(absence of glucose)	(In presence of 0.5 M glucose)	
AM 185	1.58 ± 0.02	0.45 ± 0.01	28.62	AM 231	0.20 ± 0.02	0.00 ± 0.00	-
AM 186	1.02 ± 0.04	0.06 ± 0.01	6.09	AM 232	0.23 ± 0.01	0.02 ± 0.00	9.57
AM 187	0.00 ± 0.00	0.00 ± 0.00	-	AM 233	0.50 ± 0.02	0.05 ± 0.01	10.70
AM 188	0.00 ± 0.00	0.00 ± 0.00	-	AM 234	0.43 ± 0.01	0.04 ± 0.00	9.31
AM 189	0.00 ± 0.00	0.00 ± 0.00	-	AM 235	0.50 ± 0.02	0.05 ± 0.01	10.32
AM 190	0.72 ± 0.02	0.04 ± 0.00	4.86	AM 236	0.00 ± 0.00	0.00 ± 0.00	-
AM 191	1.26 ± 0.04	0.21 ± 0.01	16.91	AM 237	0.07 ± 0.00	0.00 ± 0.00	-
AM 192	1.33 ± 0.04	0.07 ± 0.01	5.12	AM 238	1.19 ± 0.02	0.07 ± 0.02	5.54
AM 193	0.82 ± 0.03	0.04 ± 0.01	4.62	AM 239	0.00 ± 0.00	0.00 ± 0.00	-
AM 194	0.35 ± 0.02	0.07 ± 0.02	21.37	AM 240	0.00 ± 0.00	0.00 ± 0.00	-
AM 195	0.00 ± 0.00	0.00 ± 0.00	-	AM 241	0.12 ± 0.02	0.00 ± 0.00	
AM 196	0.02 ± 0.00	0.00 ± 0.00	-	AM 242	0.06 ± 0.01	0.00 ± 0.00	
AM 197	0.26 ± 0.01	0.00 ± 0.00	-	AM 243	0.15 ± 0.01	0.00 ± 0.00	-
AM 198	0.31 ± 0.01	0.07 ± 0.01	22.58	AM 244	0.15 ± 0.02	0.03 ± 0.01	18.00
AM 199	0.51 ± 0.02	0.00 ± 0.00	-	AM 245	0.00 ± 0.00	0.00 ± 0.00	-
AM 200	0.06 ± 0.00	0.00 ± 0.00	-	AM 246	0.93 ± 0.06	0.04 ± 0.00	4.33
AM 201	0.00 ± 0.00	0.00 ± 0.00	-	AM 247	1.70 ± 0.11	0.33 ± 0.01	19.65
AM 202	0.49 ± 0.02	0.04 ± 0.01	7.15	AM 248	0.00 ± 0.00	0.00 ± 0.00	-
AM 203	0.00 ± 0.00	0.00 ± 0.00	-	AM 249	1.10 ± 0.03	0.10 ± 0.01	8.90
AM 204	0.00 ± 0.00	0.00 ± 0.00	-	AM 250	0.02 ± 0.00	0.00 ± 0.00	-
AM 205	0.00 ± 0.00	0.00 ± 0.00	-	AM 251	2.19 ± 0.13	0.25 ± 0.00	11.50
AM 206	0.14 ± 0.02	0.00 ± 0.00	-	AM 252	0.00 ± 0.00	0.00 ± 0.00	-
AM 207	0.05 ± 0.00	0.00 ± 0.00	-	AM 253	0.38 ± 0.03	0.04 ± 0.01	11.21
AM 208	0.03 ± 0.00	0.00 ± 0.00	-	AM 254	0.74 ± 0.02	0.05 ± 0.00	7.35
AM 209	1.40 ± 0.06	0.46 ± 0.02	33.01	AM 255	0.07 ± 0.01	0.00 ± 0.00	-
AM 210	0.40 ± 0.04	0.00 ± 0.00	-	AM 256	0.46 ± 0.03	0.03 ± 0.00	5.78
AM 211	0.13 ± 0.02	0.00 ± 0.00	-	AM 257	1.24 ± 0.08	0.12 ± 0.01	9.25
AM 212	1.31 ± 0.11	0.06 ± 0.00	4.68	AM 258	0.14 ± 0.01	0.05 ± 0.00	39.72
AM 213	2.22 ± 0.09	0.16 ± 0.03	7.14	AM 259	8.61 ± 0.09	0.42 ± 0.04	4.88
AM 214	1.41 ± 0.10	0.27 ± 0.02	19.07	AM 260	0.81 ± 0.03	0.06 ± 0.01	7.96
AM 215	1.64 ± 0.04	0.08 ± 0.00	4.68	AM 261	0.00 ± 0.00	0.00 ± 0.00	-
AM 216	0.46 ± 0.00	0.04 ± 0.00	8.85	AM 262	0.00 ± 0.00	0.00 ± 0.00	-
AM 217	2.11 ± 0.04	0.19 ± 0.02	8.98	AM 263	0.10 ± 0.00	0.00 ± 0.00	-
AM 218	0.75 ± 0.07	0.17 ± 0.02	22.60	AM 264	0.00 ± 0.00	0.00 ± 0.00	-
AM 219	1.67 ± 0.09	0.92 ± 0.01	53.83	AM 265	0.00 ± 0.00	0.00 ± 0.00	-
AM 220	0.70 ± 0.03	0.07 ± 0.00	9.83	AM 266	0.00 ± 0.00	0.00 ± 0.00	-
AM 221	0.82 ± 0.07	0.13 ± 0.02	15.50	AM 267	0.00 ± 0.00	0.00 ± 0.00	-
AM 222	0.00 ± 0.00	0.00 ± 0.00	-	AM 268	0.63 ± 0.00	0.20 ± 0.01	31.43
AM 223	0.65 ± 0.03	0.10 ± 0.02	14.75	AM 269	0.52 ± 0.02	0.08 ± 0.01	15.33
AM 224	1.03 ± 0.02	0.08 ± 0.02	8.15	AM 270	0.07 ± 0.01	0.00 ± 0.00	-
AM 225	0.08 ± 0.00	0.00 ± 0.00	-	AM 271	2.40 ± 0.04	0.13 ± 0.02	5.49
AM 226	0.41 ± 0.03	0.05 ± 0.01	12.13	AM 272	0.36 ± 0.03	0.12 ± 0.01	33.75
AM 227	0.69 ± 0.03	0.06 ± 0.01	9.36	AM 273	0.42 ± 0.02	0.16 ± 0.01	37.97
AM 228	1.19 ± 0.01	0.22 ± 0.01	18.31	AM 274	0.00 ± 0.00	0.00 ± 0.00	-
AM 229	0.08 ± 0.01	0.00 ± 0.00	-	AM 275	0.00 ± 0.00	0.00 ± 0.00	-
AM 230	1.10 ± 0.04	0.11 ± 0.01	10.31	AM 276	0.27 ± 0.03	0.00 ± 0.00	-

Table 3.4. Continued

Strain	Enzyme activity U/ml		Activity retention %	Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)			(absence of glucose)	(In presence of 0.5 M glucose)	
AM 277	2.48 ± 0.01	0.09 ± 0.01	3.45	AM 323	0.25 ± 0.01	0.06 ± 0.01	22.46
AM 278	1.21 ± 0.01	0.04 ± 0.00	3.20	AM 324	0.07 ± 0.00	0.02 ± 0.00	24.07
AM 279	0.96 ± 0.04	0.05 ± 0.01	4.74	AM 325	2.14 ± 0.02	0.07 ± 0.01	3.43
AM 280	0.04 ± 0.01	0.00 ± 0.00	-	AM 326	2.90 ± 0.04	0.07 ± 0.03	2.29
AM 281	0.07 ± 0.00	0.00 ± 0.00	-	AM 327	1.80 ± 0.02	0.05 ± 0.01	2.54
AM 282	3.35 ± 0.02	0.40 ± 0.02	12.16	AM 328	0.16 ± 0.02	0.00 ± 0.00	-
AM 283	0.00 ± 0.00	0.00 ± 0.00	-	AM 329	0.94 ± 0.03	0.07 ± 0.02	7.45
AM 284	0.06 ± 0.02	0.00 ± 0.00	-	AM 330	0.00 ± 0.00	0.00 ± 0.00	-
AM 285	0.84 ± 0.01	0.18 ± 0.02	21.51	AM 331	0.00 ± 0.00	0.00 ± 0.00	-
AM 286	0.09 ± 0.01	0.00 ± 0.00	-	AM 332	0.57 ± 0.02	0.04 ± 0.01	7.54
AM 287	0.54 ± 0.01	0.08 ± 0.00	15.02	AM 333	0.05 ± 0.01	0.00 ± 0.00	-
AM 288	0.00 ± 0.00	0.00 ± 0.00	-	AM 334	0.18 ± 0.02	0.00 ± 0.00	-
AM 289	0.33 ± 0.01	0.05 ± 0.00	15.03	AM 335	0.00 ±	0.00 ± 0.00	-
AM 290	0.00 ± 0.00	0.00 ± 0.00	-	AM 336	0.23 ± 0.02	0.00 ± 0.00	-
AM 291	0.00 ± 0.00	0.00 ± 0.00	-	AM 337	0.79 ± 0.04	0.03 ± 0.01	3.68
AM 292	0.06 ± 0.01	0.00 ± 0.00	-	AM 338	1.17 ± 0.02	0.31 ± 0.05	26.63
AM 293	1.01 ± 0.00	0.00 ± 0.00	-	AM 339	0.00 ± 0.00	0.00 ± 0.00	-
AM 294	0.07 ± 0.00	0.00 ± 0.00	-	AM 340	0.00 ± 0.00	0.00 ± 0.00	-
AM 295	0.07 ± 0.00	0.00 ± 0.00	-	AM 341	0.23 ± 0.01	0.00 ± 0.00	
AM 296	0.46 ± 0.00	0.03 ± 0.00	6.36	AM 342	0.61 ± 0.03	0.03 ± 0.00	4.96
AM 297	0.15 ± 0.00	0.00 ± 0.00	-	AM 343	0.04 ± 0.01	0.00 ± 0.00	-
AM 298	0.75 ± 0.02	0.08 ± 0.00	11.24	AM 344	0.82 ± 0.01	0.11 ± 0.02	13.33
AM 299	0.76 ± 0.02	0.15 ± 0.02	19.37	AM 345	1.04 ± 0.03	0.00 ± 0.00	-
AM 300	1.50 ± 0.02	0.23 ± 0.01	15.49	AM 346	0.17 ± 0.01	0.04 ± 0.01	24.23
AM 301	1.32 ± 0.02	0.08 ± 0.00	6.43	AM 347	1.64 ± 0.01	0.17 ± 0.01	10.31
AM 302	0.10 ± 0.00	0.03 ± 0.00	26.52	AM 348	1.05 ± 0.02	0.03 ± 0.01	3.02
AM 303	0.43 ± 0.01	0.03 ± 0.01	7.62	AM 349	0.02 ± 0.00	0.00 ± 0.00	-
AM 304	0.25 ± 0.01	0.02 ± 0.00	5.99	AM 350	0.11 ± 0.01	0.00 ± 0.00	8.58
AM 305	0.02 ± 0.00	0.00 ± 0.00	-	AM 351	0.00 ± 0.00	0.00 ± 0.00	-
AM 306	0.84 ± 0.01	0.00 ± 0.00	-	AM 352	1.64 ± 0.03	0.12 ± 0.02	7.35
AM 307	0.19 ± 0.01	0.05 ± 0.01	27.43	AM 353	0.13 ± 0.01	0.00 ± 0.00	-
AM 308	2.18 ± 0.03	0.11 ± 0.00	4.83	AM 354	0.06 ± 0.00	0.00 ± 0.00	-
AM 309	1.07 ± 0.01	0.04 ± 0.00	4.11	AM 355	1.07 ± 0.02	0.05 ± 0.01	4.60
AM 310	0.46 ± 0.01	0.01 ± 0.00	1.24	AM 356	0.28 ± 0.00	0.04 ± 0.00	14.71
AM 311	3.78 ± 0.07	0.05 ± 0.00	1.44	AM 357	12.43 ± 0.10	0.26 ± 0.00	2.06
AM 312	2.44 ± 0.02	0.07 ± 0.01	3.02	AM 358	0.06 ± 0.01	0.00 ± 0.00	-
AM 313	0.04 ± 0.01	0.00 ± 0.00	-	AM 359	0.07 ± 0.01	0.00 ± 0.00	-
AM 314	0.66 ± 0.00	0.09 ± 0.00	14.13	AM 360	0.00 ± 0.00	0.00 ± 0.00	-
AM 315	0.42 ± 0.01	0.04 ± 0.01	8.79	AM 361	0.70 ± 0.02	0.04 ± 0.01	6.38
AM 316	0.08 ± 0.00	0.01 ± 0.00	9.71	AM 362	3.47 ± 0.08	0.37 ± 0.04	10.63
AM 317	0.33 ± 0.00	0.09 ± 0.01	26.42	AM 363	0.03 ± 0.00	0.00 ± 0.00	-
AM 318	1.92 ± 0.02	0.11 ± 0.01	5.67	AM 364	1.67 ± 0.03	0.12 ± 0.02	7.30
AM 319	3.88 ± 0.02	0.37 ± 0.02	9.52	AM 365	0.14 ± 0.01	0.04 ± 0.01	25.12
AM 320	0.42 ± 0.01	0.02 ± 0.00	4.08	AM 366	1.71 ± 0.01	0.22 ± 0.01	12.61
AM 321	1.99 ± 0.02	0.18 ± 0.01	9.09	AM 367	0.14 ± 0.01	0.05 ± 0.01	36.59
AM 322	0.16 ± 0.00	0.02 ± 0.01	14.03	AM 368	1.35 ± 0.02	0.10 ± 0.02	7.13

Table 3.4. Continued

Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)	
AM 369	0.91 ± 0.04	0.07 ± 0.02	7.71
AM 370	0.03 ± 0.00	0.00 ± 0.00	-
AM 371	2.36 ± 0.05	0.08 ± 0.01	3.44
AM 372	1.01 ± 0.01	0.03 ± 0.00	3.24
AM 373	0.64 ± 0.01	0.05 ± 0.01	8.31
AM 374	1.24 ± 0.03	0.04 ± 0.01	3.61
AM 375	0.04 ± 0.00	0.00 ± 0.00	-
AM 376	0.14 ± 0.02	0.02 ± 0.00	12.52
AM 377	0.15 ± 0.00	0.00 ± 0.00	-
AM 378	0.00 ± 0.00	0.00 ± 0.00	-
AM 379	1.53 ± 0.07	0.04 ± 0.00	2.36
AM 380	0.77 ± 0.04	0.00 ± 0.00	
AM 381	0.03 ± 0.00	0.00 ± 0.00	-
AM 382	0.80 ± 0.06	0.02 ± 0.00	3.01
AM 383	0.64 ± 0.02	0.02 ± 0.00	3.89
AM 384	0.04 ± 0.00	0.00 ± 0.00	-
AM 385	0.02 ± 0.00	0.00 ± 0.00	-
AM 386	0.05 ± 0.00	0.00 ± 0.00	-
AM 387	0.04 ± 0.00	0.00 ± 0.00	-
AM 388	0.43 ± 0.02	0.04 ± 0.01	9.89
AM 389	0.49 ± 0.00	0.05 ± 0.01	10.64
AM 390	0.72 ± 0.01	0.05 ± 0.02	7.55
AM 391	0.22 ± 0.01	0.04 ± 0.01	17.73
AM 392	0.37 ± 0.01	0.04 ± 0.01	11.26
AM 393	0.23 ± 0.02	0.05 ± 0.01	20.40
AM 394	0.08 ± 0.02	0.00 ± 0.00	-
AM 395	0.05 ± 0.00	0.00 ± 0.00	-
AM 396	0.07 ± 0.01	0.00 ± 0.00	-
AM 397	1.39 ± 0.05	0.03 ± 0.00	2.10
AM 398	0.42 ± 0.02	0.03 ± 0.01	6.89
AM 399	0.22 ± 0.00	0.00 ± 0.00	
AM 400	0.54 ± 0.02	0.03 ± 0.00	5.28
AM 401	0.00 ± 0.00	0.00 ± 0.00	-
AM 402	0.17 ± 0.01	0.00 ± 0.00	9.61
AM 403	9.05 ± 0.07	0.16 ± 0.02	1.81
AM 404	0.96 ± 0.04	0.06 ± 0.01	5.94
AM 405	0.17 ± 0.04	0.00 ± 0.00	-
AM 406	2.25 ± 0.04	0.06 ± 0.01	2.60
AM 407	1.10 ± 0.01	0.13 ± 0.05	12.02
AM 408	1.22 ± 0.06	0.06 ± 0.01	4.53
AM 409	0.21 ± 0.01	0.03 ± 0.00	13.74
AM 410	0.97 ± 0.02	0.00 ± 0.00	-
AM 411	0.72 ± 0.04	0.00 ± 0.00	-
AM 412	0.29 ± 0.03	0.00 ± 0.00	-
AM 413	1.02 ± 0.03	0.04 ± 0.02	4.21
AM 414	1.13 ± 0.05	0.03 ± 0.00	2.59
AM 415	0.37 ± 0.02	0.00 ± 0.00	-
AM 416	0.38 ± 0.03	0.00 ± 0.00	-
AM 417	0.03 ± 0.00	0.00 ± 0.00	-
AM 418	0.00 ± 0.00	0.00 ± 0.00	-
AM 419	0.48 ± 0.03	0.04 ± 0.00	8.63
AM 420	1.41 ± 0.02	0.00 ± 0.00	-
AM 421	0.00 ± 0.00	0.00 ± 0.00	-
AM 422	0.67 ± 0.05	0.05 ± 0.01	7.29
AM 423	0.00 ± 0.00	0.00 ± 0.00	-
AM 424	1.65 ± 0.09	0.06 ± 0.02	3.60
AM 425	0.26 ± 0.01	0.02 ± 0.00	5.85
AM 426	0.05 ± 0.00	0.00 ± 0.00	-
AM 427	0.12 ± 0.01	0.00 ± 0.00	-
AM 428	0.64 ± 0.02	0.06 ± 0.00	9.10
AM 429	0.00 ± 0.00	0.00 ± 0.00	-
AM 430	0.07 ± 0.00	0.00 ± 0.00	-
AM 431	0.34 ± 0.02	0.04 ± 0.01	11.16
AM 432	0.00 ± 0.00	0.00 ± 0.00	-
AM 433	0.00 ± 0.00	0.00 ± 0.00	-
AM 434	0.55 ± 0.03	0.00 ± 0.00	-
AM 435	1.99 ± 0.02	0.07 ± 0.02	3.65
AM 436	0.40 ± 0.02	0.00 ± 0.00	-
AM 437	0.00 ± 0.00	0.00 ± 0.00	-
AM 438	0.05 ± 0.00	0.00 ± 0.00	-
AM 439	0.24 ± 0.00	0.00 ± 0.00	-
AM 440	0.05 ± 0.00	0.00 ± 0.00	-
AM 441	0.00 ± 0.00	0.00 ± 0.00	-
AM 442	0.07 ± 0.01	0.00 ± 0.00	-
AM 443	0.26 ± 0.01	0.00 ± 0.00	-
AM 444	0.12 ± 0.01	0.00 ± 0.00	-
AM 445	0.00 ± 0.00	0.00 ± 0.00	-
AM 446	0.83 ± 0.03	0.00 ± 0.00	-
AM 447	0.09 ± 0.01	0.00 ± 0.00	-
AM 448	0.10 ± 0.00	0.00 ± 0.00	-
AM 449	0.07 ± 0.01	0.00 ± 0.00	-
AM 450	1.00 ± 0.03	0.03 ± 0.00	-
AM 451	0.72 ± 0.03	0.00 ± 0.00	-
AM 452	0.00 ± 0.00	0.00 ± 0.00	-
AM 453	0.00 ± 0.00	0.00 ± 0.00	-
AM 454	1.04 ± 0.04	0.00 ± 0.00	-
AM 455	0.15 ± 0.00	0.00 ± 0.00	-

Table 3.5: Screening of fungal strains isolated from aquatic fresh water ecosystem for GBGL production

Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)	
A 001	2.59 ± 0.07	0.11 ± 0.00	4.38
A 002	0.44 ± 0.00	0.10 ± 0.01	21.62
A 003	0.87 ± 0.02	0.04 ± 0.01	4.86
A 004	0.34 ± 0.01	0.09 ± 0.02	25.82
A 005	0.27 ± 0.02	0.05 ± 0.01	19.18
A 006	2.08 ± 0.06	0.04 ± 0.00	1.73
A 007	2.01 ± 0.14	0.07 ± 0.02	3.60
A 008	2.36 ± 0.06	0.05 ± 0.00	2.27
A 009	2.15 ± 0.04	0.06 ± 0.00	2.89
A 010	0.82 ± 0.05	0.13 ± 0.03	15.84
A 011	2.21 ± 0.08	0.18 ± 0.04	8.14
A 012	0.00 ± 0.00	0.00 ± 0.00	-
A 013	0.82 ± 0.03	0.02 ± 0.00	2.07
A 014	0.61 ± 0.03	0.08 ± 0.01	12.47
A 015	2.09 ± 0.14	0.18 ± 0.02	8.63
A 016	1.69 ± 0.03	0.15 ± 0.01	8.68
A 017	0.81 ± 0.07	0.00 ± 0.00	-
A 018	0.00 ± 0.00	0.00 ± 0.00	-
A 019	0.31 ± 0.04	0.00 ± 0.00	-
A 020	0.83 ± 0.09	0.06 ± 0.00	7.58
A 021	0.14 ± 0.02	0.00 ± 0.00	-
A 022	0.00 ± 0.00	0.00 ± 0.00	-
A 023	6.73 ± 0.04	0.96 ± 0.04	14.27
A 024	0.00 ± 0.00	0.00 ± 0.00	-
A 025	0.67 ± 0.06	0.00 ± 0.00	-
A 026	0.79 ± 0.03	0.00 ± 0.00	-
A 027	0.00 ± 0.00	0.00 ± 0.00	-
A 028	1.26 ± 0.04	0.15 ± 0.02	11.92
A 029	0.23 ± 0.03	0.00 ± 0.00	-
A 030	0.17 ± 0.03	0.00 ± 0.00	-
A 031	0.00 ± 0.00	0.00 ± 0.00	-
A 032	0.00 ± 0.00	0.00 ± 0.00	-
A 033	0.95 ± 0.04	0.00 ± 0.00	-
A 034	0.00 ± 0.00	0.00 ± 0.00	-
A 035	0.17 ± 0.03	0.00 ± 0.00	-
A 036	0.00 ± 0.00	0.00 ± 0.00	-
A 037	0.04 ± 0.00	0.00 ± 0.00	-
A 038	0.00 ± 0.00	0.00 ± 0.00	-
A 039	0.00 ± 0.00	0.00 ± 0.00	-
A 040	0.64 ± 0.01	0.00 ± 0.00	-
A 041	4.72 ± 0.07	0.06 ± 0.01	1.28
A 042	0.96 ± 0.05	0.10 ± 0.01	10.69
A 043	1.31 ± 0.05	0.08 ± 0.00	5.95
A 044	0.13 ± 0.01	0.07 ± 0.01	52.82
A 045	2.54 ± 0.03	0.10 ± 0.01	3.92
A 046	0.00 ± 0.00	0.00 ± 0.00	-
A 047	1.57 ± 0.01	0.19 ± 0.01	12.00
A 048	0.00 ± 0.00	0.00 ± 0.00	-
A 049	0.83 ± 0.03	0.09 ± 0.02	10.88
A 050	0.02 ± 0.00	0.00 ± 0.00	-

Table 3.6: Screening of fungal strains isolated from brackish waters for GBGL production

Strain	Enzyme activity U/ml		Activity retention %	Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)			(absence of glucose)	(In presence of 0.5 M glucose)	
SA 001	1.45 ± 0.04	0.06 ± 0.01	4.33	SA 047	0.08 ± 0.01	0.00 ± 0.00	-
SA 002	0.23 ± 0.02	0.06 ± 0.00	27.33	SA 048	0.12 ± 0.01	0.00 ± 0.00	-
SA 003	0.90 ± 0.05	0.00 ± 0.00	-	SA 049	0.02 ± 0.00	0.00 ± 0.00	-
SA 004	0.00 ± 0.00	0.00 ± 0.00	-	SA 050	0.06 ± 0.01	0.00 ± 0.00	-
SA 005	0.00 ± 0.00	0.00 ± 0.00	-	SA 051	0.26 ± 0.01	0.00 ± 0.00	-
SA 006	0.11 ± 0.01	0.00 ± 0.00	-	SA 052	0.00 ± 0.00	0.00 ± 0.00	-
SA 007	0.00 ± 0.00	0.00 ± 0.00	-	SA 053	0.10 ± 0.00	0.00 ± 0.00	-
SA 008	0.04 ± 0.04	0.00 ± 0.00	-	SA 054	0.60 ± 0.01	0.00 ± 0.00	-
SA 009	0.00 ± 0.00	0.00 ± 0.00	-	SA 055	0.08 ± 0.00	0.00 ± 0.00	-
SA 010	0.00 ± 0.00	0.00 ± 0.00	-	SA 056	0.75 ± 0.03	0.11 ± 0.01	15.19
SA 011	0.03 ± 0.00	0.00 ± 0.00	-	SA 057	0.04 ± 0.00	0.00 ± 0.00	-
SA 012	0.08 ± 0.00	0.00 ± 0.00	-	SA 058	1.45 ± 0.03	0.16 ± 0.01	11.27
SA 013	0.02 ± 0.00	0.00 ± 0.00	-	SA 059	0.33 ± 0.02	0.03 ± 0.00	9.11
SA 014	0.00 ± 0.00	0.00 ± 0.00	-	SA 060	0.68 ± 0.01	0.00 ± 0.00	-
SA 015	0.00 ± 0.00	0.00 ± 0.00	-	SA 061	0.60 ± 0.03	0.00 ± 0.00	-
SA 016	2.14 ± 0.06	0.00 ± 0.00	-	SA 062	0.47 ± 0.01	0.00 ± 0.00	-
SA 017	0.00 ± 0.00	0.00 ± 0.00	-	SA 063	0.68 ± 0.02	0.05 ± 0.01	6.85
SA 018	0.00 ± 0.00	0.00 ± 0.00	-	SA 064	0.38 ± 0.01	0.06 ± 0.01	16.01
SA 019	0.00 ± 0.00	0.00 ± 0.00	-	SA 065	0.13 ± 0.01	0.00 ± 0.00	-
SA 020	0.00 ± 0.00	0.00 ± 0.00	-	SA 066	0.50 ± 0.01	0.03 ± 0.00	5.44
SA 021	0.00 ± 0.00	0.00 ± 0.00	-	SA 067	0.56 ± 0.02	0.11 ± 0.02	19.06
SA 022	0.00 ± 0.00	0.00 ± 0.00	-	SA 068	1.64 ± 0.02	0.13 ± 0.01	8.00
SA 023	0.00 ± 0.00	0.00 ± 0.00	-	SA 069	1.28 ± 0.01	0.08 ± 0.01	5.87
SA 024	0.00 ± 0.00	0.00 ± 0.00	-	SA 070	0.04 ± 0.01	0.00 ± 0.00	28.90
SA 025	0.00 ± 0.00	0.00 ± 0.00	-	SA 071	6.72 ± 0.03	0.06 ± 0.01	0.83
SA 026	2.06 ± 0.03	0.13 ± 0.02	6.33	SA 072	1.61 ± 0.03	0.13 ± 0.00	8.32
SA 027	0.00 ± 0.00	0.00 ± 0.00	-	SA 073	0.00 ± 0.00	0.00 ± 0.00	-
SA 028	0.00 ± 0.00	0.00 ± 0.00	-	SA 074	0.00 ± 0.00	0.00 ± 0.00	-
SA 029	0.00 ± 0.00	0.00 ± 0.00	-	SA 075	0.10 ± 0.02	0.00 ± 0.00	-
SA 030	0.00 ± 0.00	0.00 ± 0.00	-	SA 076	0.76 ± 0.02	0.08 ± 0.01	10.03
SA 031	0.00 ± 0.00	0.00 ± 0.00	-	SA 077	0.02 ± 0.00	0.00 ± 0.00	-
SA 032	0.00 ± 0.00	0.00 ± 0.00	-	SA 078	0.45 ± 0.00	0.06 ± 0.01	14.06
SA 033	0.00 ± 0.00	0.00 ± 0.00	-	SA 079	1.15 ± 0.01	0.08 ± 0.00	7.36
SA 034	0.00 ± 0.00	0.00 ± 0.00	-	SA 080	1.17 ± 0.02	0.08 ± 0.01	6.66
SA 035	0.00 ± 0.00	0.00 ± 0.00	-	SA 081	0.00 ± 0.00	0.00 ± 0.00	-
SA 036	0.00 ± 0.00	0.00 ± 0.00	-	SA 082	0.17 ± 0.01	0.00 ± 0.00	-
SA 037	0.00 ± 0.00	0.00 ± 0.00	-	SA 083	0.00 ± 0.00	0.00 ± 0.00	-
SA 038	0.00 ± 0.00	0.00 ± 0.00	-	SA 084	0.00 ± 0.00	0.00 ± 0.00	-
SA 039	0.09 ± 0.01	0.00 ± 0.00	-	SA 085	0.11 ± 0.01	0.00 ± 0.00	-
SA 040	1.61 ± 0.03	0.05 ± 0.01	3.22	SA 086	0.11 ± 0.01	0.00 ± 0.00	-
SA 041	0.00 ± 0.00	0.00 ± 0.00	-	SA 087	0.02 ± 0.00	0.00 ± 0.00	-
SA 042	0.01 ± 0.00	0.00 ± 0.00	-	SA 088	0.16 ± 0.01	0.04 ± 0.00	27.22
SA 043	0.00 ± 0.00	0.00 ± 0.00	-	SA 089	0.04 ± 0.00	0.00 ± 0.00	-
SA 044	0.00 ± 0.00	0.00 ± 0.00	-	SA 090	0.26 ± 0.00	0.04 ± 0.00	15.32
SA 045	0.07 ± 0.00	0.03 ± 0.00	43.12	SA 091	0.05 ± 0.01	0.00 ± 0.00	-
SA 046	1.34 ± 0.08	0.04 ± 0.00	2.62	SA 092	0.01 ± 0.00	0.00 ± 0.00	-

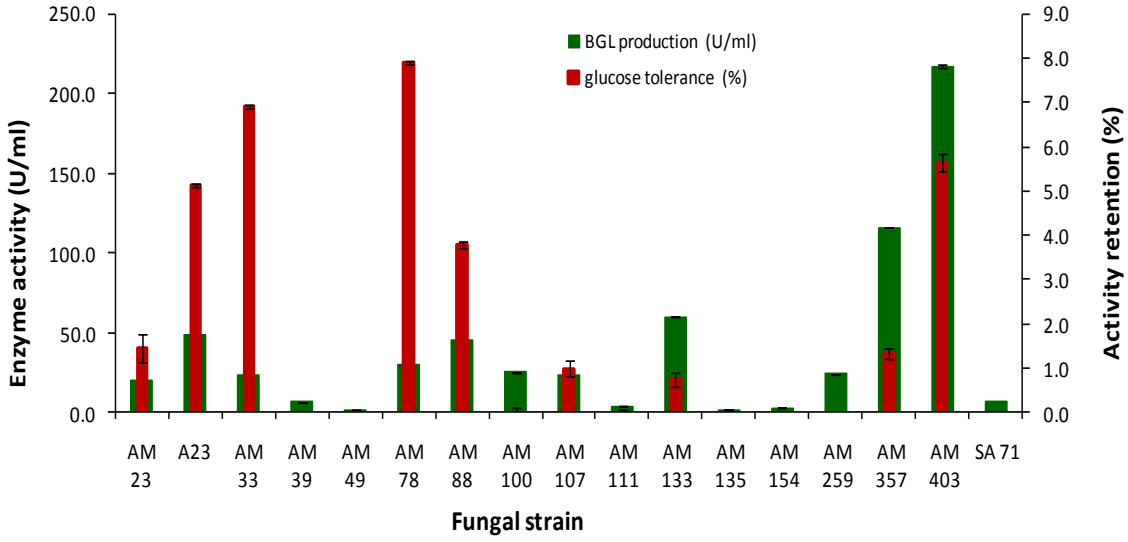
Table 3.6 Continued

Strain	Enzyme activity U/ml		Activity retention %
	(absence of glucose)	(In presence of 0.5 M glucose)	
SA 093	0.21 ± 0.00	0.02 ± 0.00	9.47
SA 094	0.00 ± 0.00	0.00 ± 0.00	-
SA 095	0.18 ± 0.01	0.02 ± 0.00	10.17
SA 096	0.00 ± 0.00	0.00 ± 0.00	-
SA 097	0.03 ± 0.00	0.00 ± 0.00	-
SA 098	0.83 ± 0.01	0.07 ± 0.01	8.42
SA 099	0.00 ± 0.00	0.00 ± 0.00	-
SA 100	0.32 ± 0.04	0.00 ± 0.00	-
SA 101	0.25 ± 0.01	0.00 ± 0.00	
SA 102	0.04 ± 0.00	0.00 ± 0.00	-
SA 103	0.75 ± 0.01	0.03 ± 0.00	4.33

3.3.3. Secondary screening of fungi

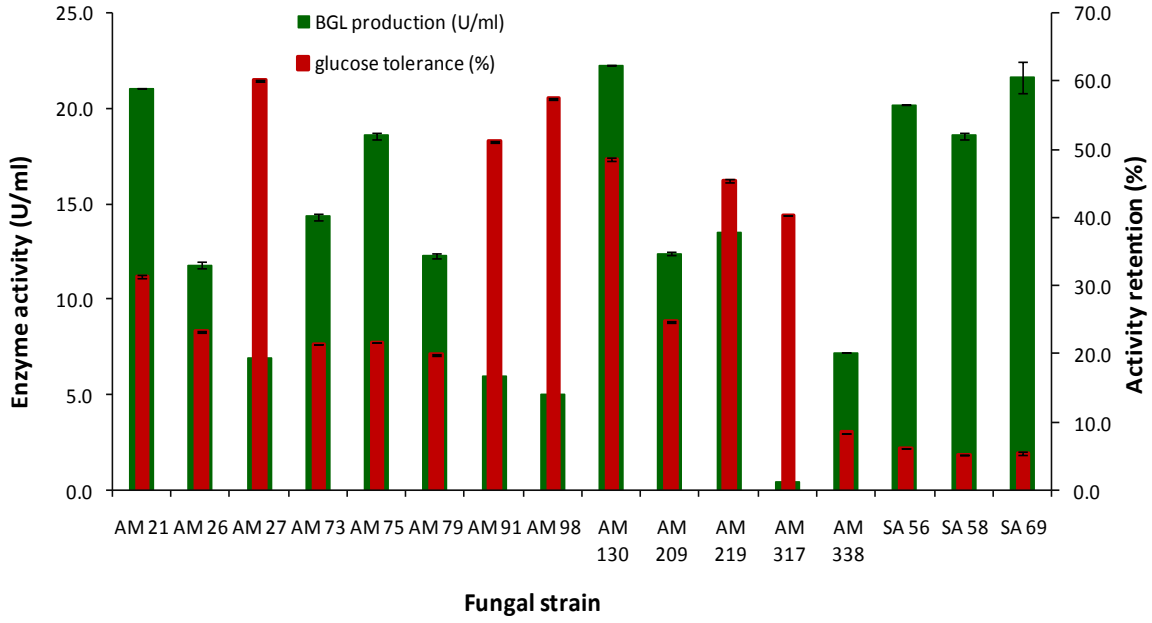
Among the 17 strains of fungi screened for high titers of BGL, AM 403 showed the maximum β-glucosidase production of 217 U/ml (Fig 3.2). However, this strain showed activity retention of only 5.65 % in 1.0 M glucose. All the fungal isolates evaluated for high BGL production showed less than 8 % activity retention in 1.0 M glucose.

Figure 3.2: Fungal strains with high titers of BGL production



Among the fungi tested for GBGL production, strain AM 130 was found to secrete 22.2 U/ml BGL, with activity retention of 48.5 % in 1.0 M glucose (Fig 3.3). Maximum activity retention was noted with BGL from AM 27 (60 %), but production of BGL was only 6.95 U/ml.

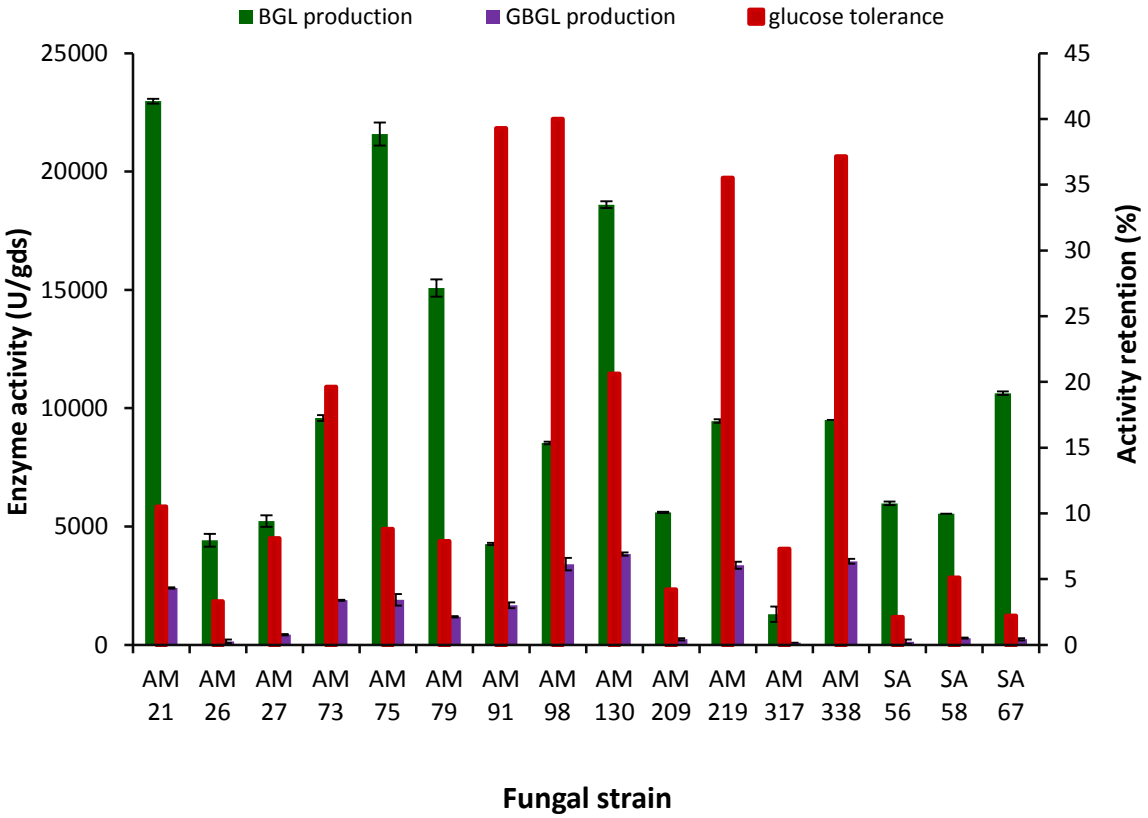
Figure 3.3: Fungal strains with high titers of GBGL production



3.3.4. Solid state fermentation yield of BGL from selected fungal strains

All the isolates that produced glucose tolerant BGL were tested for their ability for BGL production under SSF. Of the total 16 isolates tested, isolates AM 21, AM 75, AM 79 and AM 130 produced high titers of BGL $\geq$ 15000 U/gds. Strains AM 73, AM 91, AM 98, AM 130, AM 219 and AM 338 showed activity retention above 20 % (Figure 3.4).

Figure 3.4: BGL production in SSF by fungal strains

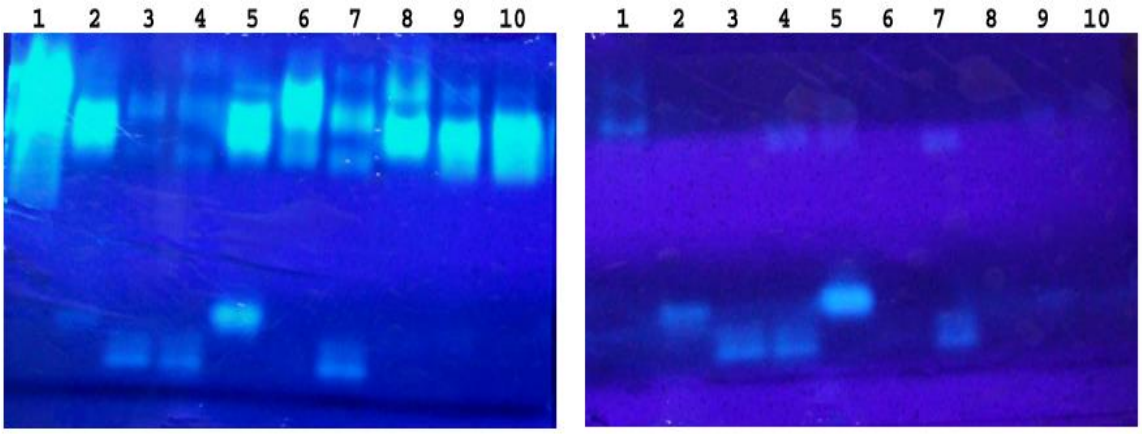


3.3.5. Native PAGE and zymogram analysis of BGL

The acetone precipitated BGL of selected strains (SSF extract of GBGL producers) on Native PAGE and Zymogram analysis revealed multiplicity of BGL. A number of isoforms were noted in these fungi, each strain producing isoforms that varied in molecular size and glucose tolerance (Fig 3.5). It was observed that in general, the low molecular weight β-glucosidase isoforms in the isolates were the ones which exibited glucose tolerance. Among the isolates, the isolate AM 130 (NII 0930) which showed highest BGL activity along with high glucose tolerance showed a low molecular weight band of β-glucosidase acitivity (Lane 5) with high glucose tolerance.

Figure 3.5: Native PAGE and zymogram analysis of acetone precipitated BGL

A. Zymogram in absence of glucose B. Zymogram in presence of 0.5 M glucose



Lane information

Lane 1 AM 27	Lane 2 AM 75	Lane 3 AM 91	Lane 4 AM 98	Lane 5 AM 130
Lane 6 AM 26	Lane 7 AM 219	Lane 8 AM 338	Lane 9 AM 79	Lane 10 AM 73

Beta-glucosidase multiplicity is a well known feature, especially in filamentous fungi. In *Penicillium aurantiogriseum* two isoforms of β-glucosidase were identified (Petruccioli et al., 1999). In *Aspergillus aculeatus*, three isoforms were reported showing similar native molecular mass of 132, 133 and 136 kDa (Murao et al., 1988). Three isoforms were also reported in *Botrytis cinerea* (Gueguen et al., 1995) and *Aspergillus niger* (Abdel-Naby et al., 1999). Four isoforms were detected in *Aspergillus terreus*, *Aspergillus caespitosus*; three isoforms in *Humicola insolens*, *Humicola fuscoatra*, *Chaetomium thermophilum*; two in *Emericella nidulans* var. *lata, Absidia corymbifera*, *Penicillium lagena,* (Sonia et al., 2008). In *Aspergillus tubingensis*, four isoforms of β-glucosidase were reported showing high diversity with respect to pH and temperature optima, temperature stability, substrate specificity and glucose tolerance. In *Paecilomyces* sp., a single BGL was reported which was a trimer with subunit molecular mass of 102 kDa (Yan et al., 2008). Absence of BGL multiplicity was also reported from *Aspergillus pulverulentus* (Mase et al., 2004), *Penicillium purpurogenum* (Jeya et al., 2010), *Sclerotinia sclerotiorum* (Kalifa et al., 2007), *Alternaria alternata* (Martínez et al., 1988) and *Aspergillus fumigatus* (Kitpreechavanich et al., 1986).

Of the four isoforms of β-glucosidase in *Aspergillus tubingensis*, BGL I and BGL II were high molecular weight proteins showing low tolerance to glucose. BGL III

and BGL IV had a similar mass of 54 kDa but were highly glucose tolerant (Decker et al., 2001). Among the two isoforms of β-glucosidase from *Aspergillus oryzae*, BGL I had a high molecular mass and was strongly inhibited by glucose. BGL II had a low molecular weight but was glucose tolerant (Günata and Vallier, 1999). Low molecular weight β-glucosidase from *Candida peltata* had high glucose tolerance (Saha and Bothast, 1996). In *Aspergillus oryzae*, major isoform had a molecular weight of 130 kDa and was highly inhibited by glucose. The other isoform though produced in lower quantities, with a molecular mass of 43 kDa was highly resistant to glucose inhibition (Riou et al., 1998). Beta-glucosidase from *Scytalidium thermophilum* had a stimulatory effect with glucose and this glucose tolerant enzyme had a low molecular mass of 40 kDa (Zanoelo et al., 2004). Similar results were obtained in the present study with lower band of β-glucosidase in fungal strains showing high levels of glucose tolerance.

3.3.6. Selection of potential strain

Among the screened fungal isolates, strain AM 130 was found to be better in terms of total BGL production and glucose tolerance. The strain showed a production of 22.24 U/ml BGL activity in secondary screening with activity retention of 48.5 % in 1.0 M glucose. In solid state fermentation, a high production of 18,600 U/gds was obtained, even before any optimization, and the crude enzyme preparation showed activity retention of 20.6 % in presence of 1.0 M glucose. The strain secreted a low molecular weight isoform showing high levels of glucose tolerance. Hence strain AM 130 was selected for further studies. The culture was deposited in NII culture collection (CSIR-NIIST) as NII 0930.

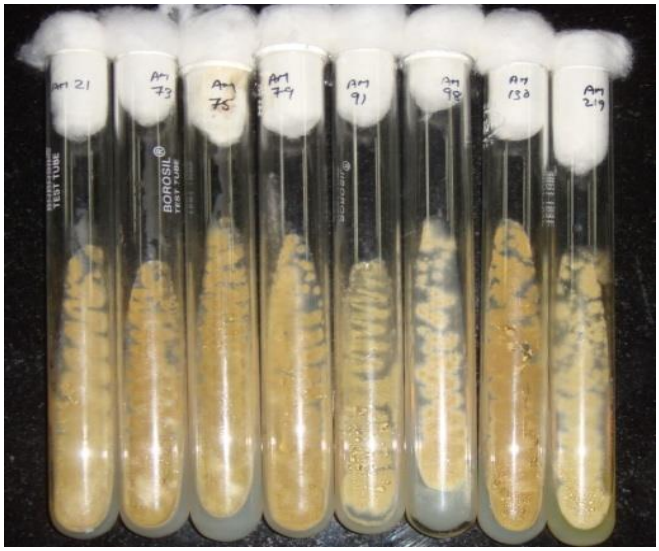
3.3.7. Identification of fungal strains

Of the 16 strains of GBGL producers selected based on a screening of 608 fungal isolates, 8 were similar in their morphological features especially in the colony color in PDA plates. AM 130 was one among the 8 strains and the others included AM 21, AM 73, AM 75, AM 79, AM 91, AM 98 and AM 219. These 8 isolates were identified by morphological and molecular techniques.

3.3.7.1 Morphological identification

In all the 8 selected isolates, the colony appeared as white mycelium, turning to yellow with conidia formation (Fig 3.6). The colonies were fast growing, flat spreading and powdery in appearance. The vegetative body consisted of branched and septate mycelium. Asexual reproduction was by conidia formation, conidia arising from erect branched conidiophores. Each conidiophore was branched twice or thrice terminating into a group of bottle shaped phialides. The phialides had inflated base, tapering into a long neck. Each phialide produced a chain of elliptical conidia in centripetal manner. The conidia were light yellow in color and later became dark yellow-brown. The asexual fructifications were very similar to that of *Penicillium* but the conidia were yellow in colour. Based on the vegetative and asexual reproductive morphology all the 8 isolates were identified as strains of *Paecilomyces*. The strains could be grouped into two sections, based on morphological similarities. Section A with AM 21, AM 73, AM 75, AM 79, AM 130 and section B with AM 91, AM 98 and AM 219. Similar morphology was reported for *Paecilomyces variotii*, isolated as heat resistant fungal taxa. It showed yellowish brown colonies with yellow pigments beneath the colony in Czapek-yeast extract agar and malt extract agar media. The colonies became patchy and granular with age and produced abundant spores. Under microscope, the phialides were flask shaped or tapering gradually to the apices (Amaeze et al., 2010).

Figure 3.6*:* Isolates identified as *Paecilomyces*



The strain AM 130 was studied further with respect to its growth and morphology. The mycelial growth was limited and sporulation occurred on the 3rd day of spore inoculation in PDA plates. The colony appeared to be yellowish but turning to yellowish brown with increase in incubation time (Fig 3.7). The conidial spores in chains and were found to be elliptical in shape (Fig 3.8). Sexual reproduction was noticed in this strain with antheridia formation (Fig 3.9) and ascospores. Since sexual reproduction is evident, strain AM 130 was identified as the telomorph of *Paecilomyces*, which is *Byssochlamys*.

Figure 3.7: Fungal colony of AM 130 (NII 0930) in PDA plate

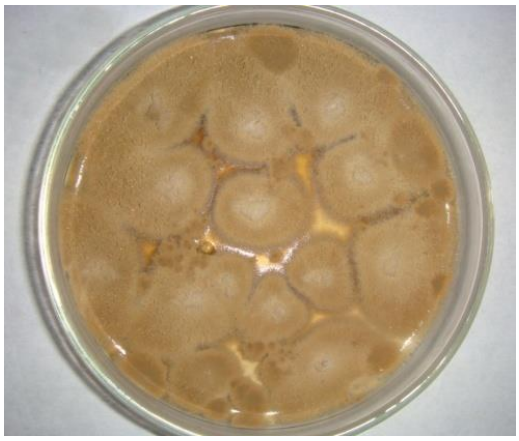


Figure 3.8: Conidiophore with conidia in AM 130
(Magnification 450X)

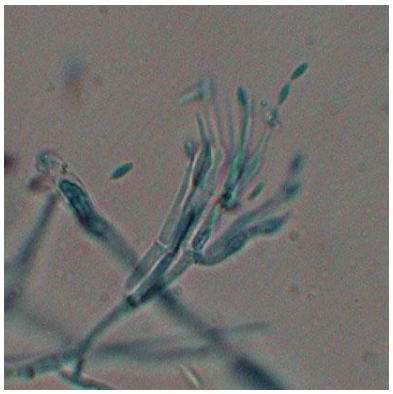
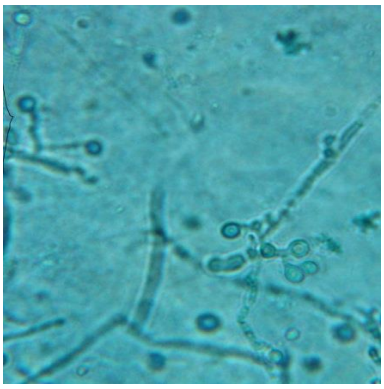


Figure 3.9: Antheridia formation in AM 130
(Magnification 450X)

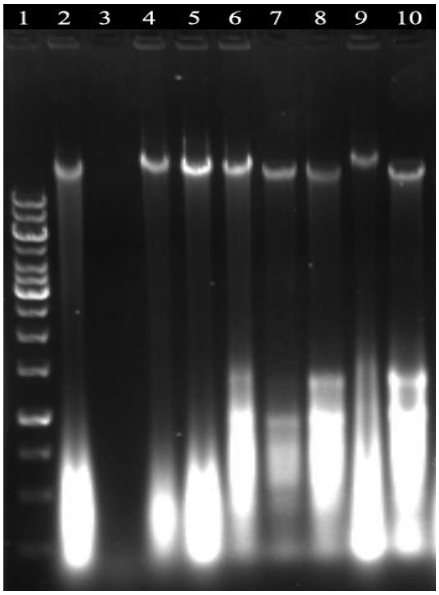


3.3.7.2. Molecular identification

3.3.7.2.1 Chromosomal DNA isolation

DNA preparation from the 8 strains was successful and high molecular weight chromosomal DNA was obtained (Figure 3.10). The DNA isolated was suspended in 10 mM Tris EDTA buffer (pH 8.0) and was used as template for PCR reactions.

Figure 3.10: Chromosomal DNA isolation from fungal strains



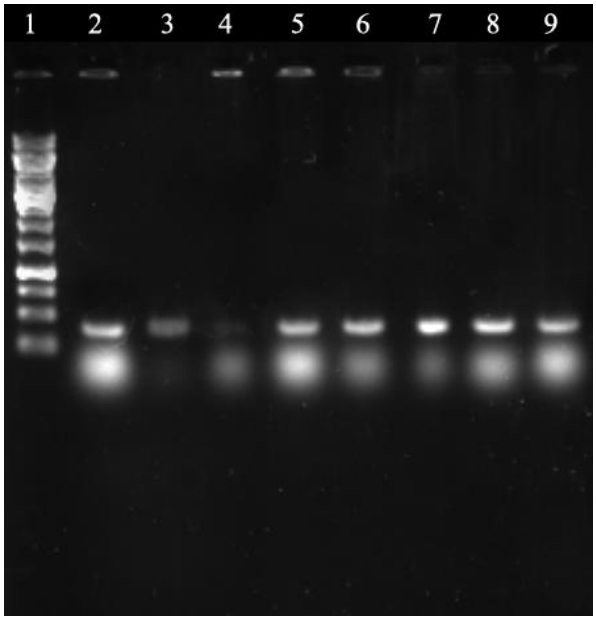
Lane Information

Lane 1 - GeneRuler ® 1kb ladder (Fermentas)
Lane 2 - AM 130
Lane 4 - AM 73
Lane 5 - AM 75
Lane 6 - AM 79
Lane 7 - AM 91
Lane 8 - AM 98
Lane 9 - AM 21
Lane 10 - AM 219

3.3.7.2.2. PCR amplification of 18S rRNA gene

PCR amplification of 18S rRNA gene was performed for the 8 fungal strains. Figure 3.11 shows the amplicons obtained with $P_{45}F$ and $P_{47}R$ primer pair. The PCR products were sequenced using the forward primer.

Figure 3.11: PCR amplification of 18S rRNA gene



Lane Information

Lane 1 - GeneRuler ® 1kb ladder (Fermentas)
Lane 2 – AM 21
Lane 3 – AM 73
Lane 4 – AM 75
Lane 5 – AM 79
Lane 6 – AM 91
Lane 7 – AM 98
Lane 8 – AM 130
Lane 9 – AM 219

3.3.7.2.3. Sequence of PCR amplicons and BLAST results

Sequences of amplicons generated for 18S rRNA gene were subjected to BLAST analysis. Sequences from different fungal strains and the BLAST analysis are given below

1. **Strain AM 21**

The nucleotide sequence obtained was 375 bp as given below

```
>AM 21
AAATCCTGTCTAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGCT
CATTAAATCAGTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAATA
CATGCTGAAAACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTT
GGTGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTAT
CAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGG
AAAGGGAGCCCTGAACAAAC
```

BLAST analysis indicated that the sequence had 99 % identity with 94 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13428 (AB 023948.1) and *Byssochlamys fulva* gene for 18S rRNA (AB 023941.1).

2. **Strain AM 73**

The nucleotide sequence obtained was 395 bp as given below

>AM 73
TTGCCAGATTGTTCTAATATTAAGCCATGCATGTCTAAATAAAAACAATCTATACGGTGAAACTGCGAATG
GCTCATTAAATCACTTAAACAAATTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGC
TAATACATGCTGAAAACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGC
TCCTTGGTGATTCATAATAACTTAACAAAATCACATGGCCTTGCGCCGGCAATGGTTCATTCAAATTTCTG
CCCTATCAACTTTCAATGGTAGGATAATGGCCTAACCATGGGTGGCAACGGGTAAACGGGGAAATTTAAGG
TTCGAATTCCCAGAACAAAGGGAACCCTTGAAAAAAACTT

The sequence had 93 % identity with 88 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13428 (AB 023948.1) and *Byssochlamys fulva* gene for 18S rRNA (AB 023941.1)

3**. Strain AM 75**

The nucleotide sequence obtained was 573 bp as given below

>AM 75
ACAGCGACTGTATTCAGATAGCCATGCATGTCTAAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGC
TCATTAAATCAGTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAAT
ACATGCTGAAAACCCCGACTTCGGAAGGGGTGTATTTATTAAATAAAAAACCAATGCCCTTCGGGAGCTGC
TTGGTGATTCATTAATAACTTTAACGAAATCGCATTGGACACATTGACGTCCGGACGAAATGGTTTTCATA
TCAAAACTTTTTCATACCTCTTAATACATACATTATCAAATGGGGTTAAGAAAATATAAAGAGAAGATTAA
ACCAATTATGTTGTGTCTTTTCAGCGGTGTCAAAACGGCAGAGGATTTTTAAAAGAAGCGTAGGAAAAATA
TAAAGCTTTTCGAAAAAAAGGGGATGATATTCCGATAAAATTCATATGAAAAGTACCAAAAAATGGCGATT
TTATAGTAGAACGTAGGGAGGGAAACGGAACAAACAAAGAAAAAAAGTGATTGGGATTCGGGATATAGGAA
GTAGT

BLAST analysis indicated the sequence to have 97 % identity with 39 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13428 (AB 023948.1) and *Byssochlamys fulva* gene for 18S rRNA (AB 023941.1).

4. **Strain AM 79**

The nucleotide sequence obtained was 390 bp as given below

>AM 79
CTAAAGTATAAGCAATCTTTACGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGT
ACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAATACATGCTGAAAACCCCGACTTCGGAAGGGGT
GTATTTATTAAATAAAAAACCAATGCCCTTCGGGAGCTGCTTGGTGATTCATTAATAACTTTAACGAAATC
GCATTGGACACATTGACGTCCGGACGAAATGGTTTACATATCAAAACTTTTTCATACCTCTTAATACATAC

ATTATCAAATGGGGTTAAGAAAATATAAAGAGAAGATTAAACCAATTATGTTGTGTCTTTTCAGCGGTGTC
AAAACGGCAGAGGATTTTTAAAAGAAGCGTAGGAA

BLAST analysis indicated that the sequence has 97 % identity with 54 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13428 (AB 023948.1) and *Byssochlamys fulva* gene for 18S rRNA (AB 023941.1).

5. **Strain AM 91**

The nucleotide sequence obtained was 368 bp as given below

>AM 91
TAAATTATGTTTAGATAGCCATGCATGTCTAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGCTCAT
TAAATCACTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAATACAT
GCTGAAAACCTCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGT
GATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAA
CTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGA
GGGAGCCTGAAAA

The sequence has 99 % identity with 95 % coverage to *Paecilomyces variotii* gene for 18S rRNA, (AB 023946.1) and *Penicilliopsis clavariiformis* gene for 18S rRNA (AB 003945.1).

6. **Strain AM 98**

The nucleotide sequence obtained was 368 bp as given below

>AM 98
AAAGAATTGTCCAGATAGCCATGCATGTCTAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGCTCAT
TAAATCAGTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAATACAT
GCTGAAAACCTCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGGT
GATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCAA
CTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGA
GGGAGCCTGAAAA

BLAST analysis indicated that the sequence has 99 % identity with 95 % coverage to *Paecilomyces variotii* gene for 18S rRNA, (AB 023946.1) and *Penicilliopsis clavariiformis* gene for 18S rRNA (AB 003945.1).

7. **Strain AM 130**

The nucleotide sequence obtained was 369 bp as given below

>AM 130
CAATGGCTGTTCAGATTAGCCATGCATGTCTAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGCTCA
TTAAATCAGTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAATACA

TGCTGAAAACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCTTGG
TGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCA
ACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAG
AGGGAGCCTGAAAA

BLAST analysis indicated the sequence to have 99 % identity with 96 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13428 (AB 023948.1) and *Byssochlamys fulva* gene for 18S rRNA (AB 023941.1). *Paecilomyces* is an anamorph representing asexual phase. Since the fungus showed sexual reproduction, strain AM 130 was identified as *Byssochlamys fulva.*

8. **Strain AM 219**

The nucleotide sequence obtained was 380 bp as given below

>AM 219
AAATGGATTTTCCTAAGATTAGCCATGCATGTCTAAGTATAAGCAATCTATACGGTGAAACTGCGAATGGC
TCATTAAATCAGTTATCGTTTATTTGATAGTACCTTGCTACATGGATACCTGTGGTAATTCTAGAGCTAAT
ACATGCTGAAAACCTCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTCCT
TGGTGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTA
TCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAAATTAGGGTTTCAATTC
CGGAAGAGGGAAGCCCTGGAAAAAA

BLAST analysis indicated that the sequence has 99 % identity with 92 % coverage to *Paecilomyces variotii* gene for 18S rRNA, strain: IAM 13426 (AB 023946.1) and *Penicilliopsis clavariiformis* gene for 18S rRNA (AB 003945.1).

3.3.8. Systematic position of strain AM 130 (NII 0930)

Kingdom	-	Mycota
Division	-	Eumycota
Subdivision	-	Ascomycotina
Class	-	Ascomycetes
Subclass	-	Plectomycetes
Order	-	Eurotiales
Family	-	Eurotiaceae
Genus	-	*Byssochlamys* Westling
Species	-	*Byssochlamys fulva* Olliver & G. Sm.
Anamorph	-	*Paecilomyces* Bainier

Blast analysis also supported the classification of the 8 strains into two sections, section A with AM 21, AM 73, AM 75, AM 79, AM 130 showing similarities with *Paecilomyces* and *Byssochlamys* and section B with AM 91, AM 98 and AM 219 showing similarities to *Paecilomyces* and *Penicilliopsis.*

The hyphomycete genus *Paecilomyces* was established by Bainier for *Paecilomyces variotii* characterized by verticillate conidiospores with divergent whorls of phialides with cylindrical or inflated base, tapering into a long neck. They are similar to *Penicillium* but differ in the absence of green colored colonies and short cylindrical phialides (Samson, 1974). The genus was revised by several authors, and at present over 40 species has been recognized under *Paecilomyces*. However, the genus *Paecilomyces* is polyphyletic across two subclasses - Sordariomycetidae and Eurotiomycetidae (Luangsa-ard et al., 2004). Within the order Eurotiales, *Paecilomyces* is monophyletic, characterized by *Byssochlamys* telomorph. The genus *Byssochlamys* is characterized by naked ascomata with globose asci and elliptical ascospores. Antheridium is swollen and ascogonium is coiled. All *Byssochlamys* have anamorphs in *Paecilomyces* which may be mesophilic, thermophilic or thermotolerant (Samson et al., 2009).

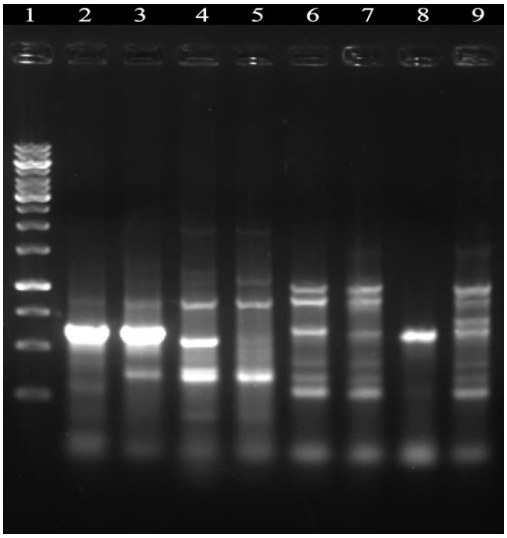
Byssochlamys is considered as the major fungal taxa involved in spoilage of heat processed food. They are capable of degrading processed fruits even under reduced oxygen tension (Amaeze et al., 2010). *Byssochlamys* species produce ascospores that are highly resistant to heat that can survive over temperatures above 85 °C for long duration (Samson et al., 2009). *B. nivea* and *B. zollerniae* were isolated from bottled *Raphia* palm wine and could survive pasteurization at 80 °C for 20 min and 85 °C for 15 min (Eziashi et al., 2010). *B. fulva* was found to degrade canned fruits without gas formation, flavor change and color change (Olliver and Rendle, 1934).

3.3.9. PCR amplification of ITS region

The ITS region is one of the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). It has higher degree of variation than

other genic regions of rDNA. ITS amplification revealed differences among the 8 *Paecilomyces* strains (Fig 3.12). The amplicons generated clearly indicated that the eight strains of *Paecilomyces* isolated in the present study differ from each other. However some similarities did exist between the strains. Banding pattern showed some similarities between AM 21, AM 73 and AM 130. Also pattern of amplification is similar in AM 75 and AM 79. Similarly AM 91, AM 98 and AM 219 are also similar. ITS analyses were employed to investigate molecular variations within samples of *Paecilomyces* isolates and to identify atypical isolates resembling *P. fumosorosus* (Azevedo et al., 2000).

Figure 3.12: PCR amplification of ITS region



Lane information

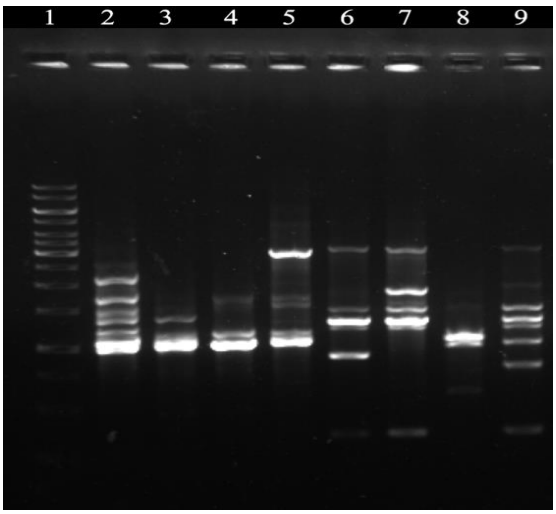
Lane 1 - GeneRuler ® 1kb ladder (Fermentas)
Lane 2 – AM 21
Lane 3 – AM 73
Lane 4 – AM 75
Lane 5 – AM 79
Lane 6 – AM 91
Lane 7 – AM 98
Lane 8 – AM 130
Lane 9 – AM 219

3.3.10. RAPD analysis

RAPD is a choice of detecting genetic polymorphism where no prior knowledge of DNA sequence is required. The genomic DNA is amplified using a single arbitrary decamer primer and the pattern of amplicons generated can be compared. By comparing the pattern of amplicons, the genetic diversity within species level can be detected. In the present study, all the 8 strains of *Paecilomyces* differed in their RAPD banding pattern with primer OPX 06 (Fig 3.13). Cluster analyses based on banding pattern revealed variations among the strains (Fig 3.14). The study further supported the classification of the strains into two sections. The strains could be clearly grouped into two clades, one with strains AM 21, AM 73, AM 75, AM 79, AM 130 and the other with AM 91, AM 98

and AM 219. Study on genetic variability and phylogenetic relations among isolates of entomopathogenic strains of *Paecilomyces* indicated monophyly and the analysis was found to be useful for intraspecific and interspecific relations among entomopathogenic fungi (Oborník et al., 2000). RAPD analysis in *Monascus* strains revealed unique RAPD pattern for each strain that can help in strain level identification (Shinzato et al., 2009). Patulin producing strains of *Paecilomyces* showed identical RAPD pattern. This can be used in detecting patulin production potential among *Paecilomyces variotii* isolates (Bokhari et al., 2009).

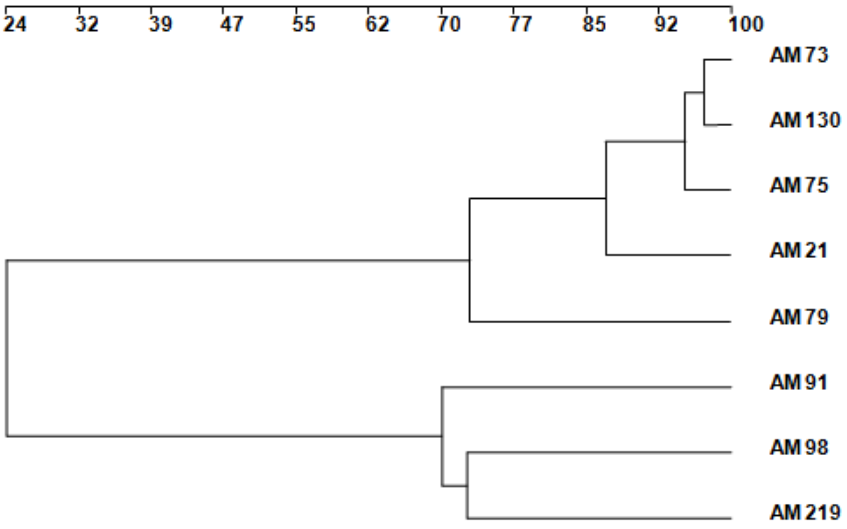
Figure 3.13: RAPD analysis of fungal strains



Lane information

Lane 1 - GeneRuler®1kb ladder (Fermentas)
Lane 2 – AM 21
Lane 3 – AM 73
Lane 4 – AM 75
Lane 5 – AM 79
Lane 6 – AM 91
Lane 7 – AM 98
Lane 8 – AM 130
Lane 9 – AM 219

Figure 3.14: Cluster analysis of strains based on RAPD banding pattern



3.3.11. Paecilomyces as producers of glucose tolerant β-glucosidases

Of the 16 strains screened for production of glucose tolerant β-glucosidase, 8 were identified as *Paecilomyces* by morphological and molecular techniques. By morphological analysis, the strains could be grouped into two sections- section A with AM 21, AM 73, AM 75, AM 79, AM 130 and section B with AM 91, AM 98 and AM 219. Section B grouping was also supported by similar banding pattern of BGL isoforms, especially the lower molecular weight isoform in zymogram analysis (Fig 3.5). The classification of *Paecilomyces* strains into the two sections was supported further by the similarities revealed by BLAST analysis of amplified regions of 18S rRNA gene. Strains in section A showed similarities to *Byssochlamys fulva*, while strains in section B had similarities to *Penicilliopsis clavariiformis* in addition to *Paecilomyces*. Genetic diversity among strains was revealed through RAPD analysis and by the banding patterns of ITS amplicons. These studies clearly indicate that the eight strains of *Paecilomyces* isolated in the present study were diverse.

Sixteen strains were selected from 608 fungal isolates and eight out of the 16 were found to be *Paecilomyces variotii*. This indicates the possibility of *Paecilomyces variotii* to be a glucose tolerant β-glucosidase producing taxa. Reports from literature also suggest *Paecilomyces* to be efficient producers of BGL. A highly glucose tolerant β-glucosidase has been reported from *Paecilomyces variotii* and process parameters were optimized for BGL production in solid state fermentation (Job et al., 2010). *Paecilomyces variotii* was found to secrete β-glucosidases in borax treated saw dust and was thus able to colonize a resource otherwise inaccessible to many fungi due to the toxicity of borax (Parker et al., 1999). Immobilized mycelia of *Paecilomyces variotii* was used as a microbial bioassay system for determination of boric acid. Beta-glucosidase secretion by the fungi was inhibited by boric acid and thus changes in β-glucosidase concentration was used as an indicator for boric acid concentration (Ang et al., 2012). A novel ginsenoside hydrolyzing β-glucosidase was isolated from *Paecilomyces*. The enzyme was a trimer with subunit molecular mass of 102 kDa showing optimum activity at pH 3.5 and temperature 55 °C (Yan et al., 2008). *Paecilomyces thermophila* was found to secrete a β-glucosidase showing a moderate *Ki* value of 73 mM of glucose (Yang et al., 2008).

3.4. Conclusion

A total of 608 fungi were isolated in the present study of which 16 strains of were found to secrete high titers of glucose tolerant β-glucosidase. Based on production under SmF and SSF and also due to presence of a highly glucose tolerant isoform, strain AM 130 (NII 0930) was selected as a potent strain for further studies. Strain AM 130 was identified as *Byssochlamys fulva* based on morphological and molecular features. Eight strains (including AM 130) out of the 16 strains were found to be *Paecilomyces variotii* and their genetic diversity was established through ITS amplification and RAPD analysis. It is concluded that glucose tolerant β-glucosidase production could be a common character exhibited by *P. variotii*. This finding opens up the possibility of selective screening of fungal taxa for glucose tolerant β-glucosidases.

Chapter 4

Fermentative production of β-glucosidase and SSF reactor design

Chapter 4

Fermentative production of β-glucosidase and SSF reactor design

4.1. Introduction

Microorganisms obtain energy for support of biosynthesis and growth from their environment. In the industrial production of metabolites using microbes, all the nutritional requirements have 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 preferred for fungal fermentations as conditions similar to natural habitat can be provided. SSF has many advantages such as high fermentation productivity, higher concentration of products, lower catabolite repression, higher product stability, cultivation of microbes in water insoluble substrates, mixed cultivation of fungi and lower demand of sterility due to low water activity (Hölker et al., 2004).

Major challenge in scaling the SSF processes lies in developing suitable bioreactors for the fermentations, especially the ones requiring sterile operation and forced supply of oxygen/air. Compared to SmF, one of the major differences in the SSF system is the existence of a gas phase between the solid particles which, due to the poor thermal conductivity causes problems in heat transfer. Also oxygen transfer becomes a great challenge in SSF use to the limited possibilities for aeration and mixing (Durand, 2003). Important parameters that affect the design of SSF reactors include the morphology of organism used for fermentation (susceptibility to mechanical shear), necessity whether to have a sterile operation or not, nature and end use of the target

product etc., in addition to the above. Excellent reviews are available on SSF bioreactors detailing these as well as the reactor type's configurations and operational aspects in Ali and Zulkali (2011), Durand (2003) and Mitchel et al. (2006). Though different categories of reactors catering to different requirements have been described, continuously mixed and aerated reactors with heat exchange and those which are capable of sterile operation are limited both at laboratory and industrial scales. The current breed of SSF reactors are generally categorized into four major groups, Group I bioreactors where the bed is static or mixed very infrequently (e.g. Tray reactors), Group II reactors where bed is static but is aerated forcefully (e.g. Packed bed reactors), Group III reactors where the bed is continuously or intermittently mixed and air is circulated around the bed but not forcefully aerated through the bed (e.g. Rotating drum bioreactors) and Group IV reactors where the bed is agitated and also forcefully mixed, like the gas–solid fluidized bed reactors (Mitchel et al., 2006). The choice of a particular reactor type often depends on several parameters like the scale of operation, whether the organism is sensitive to shear, requirement of air, water activity of the substrate, rate of growth of the culture etc. Hence, though it is impossible to design a reactor which cater to all needs, it is still possible to design one with most of the critical parameters addressed at least in the laboratory or pilot scale.

Another important aspect in the large scale production of enzymes or any other microbial metabolite is the optimization of process parameters. The traditional approach to optimization is the 'one-factor at a time' method. In this process, all variables but one are held constant and optimum level of this variable is determined. Incorporating this optimum value, the second variable's optimum is found. This is continued for all the variables and the optimum value determined for each variable is used in the production. Though simple, this technique requires numerous sequential experimental runs, is time consuming and ineffective when numerous variables are to be optimized. The process fails if there is interaction between the variables.

An alternative and more efficient approach is the use of statistical methods. When several factors are dealt with, a factorial experiment is the most effective where factors are varied together instead of one at a time. As the number of factors increase, a

fractional factorial experiment can be considered in which only a subset of the runs are made. Plackett-Burman design (Plackett and Burman, 1946) is a common non-geometric design used to identify the most significant variables and is preferred when one needs to screen a large number of factors. In such situations a design that allows one to test the largest number of factor main effects with the least number of observations is desired. To enable this, the Plackett-Burman design has the interaction effects of variables confounded with new main effects. Here each main effect is partially aliased with every two factor interaction not involving itself. Because the added factors are created by equating (aliasing) the "new" factors with the interactions of a full factorial design, these designs always will have 2^k runs, e.g., 4, 8, 16, 32 and so on. Full factorial design is fractionalized in a different manner, to yield saturated designs where the number of runs is a multiple of 4, rather than a power of 2. Each variable in the design is tested at two levels, i.e., a high (+) and low (-) level. Response surface methodology or RSM is a collection of statistical techniques that are useful for modeling and analysis of problems in which a response is influenced by several variables and the objective is to optimize this response. Box-Behnken design (Box and Behnken, 1960) is a three-level spherical design for fitting response surfaces. The designs are formed by combining 2^k factorials with incomplete block designs (Montgomery, 2001). Each independent variable thus will be studied at three different levels [low (-), medium (0) and high (+1)].

The present chapter describes the time course study on β-glucosidase production in SmF using various carbon sources and statistical optimizations to enhance BGL production by *Byssochlamys fulva* in SmF and SSF. A SSF bioreactor was designed that could be used to generate a prototype, to be tested for production of enzymes

4.2. Materials and Methods

4.2.1. Time course study on β-glucosidase production under different carbon sources

Time dependent BGL and GBGL production in presence of specific carbohydrates were studied. Mandel and Weber medium with composition as given under section 2.2.2 was used with supplementation of one the carbon sources. The

carbohydrates used were cellulose, avicel, pectin, xylan, starch, inulin, glucose, galactose, fructose, rhamnose, arabinose, ribose, xylose, mannose, lactose, sucrose, cellobiose, maltose, melibiose, trehalose, raffinose, adonitol, inositol, dulcitol, mannitol, sorbitol, glycerol, ascorbic acid, D-galacturonic acid, salicin, rutin and quercetin. Fifty milliliters of medium was taken in 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121 °C, 15 lbs pressure for 15 min. Polysaccharides, rutin and quercetin were added to the Mandel and Weber media at 0.05 % w/v prior to autoclaving. Other sugars and sugar derivatives were filter sterilized through 0.22 µ syringe filter and added to autoclaved medium to a final concentration of 0.05 M. Enzyme production was carried out as outlined under section 2.3.2 and 2.0 ml of samples were withdrawn at intervals of 24 h. Enzyme extraction was done as explained in section 2.3.2. Enzyme assays were conducted to determine BGL activity in presence and absence of 1.0 M glucose as indicated in section 2.5.1.

4.2.2. Beta-glucosidase production in SmF with wheat bran as carbon source.

The effect of wheat bran on BGL and GBGL production in SmF was studied. Enzyme production was carried out as outlined under section 2.3.2 with 1 % w/v wheat bran as carbon source. Enzyme extraction was done as explained in section 2.3.2. Enzyme assays were conducted to determine the activity in presence and absence of 1.0 M glucose as indicated in section 2.5.1.

4.2.3. Optimization of the SmF production of β-glucosidase using Statistical Designs

Optimization of BGL and GBGL production by *B. fulva* NII 0930 was performed by a sequential two step method employing Fractional Factorial (Plackett-Burman) and Response Surface (Box-Behnken) experimental designs. A Placket and Burman (1946) design was used to identify the most critical parameters influencing BGL/GBGL production by the fungus whose levels were optimized using the Box-Behnken (1960) design.

4.2.3.1. Plackett-Burman design

Submerged fermentation production of BGL was done as outlined under section 2.3.2. A Plackett-Burman design (Plackett and Burman, 1946) consisting of 12 experiments was used to determine the relative significance of 9 factors in β-glucosidase production by the fungus and also of glucose tolerance (% activity retention in 1.0 M glucose) of the enzyme produced. Along with the nine factors, two dummy variables (D1, D2) were also used, that will give an estimate of variability and standard error of factor effects. Each run was carried out in triplicate and average values of response were taken. The complete experimental design is shown below (Table 4.1). The software - Design-Expert (Version 6.0.6, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design and data analysis.

Table 4.1: Plackett-Burman design for optimization of BGL/GBGL production under SmF

Run	**Wheat Bran (% w/v)**	**Time (d)**	**Agitation (rpm)**	**Sucrose (M)**	**Glycerol (M)**	**$(NH_4)_2SO_4$ (M)**	**pH**	**Temp (°C)**	**Tween 80 (% v/v)**	**D1**	**D2**
1	1	4	100	0.01	0.01	0.03	4	37	0.05	-1	1
2	3	8	250	0.00	0.01	0.03	4	37	0.01	-1	-1
3	1	4	250	0.01	0.01	0.01	8	37	0.01	+1	-1
4	3	8	100	0.01	0.00	0.01	4	37	0.05	+1	-1
5	3	8	100	0.01	0.01	0.01	8	32	0.01	-1	+1
6	3	4	250	0.01	0.00	0.03	4	32	0.01	+1	+1
7	1	8	250	0.00	0.01	0.01	4	32	0.05	+1	+1
8	1	4	100	0.00	0.00	0.01	4	32	0.01	-1	-1
9	1	8	250	0.01	0.00	0.03	8	32	0.05	-1	-1
10	3	4	100	0.00	0.01	0.03	8	32	0.05	+1	-1
11	1	8	100	0.00	0.00	0.03	8	37	0.01	+1	+1
12	3	4	250	0.00	0.00	0.01	8	37	0.05	-1	+1

Each variable was investigated at two levels, ie - a high level (+) and low level (-). The effect 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 and glucose tolerance) of trials at

which the parameter was at its higher and lower levels respectively and 'n' is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on BGL production and glucose tolerance. The most significant parameters affecting BGL production were identified.

4.2.3.2. Response surface design – (Box-Behnken design)

Response surface methodology using Box-Behnken design (Box and Behnken, 1960) was used to find the optimum levels of the significant variables identified by the Plackett-Burman design. The experimental design used is shown in Table 4.2.

Table 4.2: Box-Behnken design for optimization of BGL/GBGL production under SmF

Run #	Wheat bran (% w/v)	Agitation (rpm)	pH
1	6.0	250	6
2	3.0	300	5
3	4.5	200	4
4	4.5	250	5
5	4.5	250	5
6	4.5	200	6
7	3.0	250	4
8	3.0	250	6
9	4.5	250	5
10	4.5	250	5
11	4.5	300	4
12	3.0	200	5
13	6.0	300	5
14	6.0	250	4
15	4.5	250	5
16	6.0	200	5
17	4.5	300	6

The effect of parameter interactions on enzyme production and glucose tolerance were also determined. A total of 17 experiments were carried out for optimizing three parameters. Each independent variable was studied at three different levels (low, medium and high). The software Design-Expert (Version 6.0.6, Stat-Ease, Inc.,

Minneapolis, USA) was used for experimental design, data analysis and quadratic model building. Each run was performed in triplicate and the average β-glucosidase yield obtained was taken as response Y_1 and of glucose tolerance as response Y_2. The predicted values of the responses were obtained from quadratic model fitting techniques. A multiple regression analysis of the data was carried out to define the response in terms of the independent variables. Response surface graphs obtained were used to estimate the effect of variables individually and in combination, for determining their optimum levels for maximum β-glucosidase production and glucose tolerance.

4.2.4. Effect of moisture content of wheat bran for β-glucosidase production in SSF

Solid state fermentation using wheat bran was performed as outlined in section 2.2.4. To determine the optimum initial moisture for BGL and GBGL production, the moisture content in wheat bran was varied from 30-100 %. Enzyme production (6 days incubation) and extraction were carried out as per section 2.3.1. Enzyme assay in absence and presence of 1.0 M glucose were performed as outlined in section 2.5.1.

4.2.5. Time course study of β-glucosidase production in SSF

Solid state fermentation using wheat bran (60 % moisture) was performed as outlined in section 2.2.4. Enzyme production was carried out as outlined under section 2.3.1 and three flasks each were withdrawn at intervals of 6 h. Enzyme extraction was done as explained in section 2.3.1. Enzyme assays were conducted to determine the activity in presence and absence of 1.0 M glucose as indicated in section 2.5.1.

4.2.6. Statistical optimization of the SSF production of β-glucosidase by B. fulva

4.2.6.1. Plackett-Burman design

Using a Plackett-Burman design, 11 variables were screened in a total of 12 experiments to identify the most significant variables. Each variable was tested at two

levels i.e., a high (+) and low (-) level. The complete experimental design matrix is shown in Table 4.3.

Table 4.3: Plackett-Burman design for β-glucosidase production and glucose tolerance under SSF

Run #	Temp (°C)	pH of minimal media	Moisture (%)	Incubation (days)	Inoculum (Spore count)	Particle size (µm)	$(NH_4)_2SO_4$ (% w/v)	Peptone (% w/v)	Starch (% w/v)	Lactose (% w/v)	Tween 80 (% v/v)
1	35	4	60	4	1.00E+06	<425	0.1	0.5	0.0	0.01	0.10
2	35	7	80	4	1.00E+04	<425	0.1	0.0	0.2	0.01	0.05
3	30	4	60	8	1.00E+04	>600	0.1	0.0	0.2	0.01	0.10
4	30	7	80	8	1.00E+04	<425	0.0	0.5	0.0	0.01	0.10
5	30	7	80	4	1.00E+06	>600	0.1	0.0	0.0	0.00	0.10
6	35	7	60	4	1.00E+04	>600	0.0	0.5	0.2	0.00	0.10
7	35	4	80	8	1.00E+06	<425	0.0	0.0	0.2	0.00	0.10
8	30	4	80	4	1.00E+06	>600	0.0	0.5	0.2	0.01	0.05
9	35	7	60	8	1.00E+06	>600	0.0	0.0	0.0	0.01	0.05
10	30	4	60	4	1.00E+04	<425	0.0	0.0	0.0	0.00	0.05
11	30	7	60	8	1.00E+06	<425	0.1	0.5	0.2	0.00	0.05
12	35	4	80	8	1.00E+04	>600	0.1	0.5	0.0	0.00	0.05

Each run was carried out in triplicate and average value was taken as the response. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on BGL production and glucose tolerance. The most significant parameters affecting BGL production were identified.

4.2.6.2. Response surface design - (Box-Behnken design)

The significant variables for BGL production as identified using the Plackett-Burman experiments were further optimized using the response surface method. A Box-Behnken design was used to find the optimum levels of the significant variables - temperature, moisture, ammonium sulfate, incubation time and peptone concentration.

The interactive effect of these parameters on enzyme production and glucose tolerance were also evaluated. Each independent variable was studied at three different levels [low (-), medium (0) and high (+1)]. A total of 46 experiments were carried out with each run in triplicates. Average BGL yield was taken as response Y_1 and glucose tolerance of the enzyme was taken as response Y_2. The experimental design matrix used for the study along with the corresponding responses is shown in Table 4.4. The software Design-Expert (Version 8.0.2, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building. Multiple regression analysis of the data was carried out to define the responses in terms of the independent variables. Response surface graphs generated using the software was used to estimate the effect of variables individually and in combination, for determining their optimum levels for maximum BGL production and glucose tolerance.

Table 4.4: Box Behnken design for optimization of BGL/GBGL production under SSF

Run #	Temp (°C)	Moisture (%)	Incubation (days)	$(NH_4)_2SO_4$ (% w/v)	Peptone (% w/v)
1	32.0	75	5	0.03	0.05
2	37.0	75	5	0.03	0.05
3	32.0	85	5	0.03	0.05
4	37.0	85	5	0.03	0.05
5	34.5	80	4	0.00	0.05
6	34.5	80	6	0.00	0.05
7	34.5	80	4	0.05	0.05
8	34.5	80	6	0.05	0.05
9	34.5	75	5	0.03	0.00
10	34.5	85	5	0.03	0.00
11	34.5	75	5	0.03	0.10
12	34.5	85	5	0.03	0.10
13	32.0	80	4	0.03	0.05
14	37.0	80	4	0.03	0.05
15	32.0	80	6	0.03	0.05
16	37.0	80	6	0.03	0.05
17	34.5	80	5	0.00	0.00
18	34.5	80	5	0.05	0.00
19	34.5	80	5	0.00	0.10
20	34.5	80	5	0.05	0.10
21	34.5	80	5	0.03	0.05
22	34.5	80	5	0.03	0.05
23	34.5	80	5	0.03	0.05
24	34.5	75	4	0.03	0.05
25	34.5	85	4	0.03	0.05
26	34.5	75	6	0.03	0.05
27	34.5	85	6	0.03	0.05
28	32.0	80	5	0.00	0.05
29	37.0	80	5	0.00	0.05
30	32.0	80	5	0.05	0.05
31	37.0	80	5	0.05	0.05
32	34.5	80	4	0.03	0.00
33	34.5	80	6	0.03	0.00
34	34.5	80	4	0.03	0.10
35	34.5	80	6	0.03	0.10
36	32.0	80	5	0.03	0.00
37	37.0	80	5	0.03	0.00
38	32.0	80	5	0.03	0.10
39	37.0	80	5	0.03	0.10
40	34.5	75	5	0.00	0.05
41	34.5	85	5	0.00	0.05
42	34.5	75	5	0.05	0.05
43	34.5	85	5	0.05	0.05
44	34.5	80	5	0.03	0.05
45	34.5	80	5	0.03	0.05
46	34.5	80	5	0.03	0.05

4.2.7. Design of a semi-pilot scale reactor for solid state fermentation

Growth of *Byssochlamys fulva* was high under high aeration from the observations while cultivating this fungus under SSF. The fungus had septate mycelia and was able to grow better with intermittent agitation in flask cultures which were parameters conducive for cultivation in SSF bioreactors which are aerated and mixed (Durand, 2003). Since there are very few SSF reactors described in literature and none available in the market, it was decided to design an SSF bioreactor that might be suitable for cultivation of such fungi that are highly aerobic and respond well to cultivation in an agitated reactor under solid state cultivation. The following criteria were adopted for the design of the reactor.

1. Reactor should allow for sterilization and sterile operation.
2. Mixing of substrate should be possible either in continuous mode or intermittently.
3. It should be possible to supply the culture with air/oxygen even while operation and continuously.
4. Reactor should allow for aseptic transfer of SSF medium and inoculum into it.
5. It should be possible to remove metabolic heat by conduction.
6. Reactor should allow for easy process monitoring.
7. Reactor should be easily disassembled for cleaning.
8. Reactor design should be able to cater to a laboratory scale or a semi pilot scale.

The design was rendered using Autodesk Inventor 2010® Educational Version (www.autodesk.com).

4.3. Results and discussion

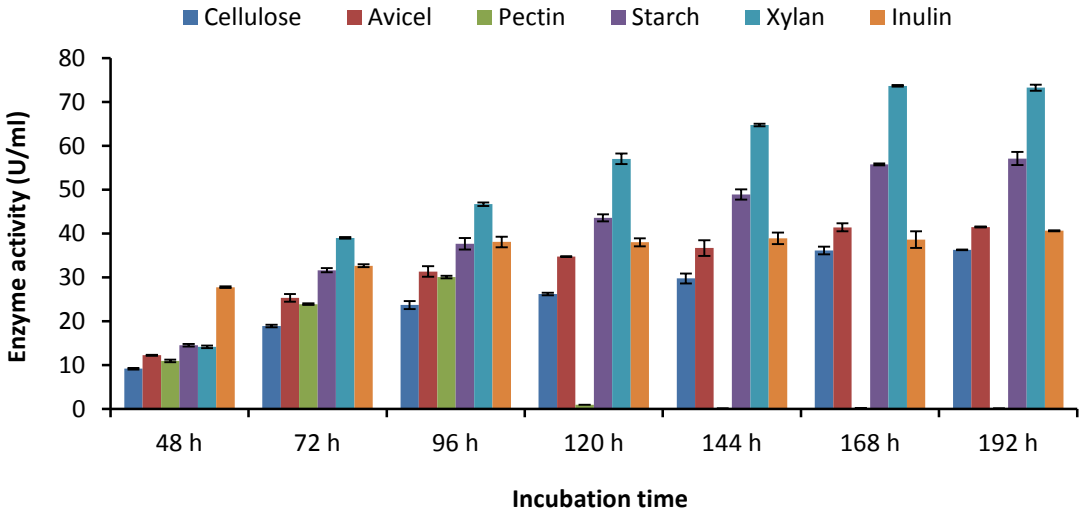
4.3.1. Time course studies on β-glucosidase production using different carbon sources

4.3.1.1. Polysaccharides as carbon source

Fungal strains vary in their nutritional requirements and hence show specificities to carbon source for BGL production. BGL production was noticed even at 48 h of incubation (Fig 4.1). Polysaccharides cannot enter the mycelium and hence extracellular enzymes has to be secreted which degrade the polysaccharides into simple sugars. These simple sugars are assimilated by the hyphae for growth and metabolite production. The extracellular enzymes may be conidia associated or may be secreted after germination of spores. At 48 h incubation, among the polysaccharides tested for BGL production, inulin supported maximum BGL production. In the induction/repression studies conducted with mycelium, inulin had a significant effect on β-glucosidase production by the fungus (section 5.3., chapter 5). Inulin is a complex polymer of fructose and glucose based oligosaccharides. In rats, administration of inulin resulted in increased β-glucosidase activity in gut indicating the stimulatory effect of inulin in β-glucosidase secretion by gut micro flora (Rowland et al., 1998). However after 72 h of incubation, xylan was found to support maximum BGL production. Starch was found to be a good carbon source and surpassed the effective role of inulin with 120 h of incubation. No further production of enzyme was noted with pectin after an incubation period of 120 h possibly due to exhaustion of carbon source. In an earlier study, pectin was found to be the best carbon source for β-glucosidase production by *Aspergillus* sp. (Elyas et al., 2010). In *Volvariella volvacea*, among the polysaccharides tested, cellulosic carbon sources at 1 % level supported maximum BGL production (Cai et al., 1998). In *Penicillium funiculosum*, maximum β-glucosidase production with avicel as carbon source was obtained at 192 h of incubation (Castro et al., 2010). In *Humicola grisea* var. *thermoidea*, avicel as carbon source supported maximum BGL production at 20 h of incubation (Nascimento et al., 2010). In the present study, non-cellulosic carbon sources were better in BGL production compared to cellulose and avicel. The role of non-

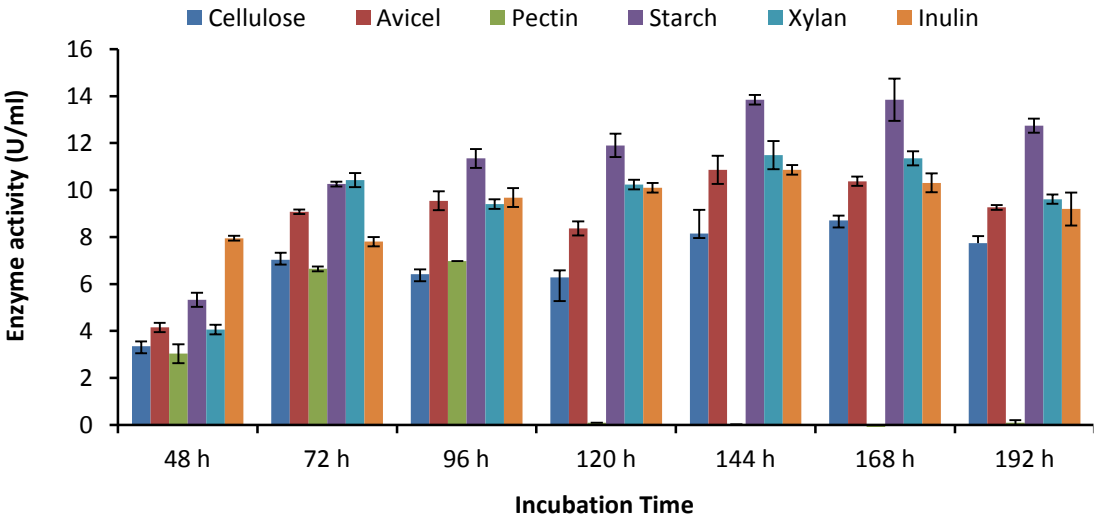
cellulosic polysaccharides in β-glucosidase production depends on the extracellular enzyme produced by the fungus. The production could be due to the increase in biomass in the case of constitutive β-glucosidase or due to the simple sugars (act as inducers) generated by hydrolysis in the case of inducible enzymes.

Figure 4.1: BGL production by *B. fulva* with polysaccharides as carbon source



For GBGL production, at 48 h of incubation, inulin was most effective as a carbon source (Fig 4.2).

Figure 4.2: GBGL production by *B. fulva* with polysaccharides as carbon source

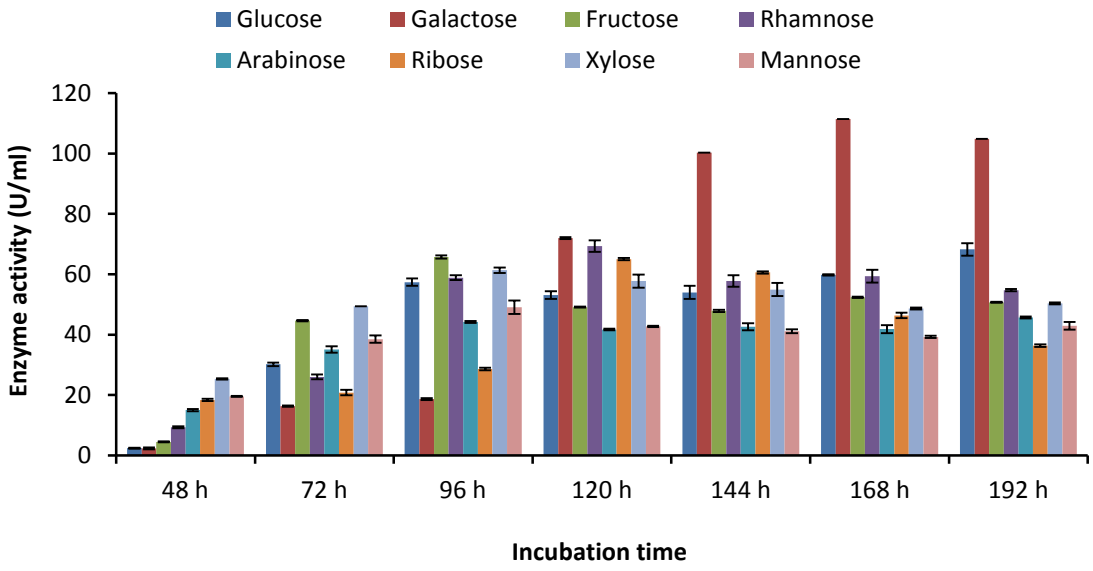


In contrast to BGL production, cellulosic substrates had significant effect in GBGL production. For GBGL production, starch was found to be the best carbon source. Xylan, avicel, inulin and cellulose had similar effect on GBGL production with 192 h of incubation.

4.3.1.2. Monosaccharides as carbon source

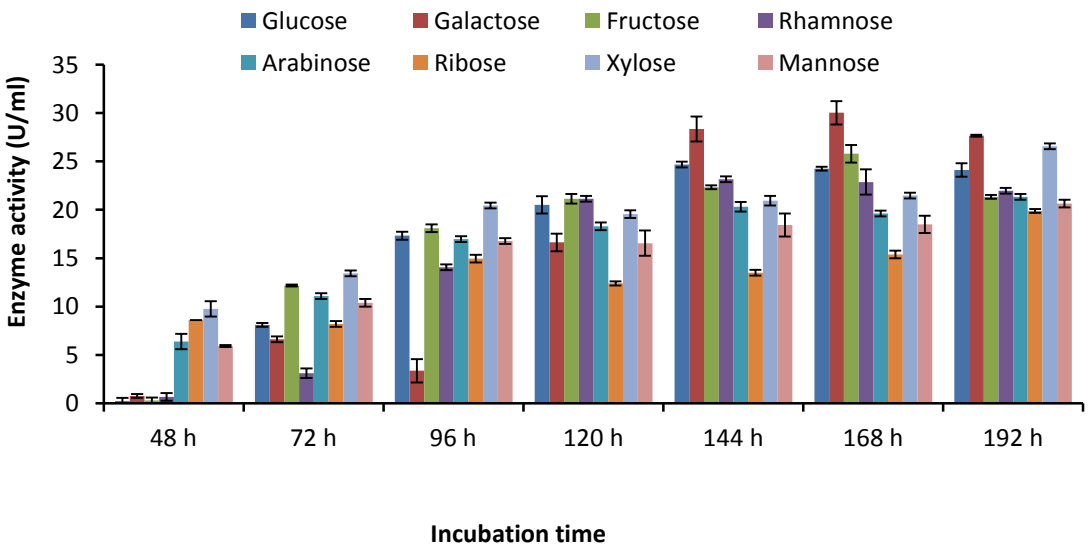
Compared to polysaccharides, monosaccharides were more effective in BGL production. Monosaccharides are readily available sugars which can easily enter the mycelium. They can thus support faster mycelial growth and production of primary metabolites. At 48 h of incubation, xylose was the best carbon source for BGL production, while glucose and galactose had minimal effect (Fig 4.3.). The effect of sugars changed with incubation period, fructose having highest effect on production at 72 h of incubation. However with increase in incubation period, galactose became the best carbon source for BGL production followed by glucose. Maximum production of 111.4 U/ml was noticed at 168 h of incubation in galactose containing medium. Glucose was found to enhance BGL production from 72 h of incubation

Figure 4.3: BGL production by *B. fulva* with monosaccharides as carbon source



In *Candida peltata*, BGL production was studied using various substrates at an incubation period of 4 days. Glucose supplementation from 1-12 % showed no significant reduction in production, maximum production was noted with 4 % glucose (Saha and Bothast, 1996). In a time course study from 0-120 h, glucose had an inhibitory effect on cell associated BGL production in *Volvariella volvacea* (Cai et al., 1998).

Figure 4.4: GBGL production by *B. fulva* with monosaccharides as carbon source

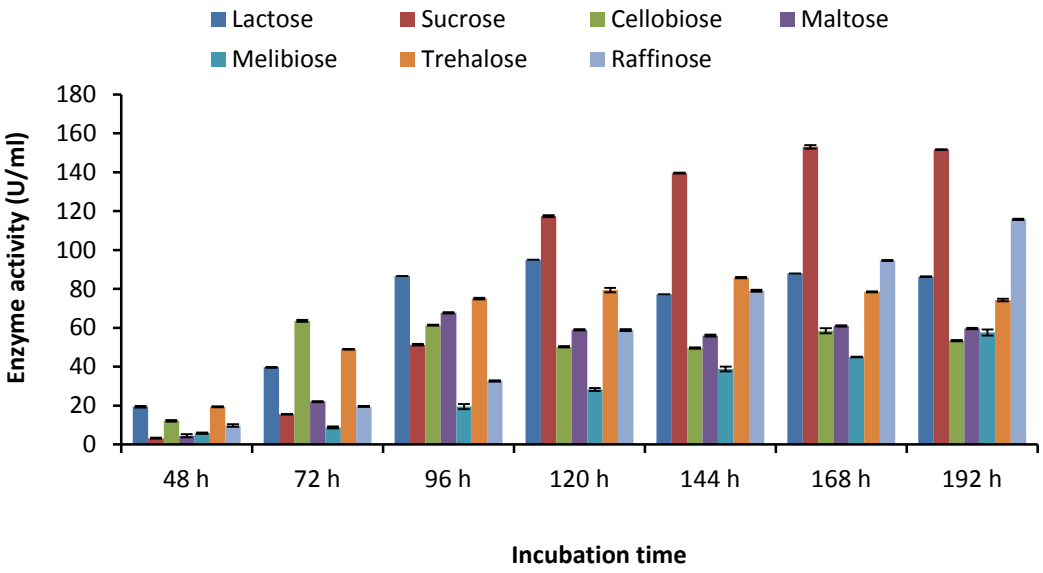


Galactose had less effect in GBGL production in the initial periods of incubation. But from 144 h onwards, galactose was the best carbon source for GBGL production (Fig 4.4). Glucose was found to support GBGL production from 72 h of incubation.

4.3.1.3. Oligosaccharides as carbon source

Among the disaccharides and trisaccharides tested as carbon source, BGL production was highest in sucrose containing flasks (Fig 4.5). A BGL production of 153.1 U/ml was recorded at 168 h of incubation with sucrose as carbon source. In *Penicillium purpurogenum*, among the various sugars and sugar derivatives tested for β-glucosidase production, sucrose was found to be the best carbon source, the optimized concentration was calculated as 1 % (Dhake and Patil, 2005). In the present study, melibiose as carbon source showed least production of BGL.

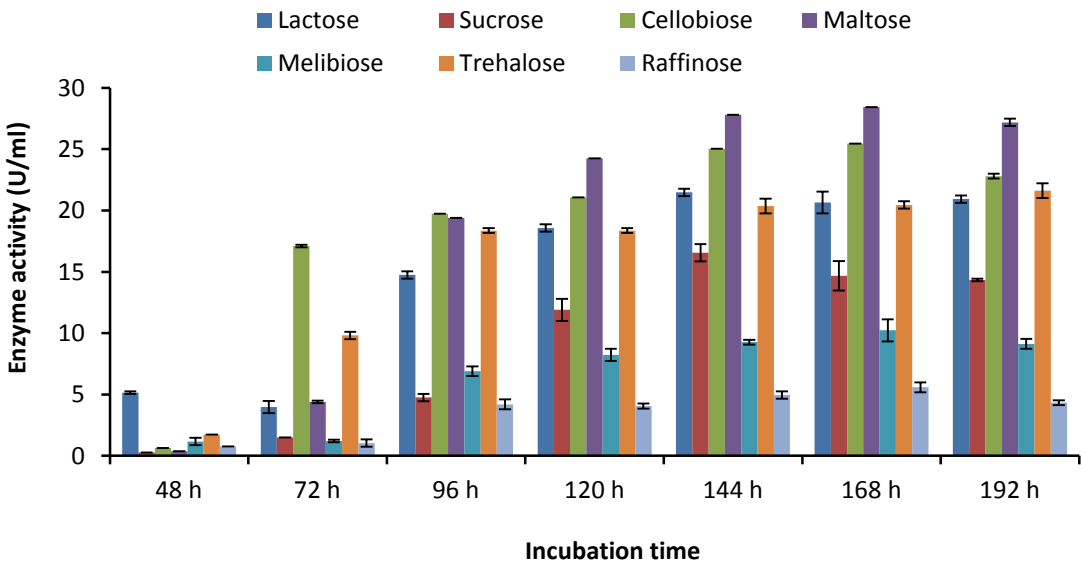
Figure 4.5: BGL production by *B. fulva* with oligosaccharides as carbon source



Cellobiose, a known inducer of BGL had significant effect at 72 h of incubation, thereafter a decrease in production was noticed with increase in incubation period. At 96 h, lactose was the best carbon source. In *Volvariella volvacea*, lactose showed varying effect in cell associated BGL production. At 48 h, increase in production was exhibited, followed by a decrease in 72 h, increase in 84 h, decrease in 96 h and maximum production at 120 h of incubation. With cellobiose as carbon source, enzyme production in *V. volvacea* showed an increase at 48 h and a decrease at 60 h with maximum BGL production at 72 h and 108 h of incubation. BGL production was absent in maltose and sucrose containing flasks even though these carbohydrates supported mycelial growth (Cai et al., 1998). In *Trichoderma harzianum*, 1 % lactose as carbon source supported maximum BGL production at 48 h of incubation and thereafter a decrease in production was noticed with increase in incubation period (Aslam et al., 2010). In *Debaryomyces pseudopolymorphus*, cellobiose at 2 % level was effective in β-glucosidase production while glucose at same concentration showed repressive effect (Arévalo-Villena et al., 2006). In *Monilia*, concentrations of glucose and cellobiose higher than 1 g/l repressed synthesis of extracellular β-glucosidase and de-repression of synthesis occurred only when the concentration of these sugars came down to 0.5 g/l (Berry and Dekker, 1984).

For GBGL production, maltose was found to be the best carbon source from 96 h of incubation (Fig 4.6). Similar to BGL production, cellobiose was the best carbon source for GBGL production at 72 h and 96 h of incubation. Lactose was also found to be effective followed by trehalose. In fact, within 48 h of incubation, GBGL production was maximum with lactose. With increase in incubation period, cellobiose remained effective in GBGL production and a production of 25.4 U/ml was observed at 168 h of incubation. However, in *Aspergillus* strains, glucose tolerant isoform production was minimum with cellobiose (Günata and Vallier, 1999).

Figure 4.6: GBGL production by *B. fulva* with oligosaccharides as carbon source

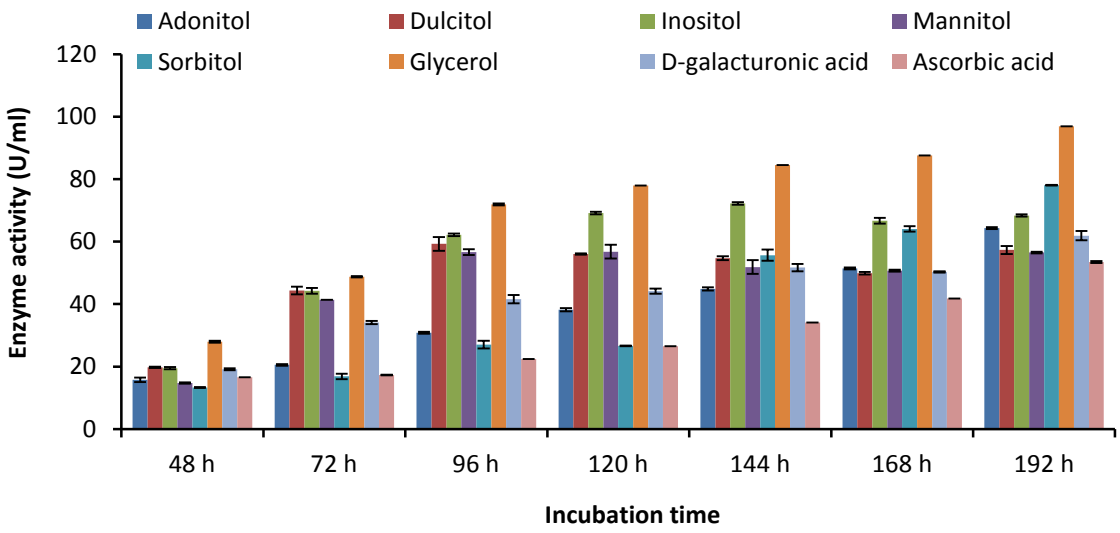


4.3.1.4. Sugar derivatives as carbon source

Among the sugar derivatives, glycerol was found to be the best carbon source. At all the tested incubation periods, maximum BGL production was noticed in glycerol containing flasks (Fig 4.7). In *Mucor racemosus*, effect of various carbon sources on β-glucosidase production at 1 % level was studied. Glycerol was found to be the best carbon source, followed by xylose, ribose, maltose and cellobiose. Very low β-glucosidase activity was noted with glucose, fructose, mannose and galactose (Borgia

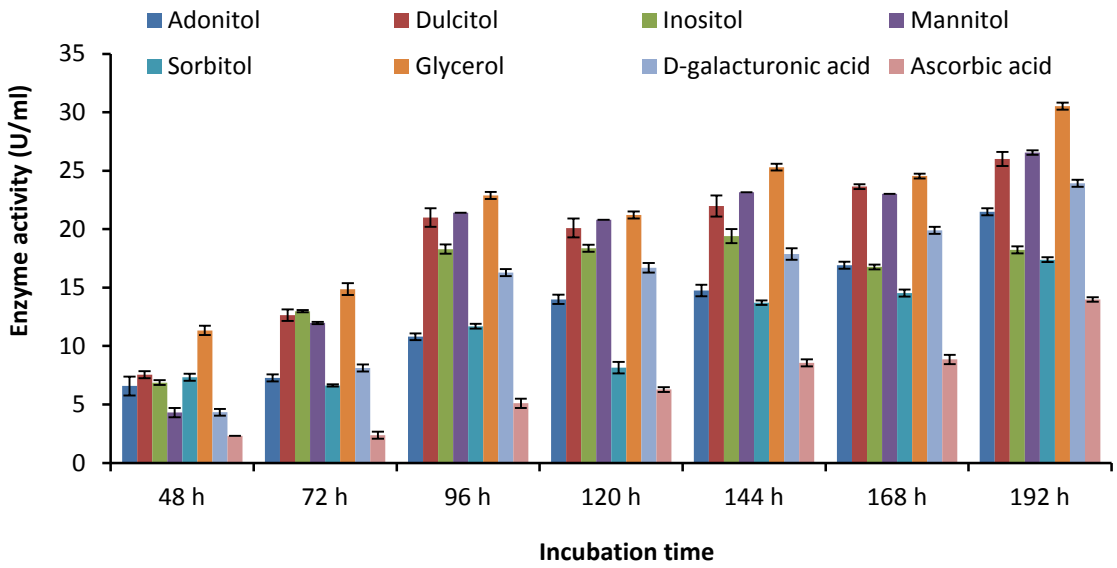
and Sypherd, 1977). Glycerol is a by-product of many industries and this offers a great potential for economic production of BGL. Inositol, sorbitol, mannitol and dulcitol were also effective in BGL production. D-galacturonic acid, the monomer of pectin had only medium effect in BGL production. Ascorbic acid as carbon source had least effect in BGL production. In *Penicillium purpurogenum*, addition of additives like ascorbic acid to sucrose containing production media had no effect on β-glucosidase production (Dhake and Patil, 2005).

Figure 4.7: BGL production by *B. fulva* with sugar derivatives as carbon source



Glycerol was also the best carbon source for GBGL production (Fig 4.8). In fact among all the carbohydrates tested, maximum GBGL production of 30.5 U/ml was noted with glycerol as carbon source. Mannitol and dulcitol were also good carbon sources for GBGL production. Least GBGL production was seen in ascorbic acid containing flasks.

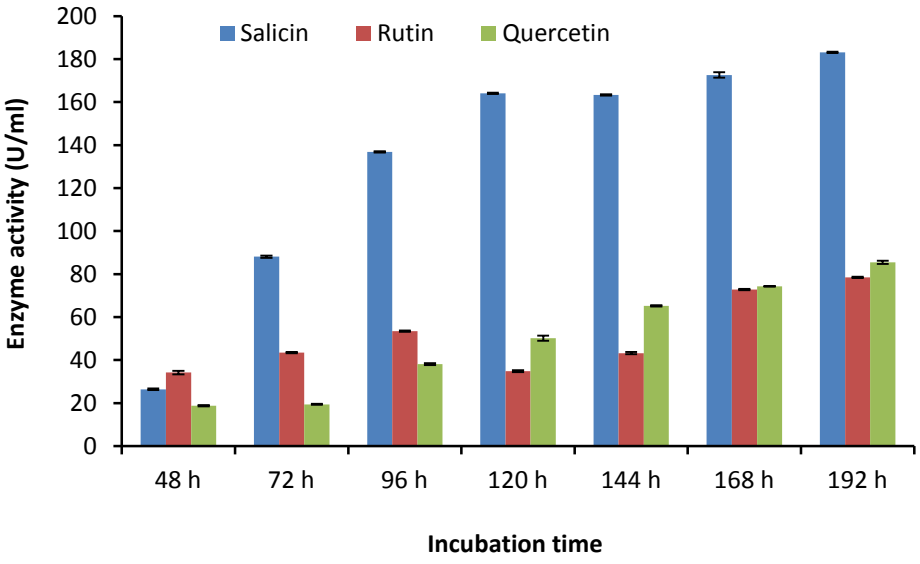
Figure 4.8: GBGL production by *B. fulva* with sugar derivatives as carbon source



4.3.1.5. Sugar glucosides as carbon source

Among sugar glucosides, salicin had a profound effect in BGL production. In fact among all the carbohydrates tested, maximum BGL production of 183.2 U/ml was noticed with salicin as carbon source (Fig 4.9). Salicin is a substrate for β-glucosidase and hence might have a direct effect in BGL production.

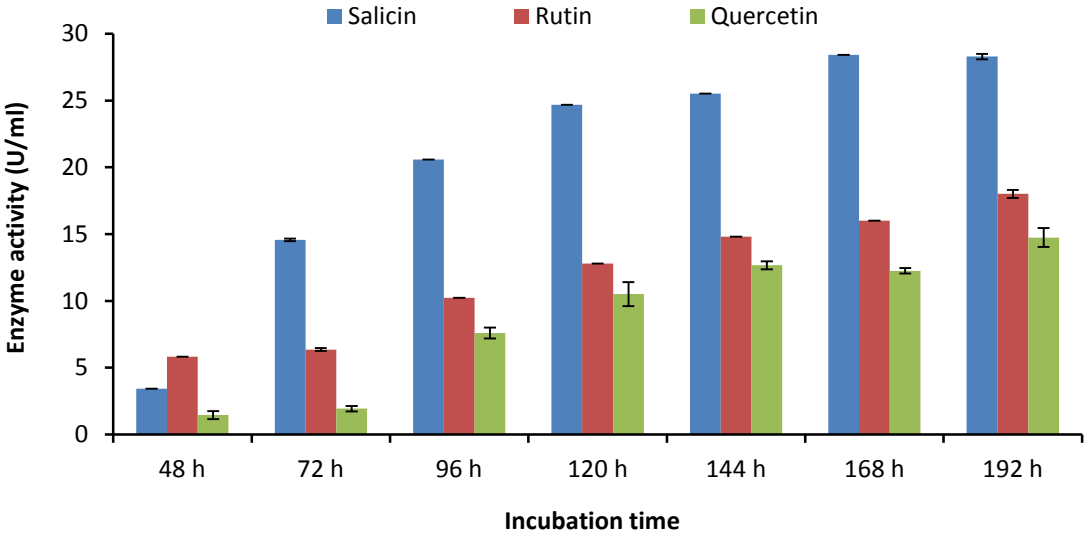
Figure 4.9: BGL production by *B. fulva* with sugar glucosides as carbon source



In a 6 h induction study in *Neurospora crassa*, salicin was found to induce aryl β-glucosidase while quercetin had no significant effect on both cellobiase and aryl β-glucosidase induction (Eberhart and Beck, 1973). Among the various carbohydrates tested for β-glucosidase production in *Volvariella volvacea*, 1 % salicin was found to be the best at 4.5 days of incubation (Cai et al., 1998).

Salicin was also found to be effective for GBGL production compared to quercetin and rutin (Fig 4.10). In *Aspergillus oryzae* and *A. niger*, total β-glucosidase production was high with rutin, while quercetin was found to have more inductive effect on the glucose tolerant BGL II production (Günata and Vallier, 1999). In the present study it was rutin that favored GBGL production than quercetin.

Figure 4.10: GBGL production by *B. fulva* with sugar glucosides as carbon source



4.3.2. Beta-glucosidase production in SmF with wheat bran as carbon source

With 1 % wheat bran as carbon source, a BGL production of 710 ± 23.2 U/ml and GBGL activity of 125 ± 2.1 U/ml were noted. However glucose tolerance was low with 17.66 ± 0.6 % activity retention in 1.0 M glucose. This activity retention of 17.66 % in 1.0 M glucose (18 % w/v glucose) is high compared to BGL from

Trichoderma sp. that was completely inhibited by 1 % glucose (Saha and Bothast, 1996). Usually complex substrates like wheat bran can enhance BGL production. However in *Trichoderma harzianum*, CMC was found to be a better substrate than wheat bran, corn cobs and birch wood xylan (Ahmed et al., 2009). In *Candida peltata*, glucose was found to be the best carbon source for BGL production (Saha and Bothast, 1996). Beta-glucosidase production by *Penicillium funiculosum* was lower in sugarcane bagasse than in avicel but pretreated sugarcane bagasse showed enhancement in BGL production (Castro et al., 2010). In *Penicillium purpurogenum*, rice straw was found to be the best carbon source than pure carbohydrates like avicel, CMC, cellobiose, glucose, lactose, sucrose, maltose, cellulose and xylan for BGL production (Jeya et al., 2010). In *Aspergillus terreus* (Nazir et al., 2009) and *Paecilomyces thermophila* (Yang et al., 2008) among the various carbohydrates and agro residues, corn cob was the best carbon source for BGL production. However in *Humicola grisea* var. *thermoidea*, among the agro-residues, wheat bran as carbon source showed maximum BGL production (Nascimento et al., 2010). Wheat bran is a complex substrate rich in proteins (14 %), carbohydrates (27 %), minerals (5 %), fat (6 %) and vitamins especially vitamin B (Haque et al., 2002). Since high production was noticed with 1 % wheat bran, this substrate was used for optimization studies.

4.3.3. Statistical optimization of the SmF production of β-glucosidase by B. fulva

4.3.3.1. Plackett-Burman design

Though a number of factors may be optimized using the classical one factor at a time approach, it is difficult to determine the most important variables. Plackett-Burman designs are very useful for screening process variables from a large number of factors, and require fewer runs than other factorial designs (Stowe and Mayer, 1966). The Plackett-Burman design matrix used for screening the factors that affect BGL production and also its glucose tolerance is given in Table 4.5 along with the responses obtained.

Table 4.5: Plackett-Burman design matrix with along with results for β-glucosidase production and glucose tolerance

Run #	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	BGL (U/ml)	% Activity retention
1	1.0	4	100	0.01	0.01	0.03	4	37	0.05	-1	+1	332	11.2
2	3.0	8	250	0.00	0.01	0.03	4	37	0.01	-1	-1	1371	5.0
3	1.0	4	250	0.01	0.01	0.01	8	37	0.01	+1	-1	268	30.2
4	3.0	8	100	0.01	0.00	0.01	4	37	0.05	+1	-1	767	16.3
5	3.0	8	100	0.01	0.01	0.01	8	32	0.01	-1	+1	340	36.2
6	3.0	4	250	0.01	0.00	0.03	4	32	0.01	+1	+1	855	20.2
7	1.0	8	250	0.00	0.01	0.01	4	32	0.05	+1	+1	870	10.9
8	1.0	4	100	0.00	0.00	0.01	4	32	0.01	-1	-1	411	19.9
9	1.0	8	250	0.01	0.00	0.03	8	32	0.05	-1	-1	480	21.8
10	3.0	4	100	0.00	0.01	0.03	8	32	0.05	+1	-1	694	28.4
11	1.0	8	100	0.00	0.00	0.03	8	37	0.01	+1	+1	297	31.7
12	3.0	4	250	0.00	0.00	0.01	8	37	0.05	-1	+1	406	26.1

X1- Wheat bran concentration (% w/v), X2- Incubation time (days), X3- Agitation (rpm), X4- Sucrose concentration (M), X5- Glycerol concentration (M), X6- $(NH_4)_2SO_4$ concentration (M), X7- pH, X8- Temperature (°C), X9- Tween 80 concentration (% v/v), X10-Dummy variable 1, X11-Dummy variable 2

Table 4.6 shows the effect estimate of each variable for both responses (BGL and glucose tolerance). The positive or negative sign of the effect of a tested variable indicates whether an increase in level of the tested variable enhances or lowers the response within the test limits. Variables *X*1, *X*2, *X*3, *X*5, *X*6, *X*9 and *X10* had positive effect on β-glucosidase production while the rest had a negative influence. Glucose tolerance of the enzyme was influenced positively by the variables *X1*, *X4*, *X7*, *X10 and X11* whereas the remaining had a negative effect. The magnitude of the coefficient of each variable indicated the intensity of its effect on the studied response. For β-glucosidase production the most significant factor was pH with increasing pH causing a lower enzyme production. Wheat bran concentration and agitation also showed high levels of significance both having positive effect. Increasing wheat bran concentration

increases the available carbon and nitrogen while higher agitation provides higher oxygen availability for growth. In *Aspergillus niger*, Plackett-Burman design was employed to screen the important variables affecting β-glucosidase production. Among the 10 variables, wheat bran concentration, KH_2PO_4 concentration and stirring speed had significant impact in BGL production (Hu et al., 2008).

Table 4.6: Effect estimate of factors for β-glucosidase production and glucose tolerance

Factor	Effect Estimate	
	BGL production	Glucose tolerance
Wheat Bran (X1)	+147.9	+0.54
Time (X2)	+96.6	-1.18
Agitation (X3)	+117.4	-2.46
Sucrose (X4)	-83.9	+1.16
Glycerol (X5)	+54.9	-1.18
Ammonium sulfate (X6)	+80.6	-1.78
pH (X7)	-176.8	+7.58
Temperature (X8)	-17.4	-1.41
Tween 80 (X9)	+0.6	-2.38
Dummy 1 (X10)	+34.3	+1.46
Dummy2 (X11)	-74.3	+1.23

4.3.3.2. Box-Behnken design

Based on the results of screening experiments for β-glucosidase production, three variables –wheat bran concentration, agitation and pH, having significant effect on the response (BGL production) were selected and further optimized using a Box-Behnken design. The Box-Behnken design matrix along with the corresponding experimental and predicted values for β-glucosidase yield and glucose tolerance is given in Table 4.7. 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 yield/glucose tolerance to the parameters tested

$$Y_1 = + 2372.40 + 415.08X_1 - 135.47X_2 + 150.95X_3 - 200.15X_1^2 + 223.45X_2^2 - 534.20X_3^2 - 0.30X_1X_2 + 297.50X_1X_3 - 43.00X_2X_3$$

$$Y_2 = + 14.49 + 2.27X_1 + 0.70X_2 - 0.17X_3 + 0.098X_1X_2 - 5.27X_1X_3 + 1.42X_2X_3$$

where Y_1 is the predicted value of β-glucosidase yield, Y_2 the predicted value for glucose tolerance and X_1, X_2 and X_3 are the coded values for wheat bran concentration, agitation and pH, respectively.

Table 4.7: Box-Behnken design with experimental and predicted results for β-glucosidase production and glucose tolerance

Run #	Wheat Bran (% w/v)	Agitation (rpm)	pH	BGL Activity (U/ml)		% Activity retention	
				Experimental	Predicted	Experimental	Predicted
1	6.0	250	6	2414	2502	14.5	11.6
2	3.0	300	5	1592	1845	12.6	12.5
3	4.5	200	4	2170	2003	13.7	15.4
4	4.5	250	5	2402	2372	15.2	14.5
5	4.5	250	5	2311	2372	14.6	14.5
6	4.5	200	6	2225	2391	12.8	12.2
7	3.0	250	4	1457	1370	8.5	6.8
8	3.0	250	6	1497	1077	16.4	17.0
9	4.5	250	5	2355	2372	13.9	14.5
10	4.5	250	5	2328	2372	14.6	14.5
11	4.5	300	4	1985	1818	12.1	13.9
12	3.0	200	5	1862	2116	11.2	11.3
13	6.0	300	5	2929	2675	14.4	17.9
14	6.0	250	4	1184	1605	27.7	22.5
15	4.5	250	5	2466	2372	14.8	14.5
16	6.0	200	5	3200	2947	12.7	16.3
17	4.5	300	6	1868	2034	16.9	16.4

The experimental data were statistically analyzed by the analysis of variance (ANOVA) and the results are shown in Table 4.8 and 4.9. The ANOVA of the quadratic regression model for β-glucosidase production indicated that the model was highly significant with a model F-value 3.75 (Table 4.8). Among the independent variables studied, wheat bran concentration had a significant effect on β-glucosidase production while the pH influenced enzyme production following a quadratic relationship. The value of R^2 (0.8283) indicated a good agreement between the experimental and predicted values of β-glucosidase yield.

Table 4.8: ANOVA of the response surface model for β-glucosidase production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	3.63E+06	9	4.04E+05	3.75	0.0476
X_1	*1.38E+06*	1	*1.38E+06*	*12.81*	*0.009*
X_2	*1.47E+05*	1	*1.47E+05*	*1.36*	*0.2809*
X_3	*1.82E+05*	1	*1.82E+05*	*1.69*	*0.2342*
X_1^2	*1.69E+05*	1	*1.69E+05*	*1.57*	*0.2507*
X_2^2	*2.10E+05*	1	*2.10E+05*	*1.95*	*0.2048*
X_3^2	*1.20E+06*	1	*1.20E+06*	*11.17*	*0.0124*
X_1X_2	*0.36*	1	*0.36*	*3.35E-06*	*0.9986*
X_1X_3	*3.54E+05*	1	*3.54E+05*	*3.29*	*0.1125*
X_2X_3	*7396*	1	*7396*	*0.069*	*0.8007*
Residual	7.53E+05	7	1.08E+05		
Lack of Fit	*7.37E+05*	3	*2.46E+05*	*63*	*0.0008*
Pure Error	*15606.02*	4	*3901.5*		
Cor Total	4.39E+06	16			

X1- wheat bran concentration (% w/v), X2-agitation (rpm), X3- pH

The interactive effect of wheat bran concentration, agitation and pH on BGL production by *B. fulva* is illustrated in Figs. 4.11–4.13. Maximum BGL production (3200 U/ml) was noticed with wheat bran concentration of 6 % w/v, agitation of 200 rpm and pH of 5.0. The interaction between the parameters was not very significant. It may be seen that, irrespective of agitation level, an increase in wheat bran concentration caused increased production (Fig 4.11). Similarly, at higher pH, production increased with increase in wheat bran concentration (Fig 4.12). But at lower pH, with increase in wheat bran concentration, production increased to an optimum and further increase in wheat bran concentration resulted in a slight decrease in enzyme production. At all the wheat bran concentrations tested, maximum enzyme production was observed at a range of pH 5.0-5.5. The response surface in Fig 4.13 indicated enhanced production with increase in pH to an optimum level and then decrease with further increase of pH. The effect of pH was independent of that of the agitation. In *Aspergillus niger*, central composite design was used to study interaction of wheat bran concentration, KH_2PO_4 concentration and stirring speed. Unlike the present result, in *A. niger*, highest β-glucosidase activity was seen around the central point corresponding to wheat bran-30 g/l, KH_2PO_4-2 g/l and stirring speed-150 rpm (Hu et al., 2008).

Figure 4.11: Response surface showing interaction between agitation and wheat bran concentration

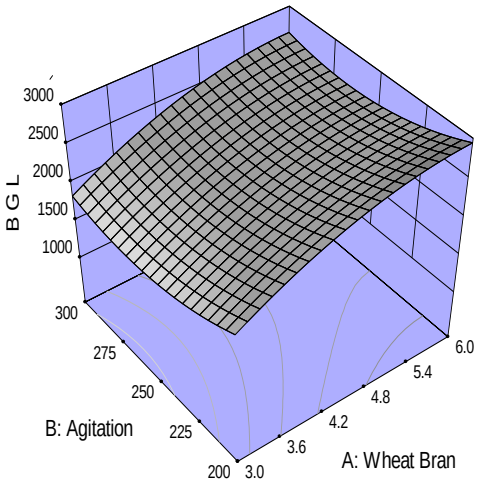


Figure 4.12: Response surface showing interaction between pH and wheat bran concentration

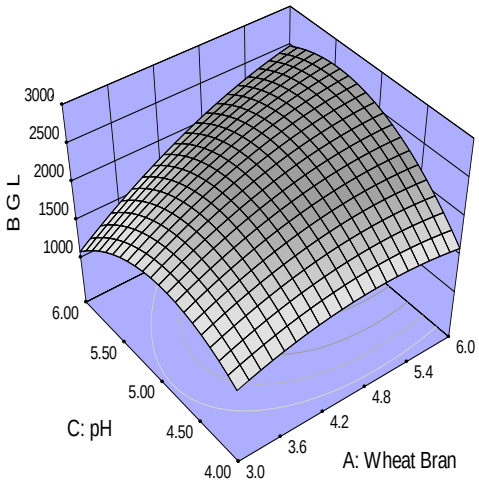
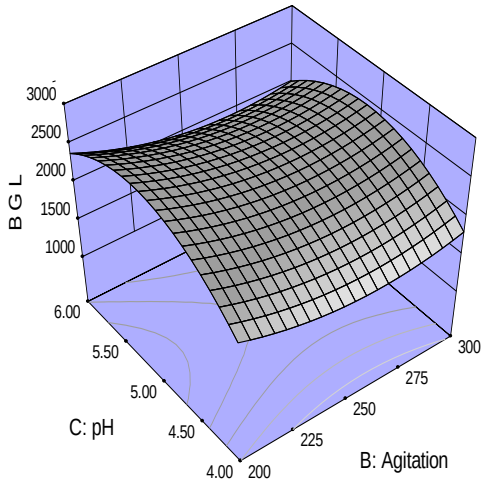


Figure 4.13: Response surface showing interaction between agitation and pH



The optimization studies in SmF had resulted in a maximum BGL yield of 3200 U/ml which was ~4 fold improvement from the starting yield of 710 U/ml indicating the success of the optimization studies.

Glucose tolerance of BGL is a highly desirable feature when the enzyme is to be used in biomass hydrolysis since it can improve the yield of sugars from a lignocellulosic feedstock. *Byssochlamys fulva* NII 0930 showed BGL multiplicity and an expression pattern dependent on the carbon sources. It was speculated that the expression of glucose tolerant β-glucosidase can be manipulated by medium optimization so as to obtain a more glucose tolerant BGL preparation and hence it was also decided to monitor the glucose tolerance of BGL preparations obtained under the different experimental conditions used for optimizing BGL production.

The ANOVA of the response model for glucose tolerance showed the model to be significant with an F-value of 4.17 (Table 4.9). Among the independent variables studied, wheat bran concentration and wheat bran- pH interaction had a significant effect on glucose tolerance. The value of R^2 (0.8544) indicated a good agreement between the experimental and predicted values for glucose tolerance.

Table 4.9: ANOVA of response surface model for glucose tolerance

Source	Sum of Squares	DF	Mean Square	F Value	Prob >F
Model	176.08	6	29.35	4.17	0.0233
X_1	*52.99*	*1*	*52.99*	*7.52*	*0.0207*
X_2	*3.89*	*1*	*3.89*	*0.55*	*0.4744*
X_3	*0.24*	*1*	*0.24*	*0.034*	*0.8568*
X_1X_2	*0.038*	*1*	*0.038*	*5.40E-03*	*0.9429*
X_1X_3	*110.88*	*1*	*110.88*	*15.74*	*0.0027*
X_2X_3	*8.04*	*1*	*8.04*	*1.14*	*0.3105*
Residual	70.43	10	7.04		
Lack of Fit	*69.59*	*6*	*11.6*	*55.04*	*0.0008*
Pure Error	*0.84*	*4*	*0.21*		
Cor Total	246.52	16			

X1- wheat bran concentration (% w/v), X2-agitation (rpm), X3- pH

The interaction effects of parameters were analyzed using response surface plots. On analyzing the interaction between wheat bran concentration and agitation, it was observed that the glucose tolerance increases with increase in both wheat bran concentration and agitation (Fig 4.14). There was a significant interaction of wheat bran concentration and pH, as evident from figure 4.15. At lower pH, increase in wheat bran concentration resulted in an improved glucose tolerance while at higher pH, the effect was reversed with the glucose tolerance decreasing on increasing the wheat bran concentration. pH can be speculated to be modulated differently with varying concentrations of wheat bran since it is known to have buffering capacity (Fadel, 1992).

Figure 4.14: Response surface showing interaction between agitation and wheat bran concentration

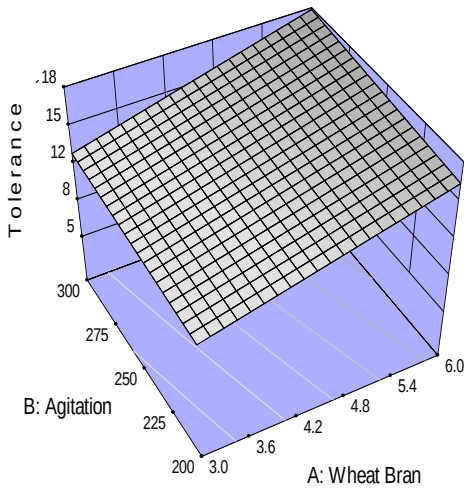
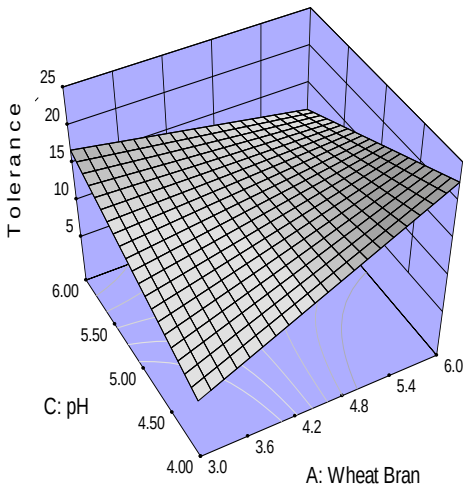
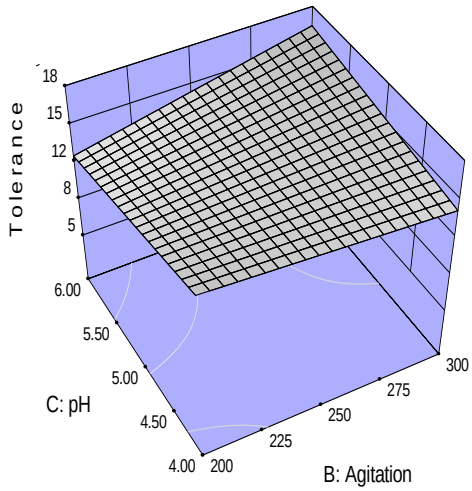


Figure 4.15: Response surface showing interaction between pH and wheat bran concentration



Interaction between pH and agitation was not significant. Nevertheless, it may be observed that the influence of agitation was different at different medium pH. At higher pH, increase in agitation resulted in improved tolerance while at lower pH, an increase in agitation resulted in lower tolerance (Fig 4.16). Variations in medium pH can affect the expression and secretion of different isoforms of an enzyme (Mansur et al., 2003) and the differences in glucose tolerance of the BGL preparations could be due to differential expression of the BGL isoforms. It is speculated that different isoforms of the BGL are expressed under different conditions of pH and aeration which results in the above observations.

Figure 4.16: Response surface showing interaction between agitation and pH



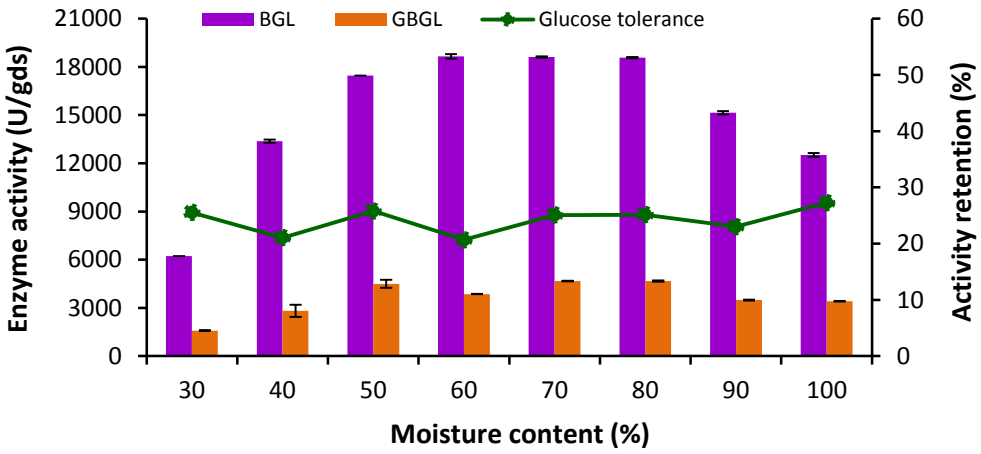
The parameters for response surface optimization though were selected to maximize the yield of BGL, also resulted in determining the conditions where an enzyme preparation with 27.7 % activity retention in presence of 1.0 M glucose was obtained (Table 4.7). Interestingly, it was observed that the conditions of maximal activity were different from those for maximal glucose tolerance which further proves that there could be differential expression of glucose tolerant and normal isoforms of the enzyme in response to the culture conditions.

4.3.4. Effect of moisture content of wheat bran for β-glucosidase production in SSF

Fungal products of biological interest like enzymes, secondary metabolites and spores were evolved for use in moist solid substrates. Consequently cultivation of fungi in aqueous suspension can impair their metabolic efficiency (Hölker et al., 2004). In *Aspergillus kawachii,* solid state fermentation using rice and barley as substrates were found to be more suitable for β-glucosidase production than liquid cultivation (Iwashita et al., 1998). Among the various agricultural by-products screened, wheat bran was the best substrate for SSF production of BGL by co-culture of *Aspergillus oryzae* and *Aspergillus niger*. Wheat bran contains adequate amounts of carbohydrates, proteins, fats, minerals and amino acids that are essential for fungal growth and enzyme production (Raza et al., 2011).

Among the initial moisture levels tested for SSF production of BGL, a moisture content from 50-80 % supported high levels of BGL production (Fig 4.17). A moisture content above 80 % caused decrease in enzyme production probably due to inefficient oxygen transfer that reduces microbial growth and enzyme production. An activity of 18661 U/gds was observed at 70 % moisture level. In *Trichoderma reesei*, maximum cellulase production was noted at 96 h of incubation with pretreated sugarcane bagasse having a moisture content of 66.4 % (Singhania et al., 2006). GBGL activity was maximum at 70 % and 80 % moisture level, but at 50 % moisture level, considerable GBGL activity was seen. This may be due to multiplicity of BGL and its differential secretion under specific environmental conditions.

Figure 4.17: Effect of moisture content on β-glucosidase production

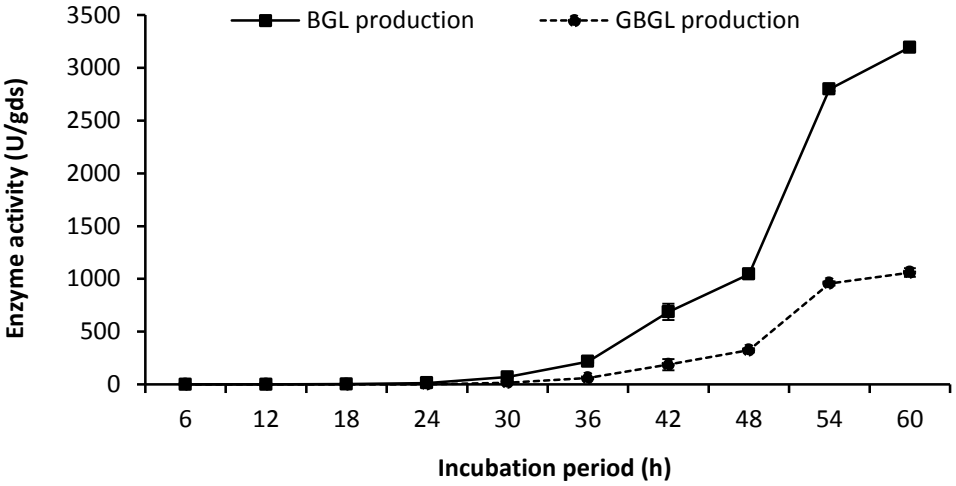


4.3.5. Time course for β-glucosidase production in SSF

With wheat bran as substrate and at an initial moisture content of 60 %, β-glucosidase production started at 18 h of incubation with an activity of 2.81 U/gds (Fig 4.18). The production increased to 1000 U/gds by 48 h and thereafter a steep rise in production was noted. The production continued to increase at a steep rate even at 60 h (time course for production was studied till 60 h of incubation) indicating the possibility of maximum production at a higher incubation period. A similar pattern was seen for GBGL activity. A glucose tolerance of 33 % activity retention in 1.0 M glucose was

observed for the enzyme recovered from 60 h incubated cultures. Spore formation was evident from 48 h of incubation.

Figure 4.18: Time course for β-glucosidase production in SSF



The optimum time for enzyme production varies with fungal taxa and culture conditions. *Thermoascus aurantiacus* in wheat bran with 60 % moisture level showed maximum BGL production in 48-72 h of incubation. But enzyme production was only 70 U/gds. *Aureobasidium pullulans* showed maximum BGL production at 120 h in wheat bran with 80 % moisture content. But here also production was low at 13 U/gds (Leite et al., 2008). Beta-glucosidase production by co-culture of *Aspergillus niger* and *Aspergillus oryzae* in wheat bran was maximum at 72 h of incubation with an activity of 1500 U/gds (Raza et al., 2011).

4.3.6. Statistical optimization of the SSF production of β-glucosidase by B. fulva

4.3.6.1. Plackett-Burman design

A Plackett-Burman design was used for identifying the most significant parameters that affect BGL production in SSF. The results of this screening experiment in terms of the responses- BGL production and its glucose tolerance are given in Table 4.10.

Table 4.10: Plackett-Burman design with corresponding β-glucosidase production and glucose tolerance

Run	A	B	C	D	E	F	G	H	I	J	K	BGL (U/gds)	% Activity retention
1	35	4	60	4	1.00E+06	<425	0.1	0.5	0.0	0.01	0.10	25274.3	17.1
2	35	7	80	4	1.00E+04	<425	0.1	0.0	0.2	0.01	0.05	44898.1	12.1
3	30	4	60	8	1.00E+04	>600	0.1	0.0	0.2	0.01	0.10	18086.8	25.3
4	30	7	80	8	1.00E+04	<425	0.0	0.5	0.0	0.01	0.10	27157.8	24.0
5	30	7	80	4	1.00E+06	>600	0.1	0.0	0.0	0.00	0.10	28312.5	15.1
6	35	7	60	4	1.00E+04	>600	0.0	0.5	0.2	0.00	0.10	36559.0	19.1
7	35	4	80	8	1.00E+06	<425	0.0	0.0	0.2	0.00	0.10	37340.3	11.1
8	30	4	80	4	1.00E+06	>600	0.0	0.5	0.2	0.01	0.05	30569.5	14.1
9	35	7	60	8	1.00E+06	>600	0.0	0.0	0.0	0.01	0.05	34753.4	15.5
10	30	4	60	4	1.00E+04	<425	0.0	0.0	0.0	0.00	0.05	23601.8	21.6
11	30	7	60	8	1.00E+06	<425	0.1	0.5	0.2	0.00	0.05	6793.39	24.7
12	35	4	80	8	1.00E+04	>600	0.1	0.5	0.0	0.00	0.05	24545.2	17.4

A- Temperature (°C), B- pH of minimal media, C-Moisture content (%), D-Incubation (days), E-Inoculum (spore count), F-Particle size (µ), G-$(NH_4)_2SO_4$ (% w/v), H- Peptone (% w/v), I- Starch (% w/v), J- Lactose (% w/v), K- Tween 80 (% v/v)

Table 4.11 shows the contribution of each variable to both responses (β-glucosidase production and glucose tolerance). The positive or negative sign in parenthesis indicates the effect of each parameter on the responses. Variables *A, B, C, F, I, J* and *K* had a positive effect on β-glucosidase production while *D, E, G* and *H* had a negative effect. On contrast, variables *B, D, G, H* and *K* had a positive effect and the rest, a negative effect on glucose tolerance of the enzyme. pH of minimal media had only minor effect in BGL production and tolerance. This can be correlated to the buffering action of wheat bran (Fadel, 1992). Incubation temperature and initial moisture content of the medium showed significant effects on BGL production while the latter affected glucose tolerance significantly. Based on these results, the parameters-temperature, moisture content, incubation time, ammonium sulphate and peptone concentrations were selected for further optimization to enhance BGL production by the fungus under SSF.

Table 4.11: Percentage contribution of factors for β-glucosidase production and glucose tolerance under SSF

Factor	% contribution	
	BGL	Glucose tolerance
Temperature (A)	36.39 (+)	33.59 (-)
pH (B)	2.79 (+)	0.49 (+)
Moisture (C)	17.51 (+)	27.74 (-)
Incubation (D)	12.62 (-)	11.35 (+)
Inoculum (E)	1.07 (-)	15.30 (-)
Particle size (F)	0.46 (+)	0.53 (-)
Ammonium sulfate (G)	13.59 (-)	1.26(+)
Peptone (H)	10.00 (-)	7.87 (+)
Starch (I)	0.86 (+)	0.58 (-)
Lactose (J)	4.27 (+)	0.027 (-)
Tween 80 (K)	0.44 (+)	1.27 (+)

4.3.6. 2. Box-Behnken design

The variables with significant effect on β-glucosidase production (incubation temperature and initial moisture content), obtained from screening experiments conducted using Plackett–Burman design were selected and further optimized using a

Box-Behnken design. The parameters- ammonium sulfate, incubation time and peptone concentration which had considerable effects on BGL production were also studied along with the significant parameters so as to obtain the best combination of these variables for enhanced production of the enzyme. Glucose tolerance of the enzyme was also measured for each experimental run and was analyzed as a second response. The Box-Behnken design matrix with corresponding experimental and predicted values for β-glucosidase yield and glucose tolerance is given in Table 4.12. The data were analyzed by multiple regression analysis using Design-Expert software and the following regression equations were obtained.

$$Y_1 = +23436 - 6915X_1 - 1886X_2 - 3718X_3 + 590X_4 + 331X_5 + 750X_1X_2 - 2070X_1X_3 - 1150X_1X_4 + 5677X_1X_5 + 1428X_2X_3 - 1870X_2X_4 + 199X_2X_5 + 573X_3X_4 + 707X_3X_5 + 1918X_4X_5 + 11272X_1^2 - 2185X_2^2 - 3304X_3^2 + 909X_4^2 - 272X_5^2$$

$$Y_2 = +22.72 + 0.38X_1 + 1.38X_2 + 1.83X_3 + 0.29X_4 + 0.54X_5 + 0.40X_1X_2 - 1.80X_1X_3 + 0.67X_1X_4 - 1.60X_1X_5 - 2.85X_2X_3 + 3.50X_2X_4 - 0.43X_2X_5 - 0.88X_3X_4 + 0.57X_3X_5 - 1.90X_4X_5 - 6.05X_1^2 + 1.40X_2^2 + 1.07X_3^2 - 0.21X_4^2 + 1.72X_5^2$$

where Y_1 is the predicted value of β-glucosidase yield, Y_2 the predicted value for glucose tolerance and X_1, X_2, X_3, X_4 and X_5 are the coded values for incubation temperature, initial moisture content, incubation time, ammonium sulphate concentration and peptone concentration respectively.

Table 4.12: Box Behnken design with results for BGL production and glucose tolerance in SSF

Run #	X1	X2	X3	X4	X5	BGL Activity (U/gds)		% Activity retention	
						Experiment	Predicted	Experiment	Predicted
1	32.0	75	5	0.03	0.05	42896	42553	18.9	16.92
2	37.0	75	5	0.03	0.05	26125	28129	16.5	16.88
3	32.0	85	5	0.03	0.05	34563	38186	20.8	18.88
4	37.0	85	5	0.03	0.05	20795	25875	20.0	20.44
5	34.5	80	4	0.00	0.05	32879	26127	19.2	20.81
6	34.5	80	6	0.00	0.05	21090	17544	25.8	26.21
7	34.5	80	4	0.05	0.05	29910	26161	22.1	23.13
8	34.5	80	6	0.05	0.05	20413	19870	25.2	25.03
9	34.5	75	5	0.03	0.00	26802	24119	24.4	23.71
10	34.5	85	5	0.03	0.00	22444	19946	27.2	27.32
11	34.5	75	5	0.03	0.10	29858	24381	23.8	25.63
12	34.5	85	5	0.03	0.10	26299	21007	24.9	27.55
13	32.0	80	4	0.03	0.05	40448	41352	13.9	13.95
14	37.0	80	4	0.03	0.05	36993	31662	15.9	18.31
15	32.0	80	6	0.03	0.05	29875	38055	25.0	21.20
16	37.0	80	6	0.03	0.05	18139	20085	19.8	18.36
17	34.5	80	5	0.00	0.00	24476	26455	22.0	21.72
18	34.5	80	5	0.05	0.00	21160	23799	22.7	26.09
19	34.5	80	5	0.00	0.10	19059	23280	31.0	26.59
20	34.5	80	5	0.05	0.10	23417	28298	24.1	23.37
21	34.5	80	5	0.03	0.05	25083	24821	23.1	22.94
22	34.5	80	5	0.03	0.05	24736	24821	22.3	22.94
23	34.5	80	5	0.03	0.05	24788	24821	22.3	22.94
24	34.5	75	4	0.03	0.05	22410	23595	18.4	18.91
25	34.5	85	4	0.03	0.05	19545	16965	24.9	27.37
26	34.5	75	6	0.03	0.05	12340	13301	28.8	28.26
27	34.5	85	6	0.03	0.05	15188	12384	23.9	25.32
28	32.0	80	5	0.00	0.05	39840	39408	14.8	16.24
29	37.0	80	5	0.00	0.05	23729	27878	16.0	15.65
30	32.0	80	5	0.05	0.05	50569	42889	13.2	15.46
31	37.0	80	5	0.05	0.05	29858	26758	17.1	17.57
32	34.5	80	4	0.03	0.00	12965	22569	28.0	23.50
33	34.5	80	6	0.03	0.00	14372	13717	24.4	26.00
34	34.5	80	4	0.03	0.10	15101	21816	27.0	23.43
35	34.5	80	6	0.03	0.10	19337	15794	25.7	28.23
36	32.0	80	5	0.03	0.00	49615	45313	14.0	15.65
37	37.0	80	5	0.03	0.00	24215	20128	20.9	19.61
38	32.0	80	5	0.03	0.10	35483	34621	17.6	19.92
39	37.0	80	5	0.03	0.10	32792	32145	18.1	17.48
40	34.5	75	5	0.00	0.05	20274	20201	23.3	25.51
41	34.5	85	5	0.00	0.05	19719	20169	21.9	21.27
42	34.5	75	5	0.05	0.05	21611	25124	20.8	19.09
43	34.5	85	5	0.05	0.05	13573	17608	33.4	28.85
44	34.5	80	5	0.03	0.05	22878	22051	22.0	22.50
45	34.5	80	5	0.03	0.05	22045	22051	24.2	22.50
46	34.5	80	5	0.03	0.05	21090	22051	22.4	22.50

X1- temperature (°C), X2-Moisture (%), X3- incubation (days), X4-$(NH_4)_2SO_4$ (% w/v), X5- Peptone (% w/v)

The results of the analysis of variance (ANOVA) tests done on the experimental models are given in Table 4.13 for BGL production. The ANOVA of the quadratic regression model for β-glucosidase production indicated that the model was highly significant with an F-value of 5.28. The multiple regression coefficient (R^2) had a value of 0.8149, indicating that the model could explain up to 81.49 % of the variability of the response. Among the parameters studied, temperature, incubation time and the interaction effect of temperature and peptone had significant effects. Influence of incubation temperature on BGL production followed quadratic relation.

Table 4.13: ANOVA of the response surface model for β-glucosidase production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	2.90E+009	20	1.45E+008	5.28	< 0.0001
X_1	7.65E+008	1	7.65E+008	27.84	< 0.0001
X_2	5.70E+007	1	5.70E+007	2.07	0.1629
X_3	2.21E+008	1	2.21E+008	8.05	0.0091
X_4	5.58E+006	1	5.58E+006	0.2	0.6564
X_5	1.75E+006	1	1.75E+006	0.064	0.8027
X_1X_2	2.26E+006	1	2.26E+006	0.082	0.777
X_1X_3	1.71E+007	1	1.71E+007	0.62	0.4374
X_1X_4	5.29E+006	1	5.29E+006	0.19	0.6648
X_1X_5	1.29E+008	1	1.29E+008	4.69	0.0405
X_2X_3	8.16E+006	1	8.16E+006	0.3	0.5908
X_2X_4	1.40E+007	1	1.40E+007	0.51	0.4823
X_2X_5	1.60E+005	1	1.60E+005	5.81E-03	0.9399
X_3X_4	1.31E+006	1	1.31E+006	0.048	0.8288
X_3X_5	2.00E+006	1	2.00E+006	0.073	0.7896
X_4X_5	1.47E+007	1	1.47E+007	0.54	0.4713
X_1^2	1.11E+009	1	1.11E+009	40.35	*< 0.0001*
X_2^2	4.17E+007	1	4.17E+007	1.52	0.2301
X_3^2	9.53E+007	1	9.53E+007	3.47	0.0748
X_4^2	7.22E+006	1	7.22E+006	0.26	0.613
X_5^2	6.48E+005	1	6.48E+005	0.024	0.8793
Residual	6.60E+008	24	2.75E+007		
Lack of Fit	6.58E+008	20	3.29E+007	78.74	0.0003
Pure Error	1.67E+006	4	4.18E+005		
Corr. Total	3.65E+009	45			

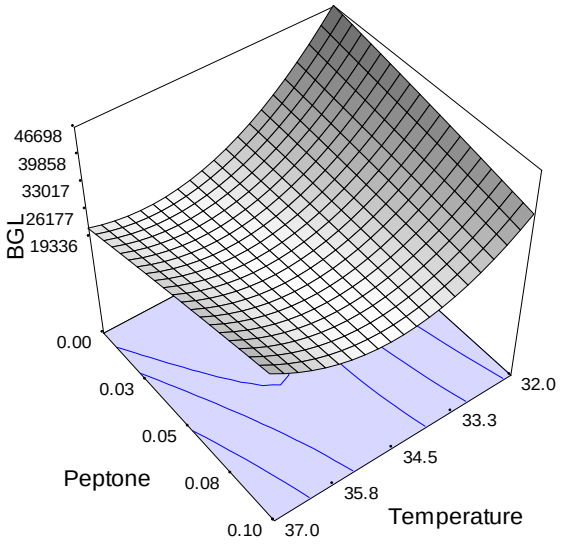
X1- temperature (°C), X2-Moisture (%), X3- incubation (days), X4-$(NH_4)_2SO_4$ (% w/v), X5- Peptone (% w/v)

Maximum β-glucosidase production (50569 U/gds) was noticed at an incubation temperature of 32 °C, moisture content of 80 %, incubation period of 5 days, ammonium

sulphate concentration of 0.05 % w/v and peptone concentration of 0.05 % w/v. Increased production of cellobiase with the increase in moisture content up to 70 % was reported in *Aspergillus niger* grown on mixture of wheat bran and ground corn cob (Tsao et al., 2000). Moisture content of the substrate can directly influence water activity which is critical in the growth and metabolite production of microbes under SSF. Achieving the optimal water activity for a fungus is dependent on the water holding capacity of the substrate (Raimbault, 1998).

There were no major interactions apart from that between temperature and peptone concentration (Fig.4.19). At higher temperatures, enzyme production increased with increase in peptone concentration while the contrary was true in the case of lower temperatures.

Figure 4.19: Response surface showing interaction between temperature and peptone



Similar analysis done on the second response (glucose tolerance) indicated that the polynomial model fitted for glucose tolerance was significant with an F-value of 4.89, and the value of R^2 (0.8031) indicated a good agreement between the experimental and predicted values for glucose tolerance (Table 4.14).

Table 4.14: ANOVA of response surface model for glucose tolerance

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	725.07	20	36.25	4.89	*0.0002*
X_1	2.33	1	2.33	0.31	0.5805
X_2	30.53	1	30.53	4.12	0.0536
X_3	53.29	1	53.29	7.19	0.013
X_4	1.32	1	1.32	0.18	0.6764
X_5	4.62	1	4.62	0.62	0.4373
X_1X_2	0.64	1	0.64	0.086	0.7713
X_1X_3	12.96	1	12.96	1.75	0.1984
X_1X_4	1.82	1	1.82	0.25	0.6244
X_1X_5	10.24	1	10.24	1.38	0.2512
X_2X_3	32.49	1	32.49	4.39	0.047
X_2X_4	49	1	49	6.61	0.0167
X_2X_5	0.72	1	0.72	0.098	0.7575
X_3X_4	3.06	1	3.06	0.41	0.5263
X_3X_5	1.32	1	1.32	0.18	0.6764
X_4X_5	14.44	1	14.44	1.95	0.1754
X_1^2	319.88	1	319.88	43.18	< 0.0001
X_2^2	17	1	17	2.3	0.1428
X_3^2	10.01	1	10.01	1.35	0.2565
X_4^2	0.39	1	0.39	0.053	0.8195
X_5^2	25.84	1	25.84	3.49	0.074
Residual	177.78	24	7.41		
Lack of Fit	174.6	20	8.73	11	0.0157
Pure Error	3.17	4	0.79		
Cor Total	905.07	45			

X1- temperature (°C), X2-Moisture (%), X3- incubation (days), X4-$(NH_4)_2SO_4$ (% w/v), X5- Peptone (%w/v)

Maximum glucose tolerance was noted for enzyme produced using an incubation temperature of 34.5 °C, 85 % moisture content, incubation period of 5 days, 0.05 % w/v ammonium sulphate concentration and 0.05 % w/v peptone concentration (Table 4.12). Optimal temperature for the growth can also be the optimal for production of primary metabolites and *Byssochlamys fulva* is reported to grow actively in the temperature range 30-35 $^{\circ}$C (Samson et al., 2004). Both production and glucose tolerance of BGL was optimal at the temperature range of 30-35 $^{\circ}$C indicating that temperature may not be a critical parameter in the differences in expression of the different BGL isoforms in SSF. Similarly, the optimal moisture content for both production and glucose tolerance of BGL was high and not much different from each

other which indicate a growth associated production of the different isoforms of the enzyme.

Figure 4.20 shows response surface plot for interaction between moisture content and incubation time. At low moisture levels glucose tolerance increased with increase in incubation time while at higher moisture levels glucose tolerance decreased with longer incubation. Higher level expression of glucose tolerant BGL isoforms at a later stage in growth might be speculated especially when the moisture levels are lower. With higher amount of moisture, glucose sensitive isoforms may be expressed more which could explain the higher activity and lower glucose tolerance. The interaction between moisture and ammonium sulfate concentration also was interesting and it displayed differences in the pattern of glucose tolerance at different moisture levels. At lower ammonium sulfate concentrations, an increase in moisture led to decrease in glucose tolerance while at higher ammonium sulfate concentrations, an increase in moisture resulted in higher glucose tolerance (Fig 4.21). Higher availability of nitrogen and moisture could have led to an increased production of all isoforms of BGL especially glucose tolerant forms which could have reflected as an increased glucose tolerance.

Figure 4.20: Response surface showing interaction between moisture and incubation time

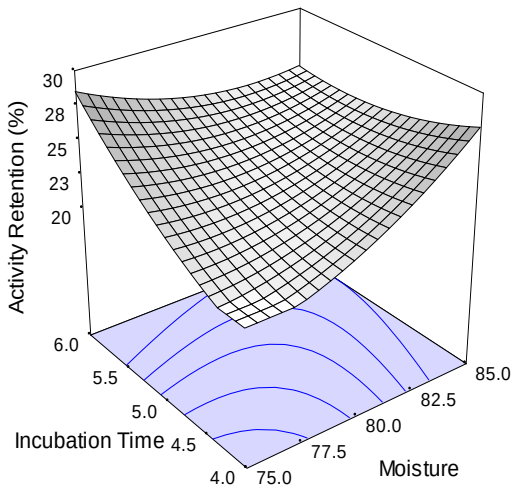
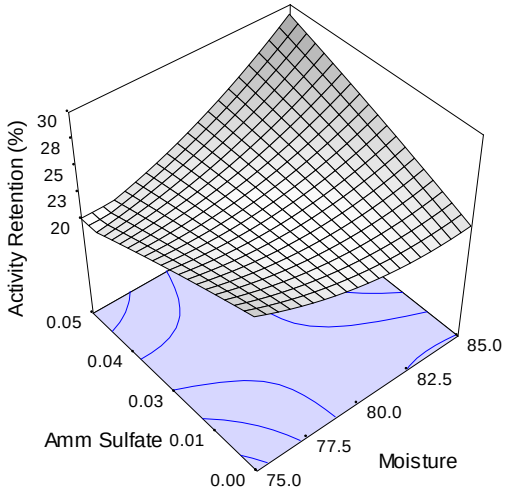


Figure 4.21: Response surface showing interaction between moisture and $(NH_4)_2SO_4$ concentration



4.3.7. Reactor design for SSF production of enzymes

The solid state fermenter design consists of multiple levels of circular perforated stainless steel platforms (discs) arranged one above the other with ample space in between for air circulation and heat dissipation (Fig 4.22 A). Each platform is fixed on to a support frame with three legs arranged at 120° angle to each other, which is designed in such a way as to fit correctly inside a stainless steel cylindrical vessel (the outer shell of the fermenter). The entire disc assembly with its accessories is mounted on the frame into a single unit which can be inserted into this pressure vessel which will then be closed steam tight since the disc assembly has a top plate which will fit onto the top of the vessel through a silicon rubber gasket (4.22 B). Each disc has a central grove to allow passage of a rotor shaft.

Figure 4.22 A: SSF bioreactor design showing the multi-tier circular platform with support structure

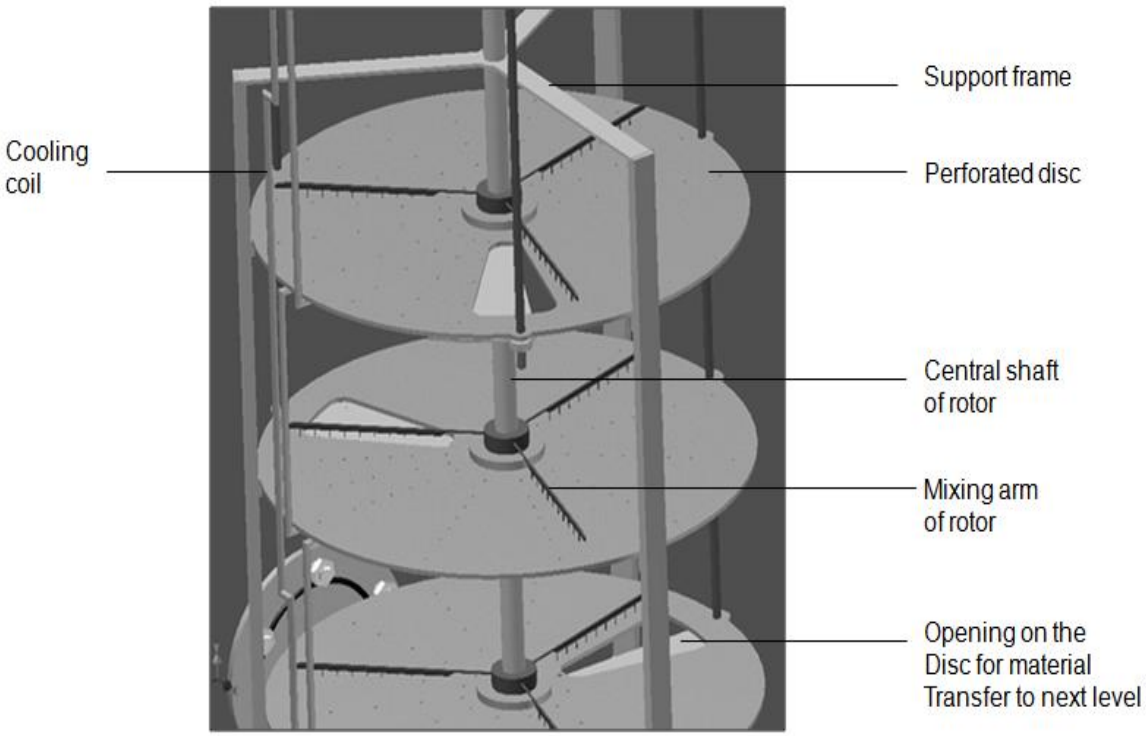
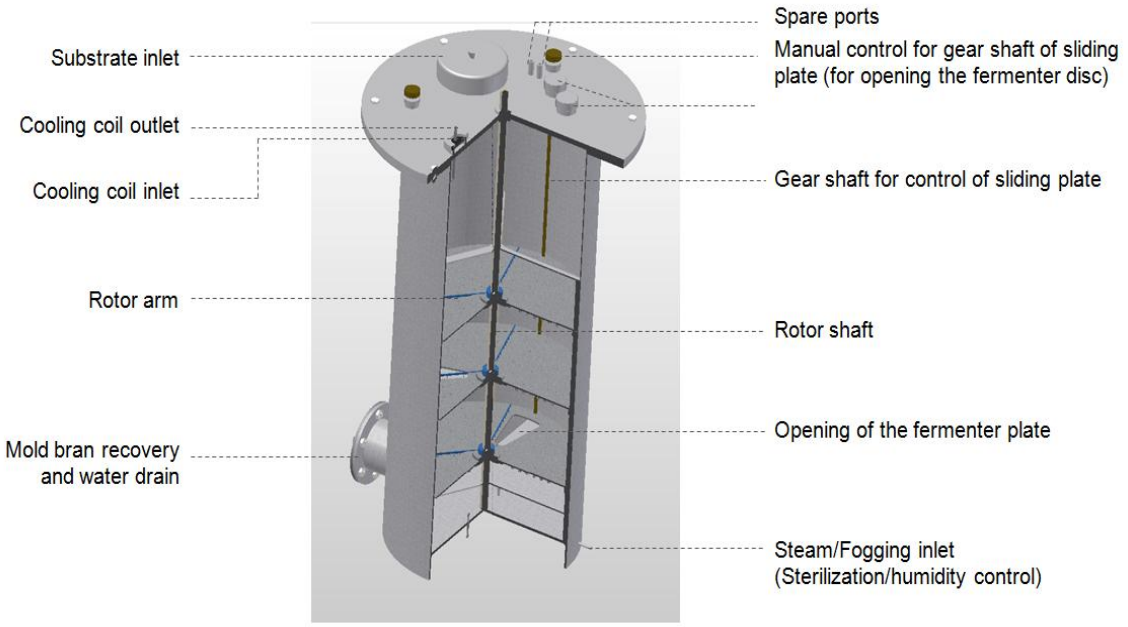
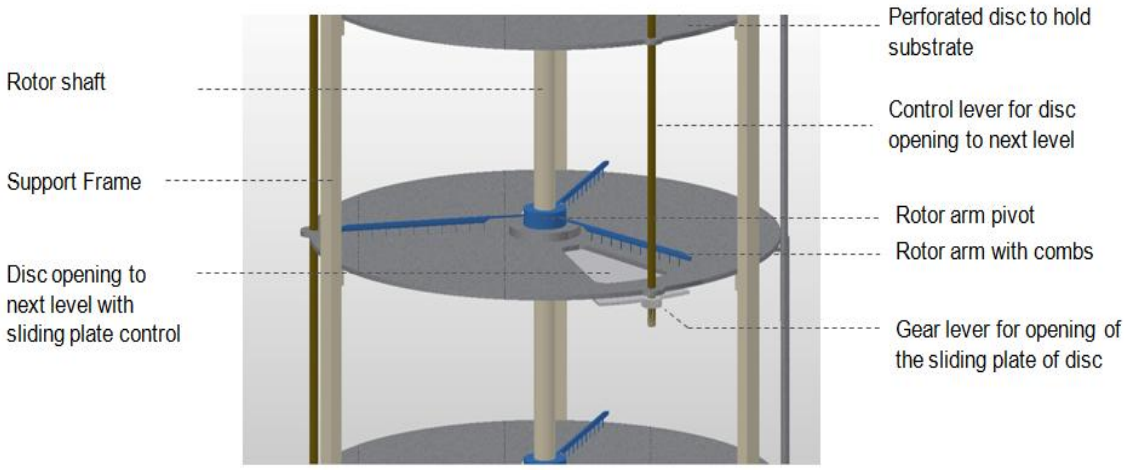


Figure 4.22 B: SSF bioreactor sectioned diagram showing entire unit with the disc assembly inserted in the pressure vessel and the top plate assembly



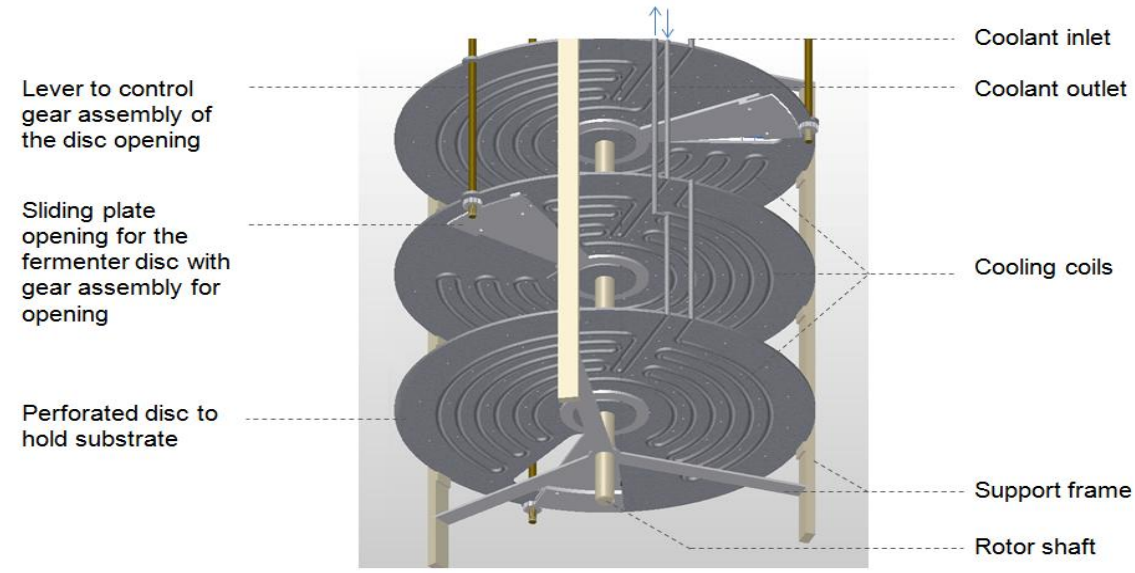
The rotor shaft can be inserted through the groves so as to pass through all the platforms/discs and can be freely removed or rotated inside the groves when assembled. In the proposed design, the rotor shaft passes out through a sealed assembly of the types which are already available in the market (and hence not shown) through the top plate (Fig 4.22 B). The vessel is therefore pressure tight and allows for steam sterilization. The rotor shaft at each level just above the disc is fitted with removable rotor arms made of steel and fixed to a central pivot ring which also has a grove to allow passage of the rotor shaft. The rotor consist of three stainless steel arms arranged at 120 ^{o} angle to each other and has a length about 0.5 cm less than the radius of the plate so as to provide a complete sweep of the fermenter discs while rotating. The rotors are provided at every level and can be fixed to the shaft through a central pivot ring which can be screwed on to the shaft after inserting the shaft (Fig 4.23- Rotor arms and pivot shown in blue color).

Figure 4.23: SSF bioreactor design showing the rotor shaft and rotor assembly



The number of the fermenter plates in this laboratory version of the reactor is limited to 3 which are equally spaced vertically and fixed to the support framework. SSF reactors in general offer limited possibility for scaling and sterile operation, which sometimes are required for containment or to maintain sterility (Khedkar et al, 2000, Suryanarayan & Mazumdar, 2000). Each plate is also provided with cooling coils made of stainless steel tubes, which are fixed on the bottom side of the perforated discs in close contact with the discs to allow efficient heat exchange (Fig 4.24).

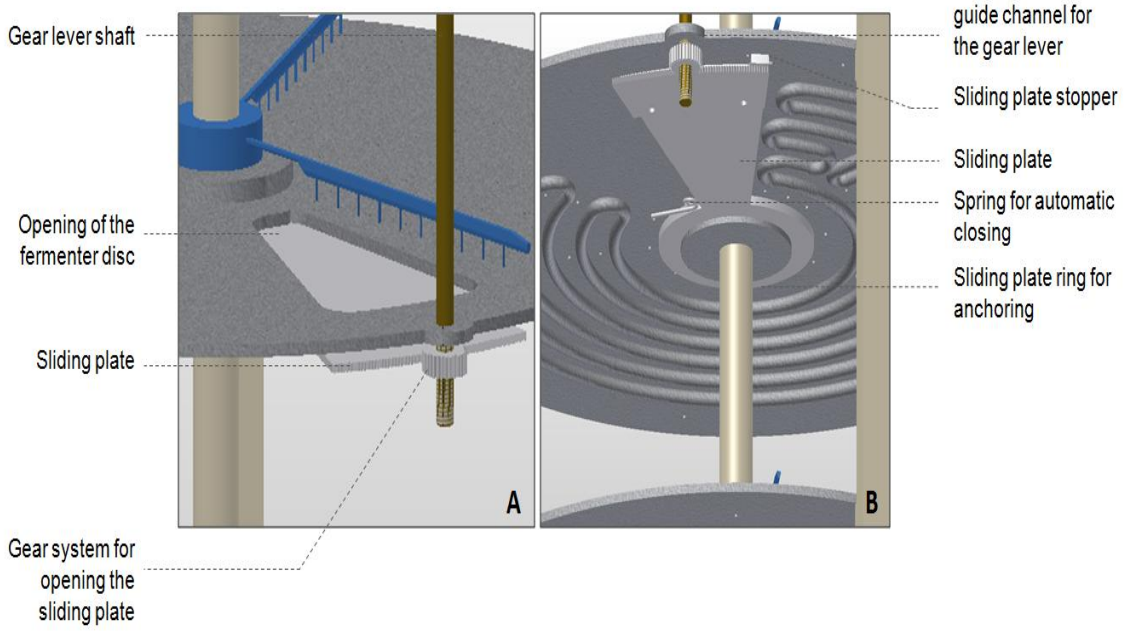
Figure 4.24: SSF bioreactor design showing heat exchanger design



The coils are arranged in loops covering a major part of the under surface of fermenter discs and is continuous with each other on the three discs so that coolant entering the inlet on the top plate passes through the coils of each disc and returns back to the outlet on the top plate. The inlet and outlet of the cooling coil are welded on to the top plate (Fig 4.22 B) and hence allow for steam sterilization of the vessel and aseptic operation.

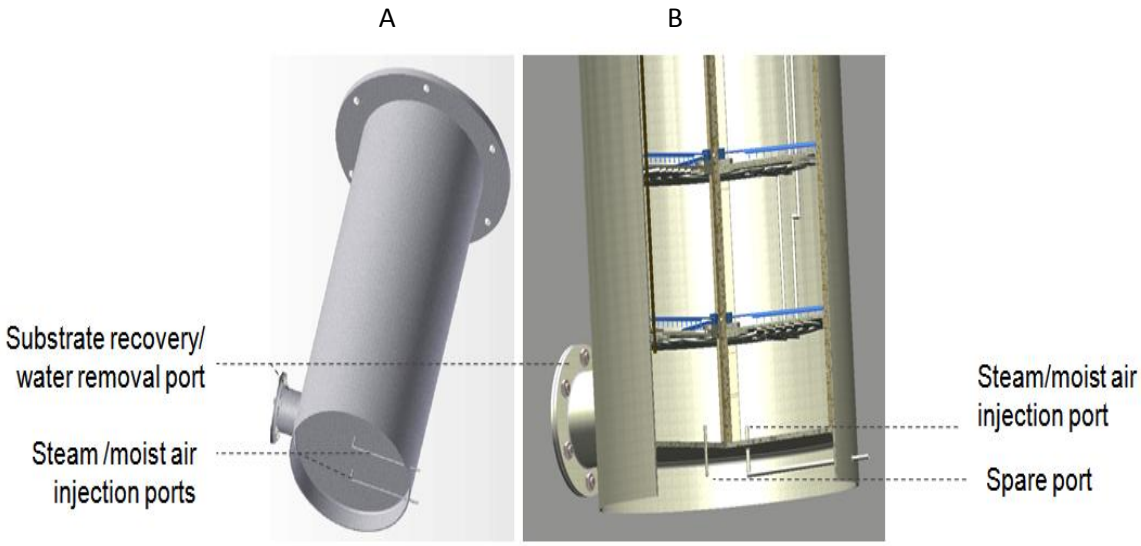
Each of the fermenter discs are also provided with a triangular opening with a length spanning almost 80 % of the radius of the plate, to allow passage of inoculated medium from one level to the other (Fig 4.25 A). This is controlled by an opening and closing system made of a sliding plate pivoted on to the bottom of the perforated plate at the centre through a ring structure and to the outer edge of the plate by use of a rail system that allows free movement of this plate. This allows opening and closing of the plate which acts as a door for the opening which can be controlled from outside the reactor, through a gear system and lever (Fig 4.25 B). The utility of such a system is to allow loading of the fermenter discs with inoculated substrate which can be loaded onto the vessel through the port on the top plate.

Figure 4.25: SSF bioreactor design showing mechanism for disc opening control



The bottom of the reactor has a bottom plate which is placed higher than the vessel bottom so as to provide a space beneath the fermentation chamber. Two stainless steel tubes are made to enter into the vessel through this area and these serve the purpose of steam or mist and air supply for sterilization/ humidification and aeration respectively (Fig 4.26 A & B). One of the tubes can be connected to the steam supply while the vessel has to be sterilized and once the reactor is in operation this can be connected to a humidification unit which will generate moist air. The other tube that enters the vessel can be used as a desiccated aeration/humidification port if the first port has to be connected permanently to the steam supply.

Figure 4.26: SSF bioreactor design showing bottom section

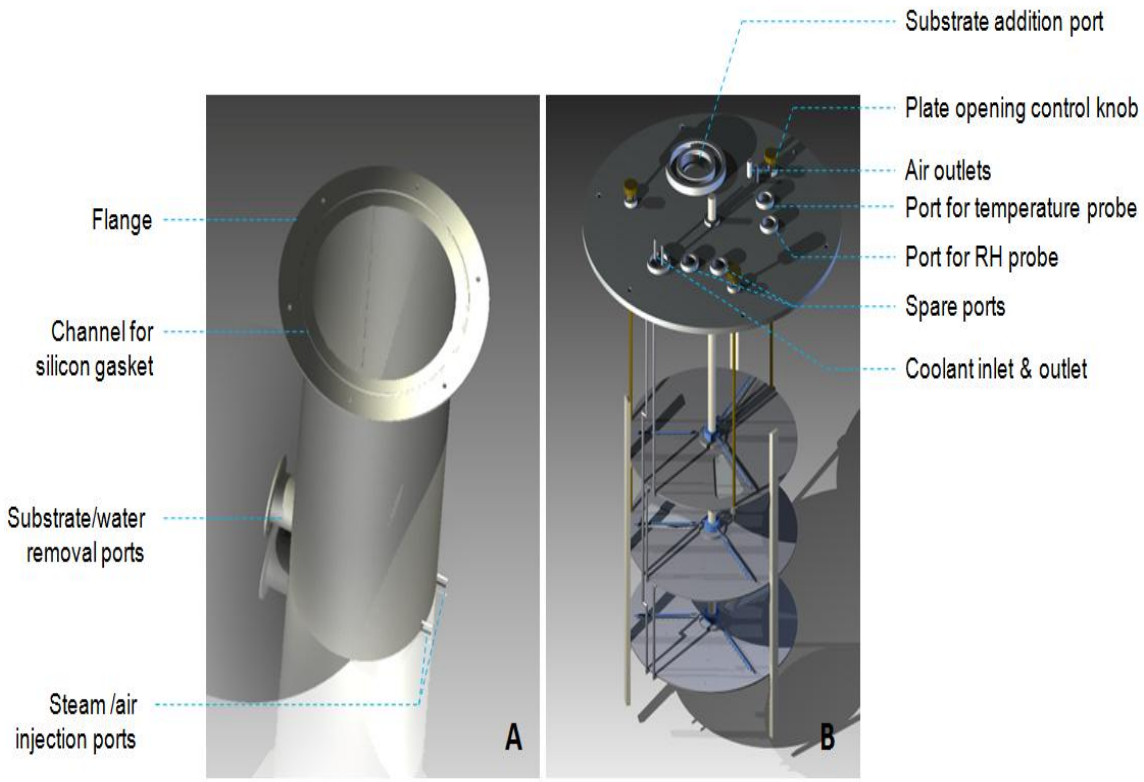


4.3.7.1. Operation of the reactor

The designed SSF reactor can operate either as a Group III or Group IV reactor which respectively are reactors where the bed is continuously or intermittently mixed and air is circulated around the bed but not forcefully aerated through the bed and where the bed is agitated and also forcefully aerated (Mitchel et al, 2006). The fermenter plate assembly needs to be assembled first as shown in figure 4.27 B and then inserted into the stainless steel fermenter shell (Fig 4.27A) when the top plate will fit on top of the shell's

flange and to make it steam tight a silicon rubber gasket is placed in between the flanges of the vessel and the top plate. The assembly is now bolted tight and can be sterilized by autoclaving (in the case of lab sized models) or by steam injection in the case of larger models. In the latter case the bottom steam port is connected to a steam outlet and all the openings are closed with their respective steam tight metal closures. Once sterilized, the reactor is allowed to cool down and the rotor shaft is connected to a drive motor. Temperature and relative humidity probes are inserted into appropriate ports for monitoring and can be connected to their respective monitoring devices or may even be connected to a computer through appropriate transducers and circuitry. Also the cooling coils are connected to a chiller circulator set at the desired temperature. A pre-designated volume of pre-inoculated solid substrate (required to occupy the fermenter plate surface, to the desired bed height) is added aseptically through the substrate addition port on the top plate while keeping fermenter discs 1 and 2 (counted from top) open and the rotor in motion. Due to the sweeping action of the rotor arms, the solid medium falls down through the openings of discs 1 and 2 and gets retained on plate 3 where it is spread evenly by the sweeping of rotor arm. Since a pre-designated volume is added, once this batch of addition is completed the level 3 is filled. Now the opening at level 2 is closed and the process is repeated to fill this level. Once accomplished, the process is now repeated for level 1 and then the substrate addition port is closed. Now the reactor can be connected to a filtered moist air supply which again can be regulated by a humidity controller. Also if required a desiccated filtered air supply can be connected to the second port for air supply. The air discharge port on top of the reactor is opened and venting is accomplished through a filter. The reactor now can be maintained at the desired operational conditions with continuous or intermittent mixing as required. At the end of the fermentation, the plate openings can be opened and the rotor set in motion, to collect the moldy bran. Since all the gates are open, the substrate will be swept from all levels and will collect on the bottom plate of the reactor from where it can be taken out through the side port.

Figure 4.27: SSF bioreactor assembly for operation



4.4. Conclusion

Nutritional requirements of microbes used in industrial fermentation processes are generally complex and varies with organism. Among the nutrients, carbon source forms a major element as it is an energy source and an important component in biomass. Carbohydrates are excellent sources of carbon, hydrogen, oxygen and metabolic energy. Among the carbohydrates tested, BGL production was highest (183.2 U/ml) with salicin as carbon source and GBGL production was maximum with glycerol as carbon source (30.5 U/ml). However use of 1 % wheat bran as carbon source in SmF resulted in a production of 710 U/ml BGL and 125 U/ml GBGL. Glucose tolerant β-glucosidases have potent application in biomass hydrolysis. High titers of GBGL produced

economically can reduce the overall cost of saccharification and therefore that of bioethanol production. The statistical optimization studies have resulted in an important finding that most variables that enhance β-glucosidase production could result in a lower glucose tolerance of the enzyme preparation from the fungus. This, points to the differential expression of BGL isoforms which vary in their glucose tolerance. The results may be used to design cultivation strategies that will result in BGL preparations that are more glucose tolerant. Optimizations performed using two step statistical design resulted in a significant (4 fold) improvement in production under SmF (750 U/ml to 3200 U/ml) and 2.7 fold improvement (18600 U/gds to 50569 U/gds) in SSF. It may be concluded that the study had resulted in an optimized process for enhanced BGL production from the fungus and has paved the way for further studies on differential expression of BGL isoforms in response to culture parameters. Besides, a lab to semi pilot scale SSF reactor capable of sterile operation was designed and its 3D model was rendered. The design may be used to generate a prototype to be tested for production of enzymes. This reactor offers the possibility of continuous or intermittent mixing along with aeration and is capable of sterile operations as required by certain fermentation processes.

Chapter 5

Induction and repression of β-glucosidase

Chapter 5

Induction and repression of β-glucosidase

5.1. Introduction

Enzymes may be classified as constitutive or inducible based on the mode of their expression. An inducible enzyme or adaptive enzyme is expressed only under some physiological state in presence of some triggering agents which are called inducers. These inducers are generally chemical substances that are assimilated by the cell or produced by it that stimulates the production of the adaptive enzyme. In contrast, constitutive enzymes are produced continuously without any influence from external inducing factors. Many enzymes are prone to repression whereby the production of enzyme is reduced or temporarily shut down as per the requirement of the cell. The factors, mostly chemical substances that take part in repression are called repressors. Induction and repression mechanisms are generally part of the cellular economy where certain enzymes are produced only when they are required which in turn are determined by certain external/internal conditions. For example, lactose assimilation enzymes are required by a microbe only when lactose is present in the medium and induction and repression mechanisms ensure that the enzymes are synthesized only when medium contains lactose and otherwise the expression of these enzymes is shut down.

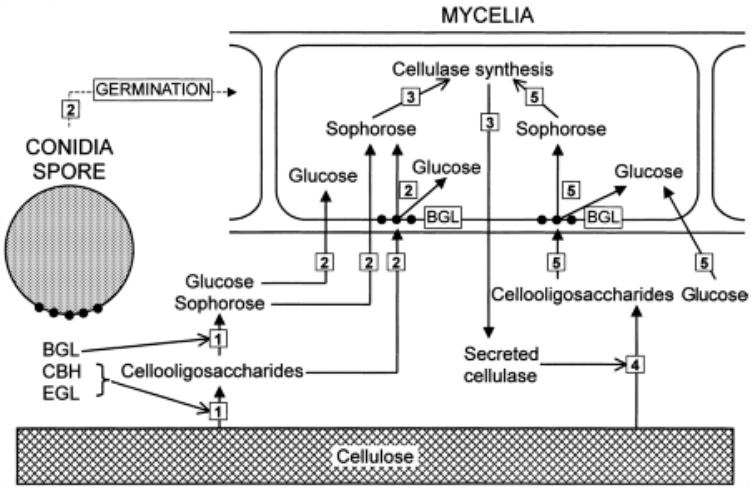
Generally cellulases are inducible enzymes produced only when cellulose is present in the medium. Bacterial cellulases as in *Clostridium thermocellum*, *Cellulomonas flavigena* and *Pseudomonas fluorescens* var. *cellulosa* are constitutive in nature while fungal cellulases are generally inducible (Suto and Tomita, 2001). However, a low level of cellulase expression in the absence of cellulose has been reported in fungi by many investigators. Cellulose is a polymer which the cells cannot assimilate. It is believed that a minor amount of constitutive cellulase expression cause initial degradation of cellulose, resulting in the production of mono or oligosaccharides that act as signal molecules for full scale induction of the cellulolytic enzymes. Beta-glucosidases play a key role in this induction by regulating the production and uptake of

cellobiose (Berry and Dekker, 1984). Beta-glucosidase with its transglycosylation activity has the ability to convert cellobiose into disaccharide sugars like sophorose which is demonstrated to have very high potential for cellulase induction (Loewenberg and Chapman, 1977; Saloheimo et al., 2002; Suto and Tomita, 2001; Umile and Kubicek, 1986).

5.1.1. Model of cellulase induction in Trichoderma reesei (Suto and Tomita, 2001)

Trichoderma reesei spores have cellobiohydrolase on the surface, which degrades cellulose to cellooligosaccharides. These oligosaccharides are then hydrolyzed into glucose and transglycosylated to sophorose by a constitutive β-glucosidase on the spore surface. The spore germinates to form mycelium and glucose (carbon source), sophorose (inducer) and cellooligosaccharides enters the mycelia. The mycelium bound β-glucosidase convert cellooligosaccharides into sophorose and glucose. The sophorose induces cellulase expression and the resultant proteins are secreted outside. The secreted cellulase degrades cellulose into glucose and cellooligosaccharides, and while the glucose is assimilated, some cellooligosaccharides are converted into inducer by the constitutive β-glucosidase continuing the cycle (Fig 5.1).

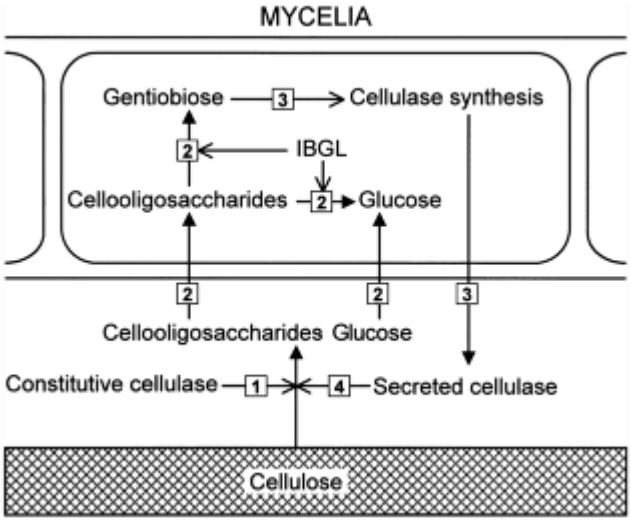
Figure 5.1: Schematic diagram showing cellulase induction in *Trichoderma reesei* (Suto and Tomita, 2001)



5.1.2. Model of cellulase induction in Penicillium purpurogenum (Suto and Tomita, 2001)

In *Penicillium purpurogenum,* constitutive cellulase degrades cellulose into cellooligosaccharides and glucose which enters the mycelium and glucose is assimilated as carbon source. The extracellular β-glucosidase of the fungus lacks transglycosylation property (Kurasawa et al., 1992) and it is believed that the intracellular β-glucosidase convert cellooligosaccharides to gentiobiose which later induces cellulase. The cellulase so produced is secreted outside which degrades cellulose, producing glucose and cellooligosaccharides. These are absorbed by the mycelia and the cycle continues (Fig 5.2).

Figure 5.2: Schematic diagram showing cellulase induction in *Penicillium purpurogenum (Suto and Tomita, 2001)*



5.1.3. Beta-glucosidase – a constitutive or inducible enzyme

Reports indicate that β-glucosidase may occur as either an inducible or a constitutive enzyme. The inducible enzymes were also subject to repression by specific sugar molecules. In *Monilia* sp., one intracellular β-glucosidase (IG 1) was constitutive

while the other intracellular isoform (IG 2) and extracellular β-glucosidase (EG 1) were inducible. Cellobiose and D-glucose was found to repress β-glucosidase (IG 2 and EG 1) formation at higher substrate levels. This repression by cellobiose was suggested to be due to accumulation of glucose within the cell due to the action of intracellular β-glucosidase especially IG 1 (Berry and Dekker, 1984). In *Neurospora*, cellobiose was found to be the inducer for β-glucosidase production. Both aryl β-glucosidase and cellobiase were induced with cellobiose, though higher concentration of cellobiose (10 mM) was found to cause significant inhibition to cellobiase production (Eberhart and Beck, 1973). In *Streptomyces*, carboxy methyl cellulose and cellobiose was found to induce two intracellular β-glucosidases. Glucose added along with cellobiose caused repression of enzyme synthesis, possibly due to inhibition of inducer transport in to the cell (Pérez-Pons et al., 1995). In *Shewanella*, three isoforms of β-glucosidase were reported when grown in cellobiose and glucose as carbon source. However, production of the three isoforms were higher in cellobiose as carbon source (Cristóbal et al., 2009).

Beta-glucosidase production in fungal strains varies in response to the chemical nature of sugar molecule. Also, chemicals reported to be an inducer in one strain may have no effect or may even act as repressor in the other strains. Sophorose is a well known inducer of cellulase. However in *Trichoderma reesei*, sophorose caused repression of β-glucosidase. The low levels of β-glucosidase in *Trichoderma reesei* may be associated with cellulase inducer sophorose which represses β-glucosidase synthesis to prevent itself from getting hydrolyzed. Thus keeping low concentrations of β-glucosidase maximizes sophorose concentration in mycelium causing induction of cellulase (Sternbergt and Mandels, 1980).

Byssochlamys fulva NII 0930 was found to produce several isoforms of the BGL and it was observed that the levels of expression of these proteins were differing when grown in different carbon source leading to the inference that at least some of the BGL isoforms might be inducible. Experiments were conducted to study the effect of carbon sources on induction/repression of BGLs in the fungus.

5.2. Materials and Methods

5.2.1. Preparation of mycelia

Mandel & Weber media was prepared as outlined in section 2.2.2. Four liters of media was added in to a 5 L fermenter and autoclaved. Filter sterilized glucose was added aseptically in to the media to a final concentration of 1 % w/v. Spores from 8 day old culture, suspended in 0.01 % tween 80 were used as inoculum. An agitation of 150 rpm was provided and the system was run at 30 °C for 48 h. Dissolved oxygen was maintained at 50 % level. The mycelia were harvested, washed thrice with sterile buffer and then suspended in sterile citrate buffer (0.05 M, pH 4.8).

5.2.2. Carbohydrates used for induction/repression studies

Monosaccharides, oligosaccharides, polysaccharides and derivatives of carbohydrates were tested as inducers or repressors. The carbohydrates used were glucose, thioglucose, cellobiose, cellobiose octaacetate, lactose, galactose, sucrose, maltose, fructose, salicin, rhamnose, adonitol, arabinose, inositol, mannitol, mannose, melibiose, sorbitol, rutin, quercetin, ribose, xylose, glycerol, trehalose, ascorbic acid, para-nitrophenyl β-D glucopyranoside (pNPG), methyl umbelliferyl β-D glucopyranoside (MUG), sophorose, cellulose, avicel, carboxy methyl cellulose (CMC), microcrystalline cellulose, pectin, xylan, starch, inulin, thiocellobiose and xylitol. To study the combined effect of two sugars, a combination of sophorose (0.5 mg/50 ml) and thioglucose (1.0 mM), thiocellobiose (0.5 mg/50 ml) and thioglucose (1.0 mM), salicin (1.0 mM) and thiocellobiose (0.5 mg/50 ml) were used.

5.2.3. Induction/repression of β-glucosidase by carbohydrates

Fresh mycelia harvested from fermenter were added to 50 ml sterile citrate buffer (0.05 M, pH 4.8) in 250 ml conical flasks so as to achieve a final biomass of 0.5 mg/ml. Polysaccharides (cellulose, avicel, carboxy methyl cellulose, microcrystalline cellulose, pectin, xylan, starch or inulin) were autoclaved separately and added to the induction medium to a final concentration of 0.01 % w/v. Monosaccharides, oligosaccharides and their derivatives were filter sterilized using 0.22 µ syringe filter. Except thiocellobiose and sophorose, all other filter sterilized sugars were added to a final concentration of 1.0 mM. Thiocellobiose and sophorose were added to a final concentration of 1.0 mg/ml. To study the combined effect of known inducers and repressors, combination of two sugars were also tested. These included sophorose (0.5 mg/50 ml) and thioglucose (1.0 mM), thiocellobiose (0.5 mg/50 ml) and thioglucose (1.0 mM), salicin (1.0 mM) and thiocellobiose (0.5 mg/50 ml). The flasks were incubated at 30 °C in an incubator shaker at 200 rpm. Samples (5 ml) were taken at 8 h, 16 h, 24 h and 32 h of incubation and centrifuged at 12000 rpm, 4 °C for 15 min. The supernatants were assayed for β-glucosidase activity and glucose tolerance as per section 2.5.1.

5.2.4. Native PAGE, zymogram analysis and silver staining

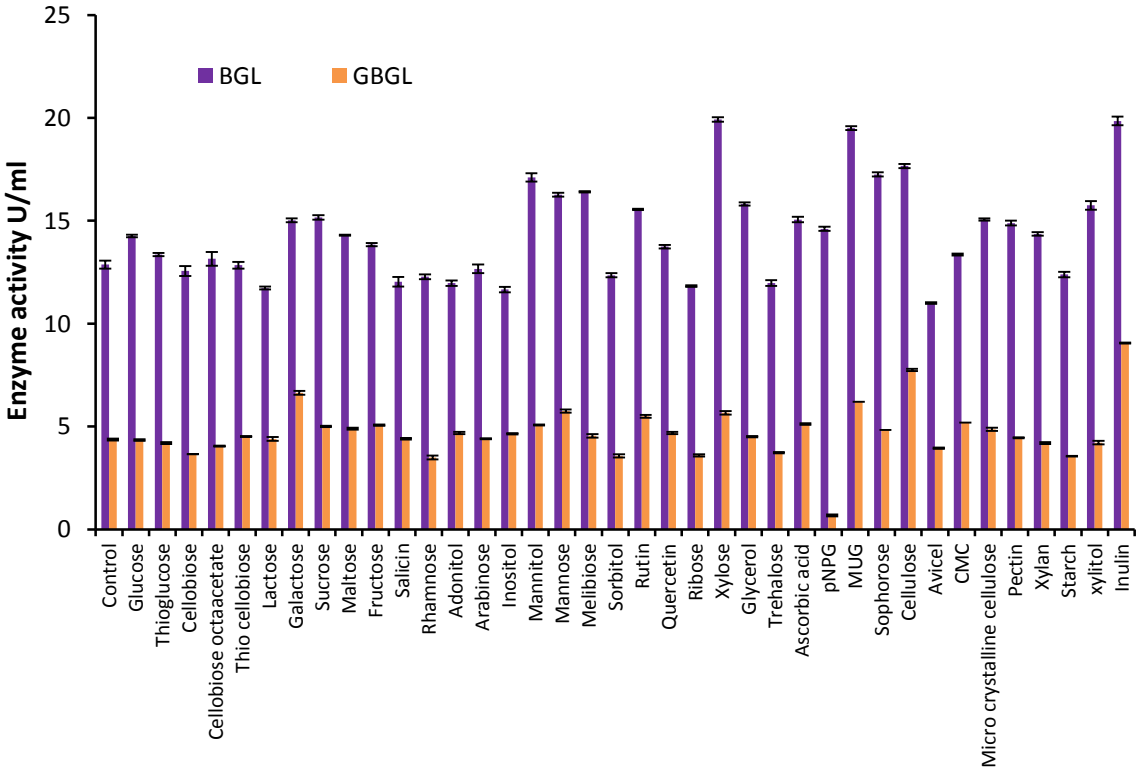
Ten milliliters of the culture supernatant from 32 h incubated flasks of the induction/repression study were precipitated using 4 volumes of acetone as outlined in section 2.4. The precipitate was air dried, dissolved in 100 µl citrate buffer and native PAGE (8 % gel) and zymogram analysis using MUG as substrate was carried out as explained in section 2.6. The gel was visualized under a UV illuminator and photographed using an imaging system (Syngene-GBox, UK). The gel was washed twice with distilled water and silver staining of protein bands were performed according to a modified protocol of Merril et al., 1981. The silver stained gel was photographed under white light using the imaging system (Syngene-GBox, UK).

5.3. Results and Discussion

5.3.1. Enzyme production after 8 h of incubation

Beta-glucosidase production was noticed in all the experimental flasks. In the control, even in the absence carbohydrates, BGL production was noticed (Fig 5.3). This indicates the constitutive nature of BGL secretion by *Byssochlamys fulva* NII 0930. In *Neurospora crassa*, a 6 h conidial induction with monosaccharides and disaccharides on cellobiase and aryl β-glucosidase was reported. As in the present study, control flasks with no added sugar showed β-glucosidase production (Eberhart and Beck, 1973).

Figure 5.3: BGL and GBGL production after 8 h of incubation



Within the first 8 h there was no significant induction or repression of BGL. However, a significant reduction in GBGL production was noticed with pNPG. BGL production was comparatively high with xylose, inulin and MUG. Compared to other

sugars, GBGL production was highest with inulin followed by cellulose, galactose and MUG.

In *Neurospora crassa* among the sugars tested, for strain '74-ORS 1a' maximum induction was noted with cellobiose. An induction of 25 % as compared to cellobiose was noted with maltose, followed by D-galactose, D-glucose and mannose. A low level of induction was noted with D-fructose, trehalose and D-xylose while enzyme activity in D-arabinose, melezitose and lactose was comparable to control. Flasks with D-fucose, L-fucose and melibiose showed absence of cellobiase. A similar pattern was noticed with '33(2-6)A' strain but lactose was found to repress enzyme production (Eberhart and Beck, 1973). In the present study, cellobiose had no significant effect in BGL induction while mannose, D-glucose, melibiose and galactose showed slight inductive effect. Lactose had a repressive effect on BGL production. In *Neurospora crassa*, for aryl β-glucosidase, cellobiose was the promising inducer, followed by galactose. Maltose, xylose, D-arabinose, mannose and D-glucose showed moderate induction. In strain '74-ORS 1a' repression was noted with D-fucose, L-fucose, melibiose, melezitose, trehalose and lactose. In '33(2-6)A' strain, D-fucose, D-fructose, D-glucose, D-mannose, melibiose was found to repress enzyme activity (Eberhart and Beck, 1973).

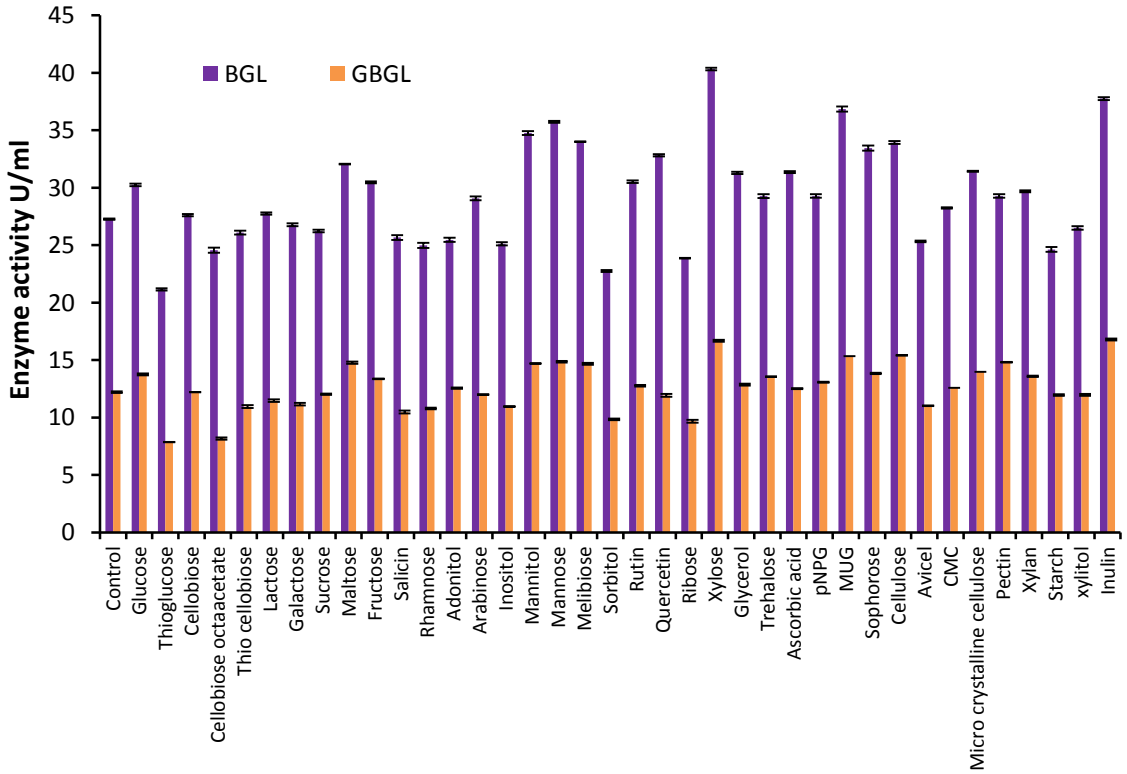
Among heteroglucosides and sugar derivatives, arbutin showed induction of both enzymes in the two strains of *Neurospora crassa*. In both strains, salicin and pNPG was found to be the inducer and quercetin to be the repressor of aryl β-glucosidase (Eberhart and Beck, 1973). In the present study, pNPG had significant effect on GBGL production and showed considerable levels of repression. The control flask recorded a GBGL activity of 4.37 U/ml while flask with pNPG showed only 0.68 U/ml GBGL activity.

5.3.2. Enzyme production after 16 h of incubation

With 16 h of incubation, induction and repression of BGL and GBGL synthesis were evident, even though the basal level expression continued. Xylose was the best

inducer for BGL, followed by inulin and MUG (Fig 5.4). Among the cellulosic substrates, cellulose and microcrystalline cellulose behaved as inducers for BGL while avicel and CMC had no significant effect on induction. Production of BGL in cellobiose flask was comparable to that of control. Repression of BGL synthesis was evident with thioglucose and sorbitol causing marked decrease in BGL production. Interestingly, glucose was found to exhibit a minor inductive effect on BGL production. For GBGL production, xylose and inulin was found to be the best inducers followed by cellulose and MUG. Repression of GBGL was evident with thioglucose and cellobiose octaacetate. Minor levels of repression to GBGL production was noted with ribose, sorbitol, salicin, rhamnose, thiocellobiose, inositol, avicel, galactose, lactose, quercetin, starch and xylose. In *Scytalidium thermophilum*, addition of glucose, fructose, cellobiose and ethanol caused induction of BGL and among the monosaccharides, cellobiose was found to be the best inducer. Sucrose, mannitol, glycerol and sorbitol behaved as repressors to BGL production (Kaur et al., 2006)

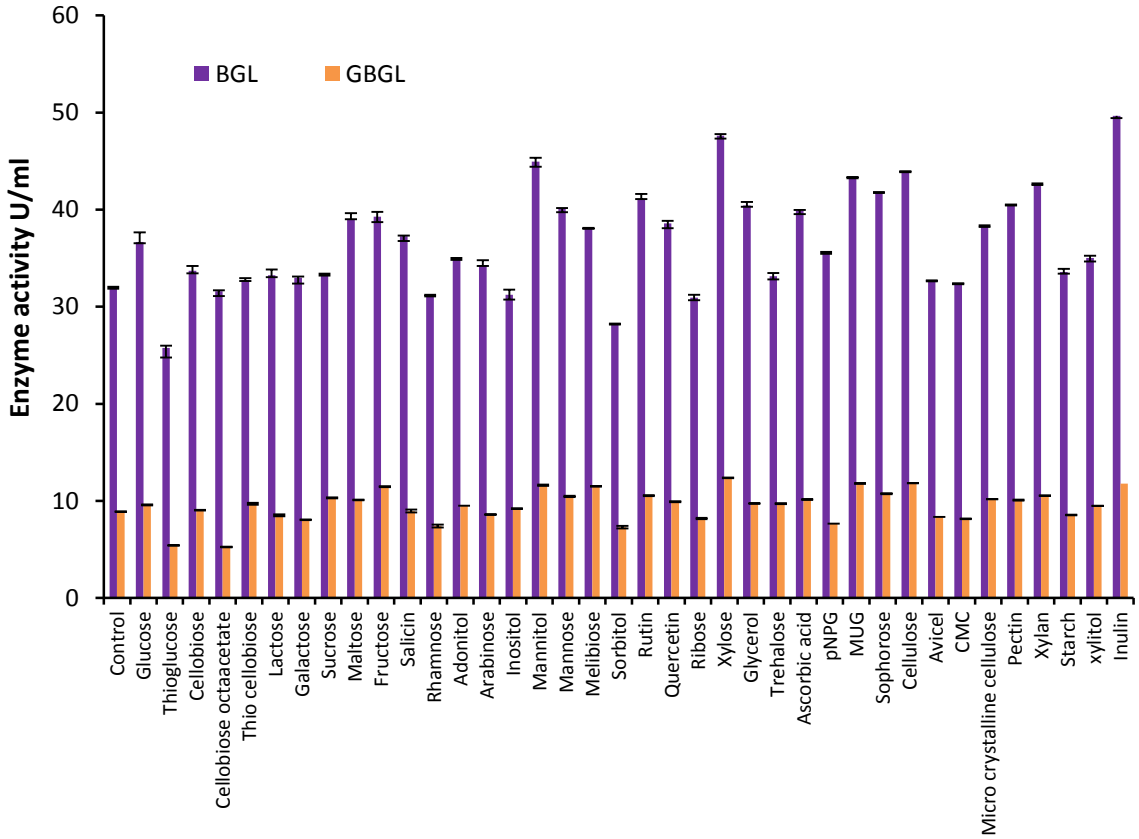
Figure 5.4: BGL and GBGL production after 16 h of incubation



5.3.3. Enzyme production after 24 h of incubation

With 24 h of incubation inulin showed high levels of BGL induction (Fig 5.5) followed by xylose, mannitol and cellulose (≥ 44 U/ml) as compared to control (31.8 U/ml). Inulin is a polymer of fructose and glucose based oligosaccharides. In rats, administration of inulin resulted in increased β-glucosidase activity in gut. This result indicates the stimulatory effect of inulin in β-glucosidase secretion by gut micro flora (Rowland et al., 1998). There was marked repression of BGL production with thioglucose (25.74 U/ml) followed by sorbitol (28.2 U/ml). As for GBGL production, among the tested chemicals xylose was the best inducer followed by cellulose, MUG, inulin, mannitol, melibiose and fructose. Cellobiose octaacetate and thioglucose showed marked repression of GBGL production.

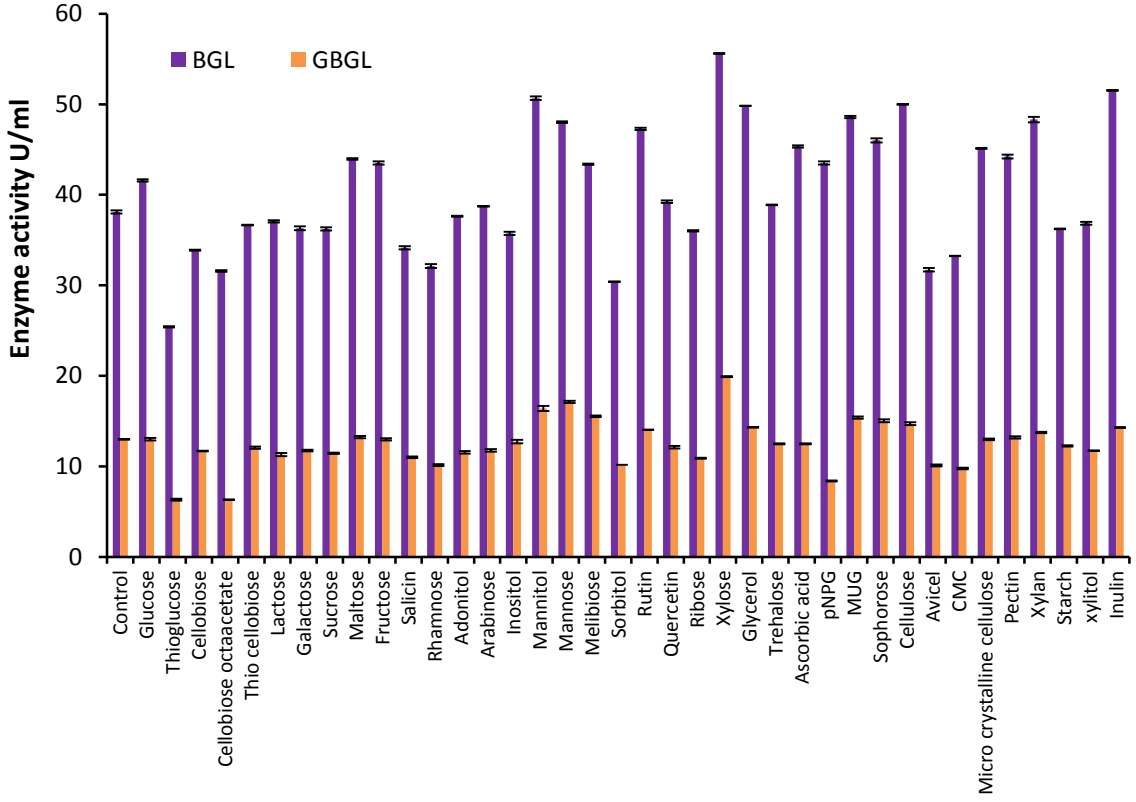
Figure 5.5: BGL and GBGL production after 24 h of incubation



5.3.4. Enzyme production after 32 h of incubation

With 32 h of incubation, xylose showed high levels of BGL induction followed by inulin, mannitol, cellulose and glycerol (≥ 50 U/ml) as compared to control (38.1 U/ml). There was marked repression of BGL production with thioglucose (25.4 U/ml) followed by sorbitol (30.4 U/ml). As for GBGL production, among the tested chemicals xylose was the best inducer followed by mannose and mannitol. Thioglucose and cellobiose octaacetate showed repression of GBGL (Fig 5.6).

Figure 5.6: BGL and GBGL production after 32 h of incubation



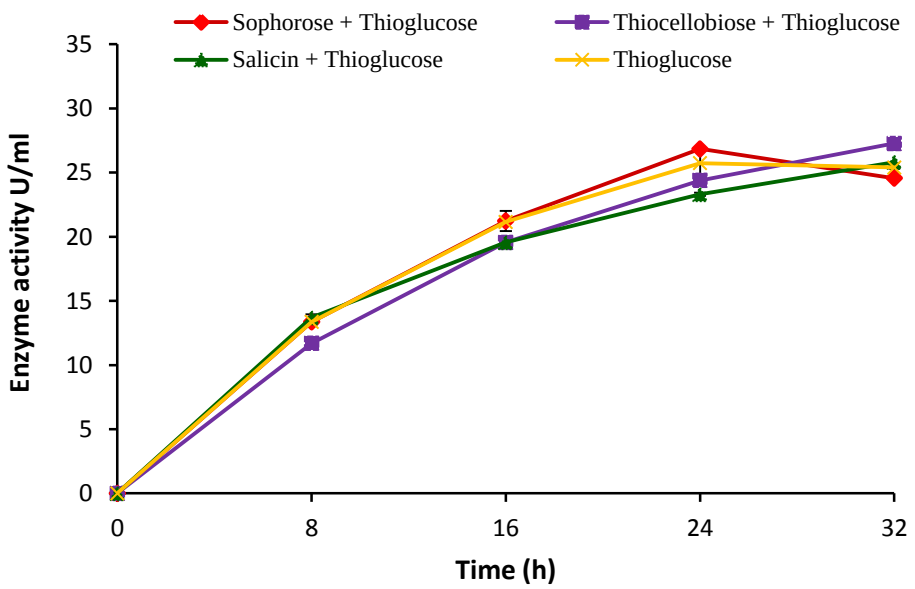
No β-glucosidase and cellulase production was seen when glucose was used as the carbon source for growth of *Trichoderma harzianum*. However, mycelial biomass were found to be the highest compared to fungi grown in CMC, sigma cell, lactose, wheat bran or corncob as carbon source indicating effect of glucose in cellulase repression (Aslam et al., 2010). In *Trichoderma harzianum*, CMC as carbon source was

found to be a cellulase inducer while glucose showed strong repression (Ahmed et al., 2009). *Scytalidium thermophilum* MTCC 4520, when grown in avicel and Solka-Floc produced high titers of BGL than in glucose, fructose, carboxy methyl cellulose, rice straw or wheat bran (Kaur et al., 2006). *Monascus purpureus* produced a constitutive level of β-glucosidase in presence of glucose and higher enzyme production occurred only after glucose depletion. This indicates the inducible nature of β-glucosidase in *M. purpureus* and the catabolic repression controlled by glucose (Daroit et al., 2007). Among rutin, quercetin, cellobiose and glucose tested for induction, quercetin was found to be the best inducer for glucose tolerant β-glucosidase production in *Aspergillus oryzae* and *Aspergillus niger* (Günata and Vallier, 1999). In *Acremonium persicinum*, extracellular β-glucosidase was found to be an inducible enzyme, prone to repression by simple sugars. Growth of fungi in sophorose containing medium resulted in a 10 fold increase in enzyme production. Gentiobiose, laminaribiose, laminaritriose, laminaritetraose, also showed inductive effect while easily metabolized sugars like glucose, sucrose, maltose, fructose, galactose, lactose showed repression (Piston et al., 1999). In *Myceliophthora thermophila,* pNPG was the best inducer for β-glucosidase production and glucose showed repression to enzyme production (Roy et al., 1988).

5.3.5. Effect of sophorose, thiocellobiose and salicin in thioglucose induced repression of BGL and GBGL production

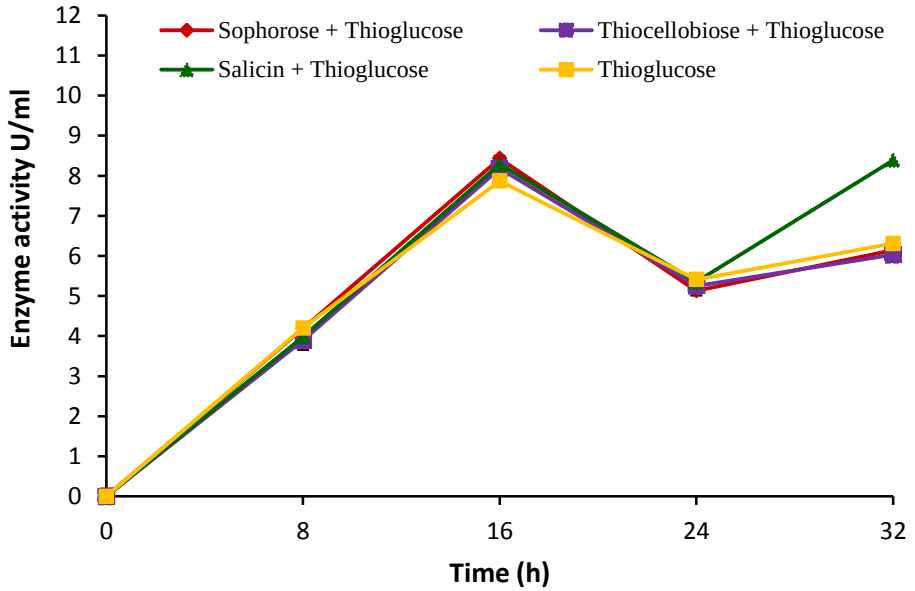
In the present study, thioglucose showed repression of both BGL and GBGL production. Addition of thiocellobiose, salicin or sophorose did not reverse the repressive effect of thioglucose on BGL production (Fig 5.7).

Figure 5.7: Effect of sophorose, thiocellobiose and salicin in thioglucose induced repression of BGL production



The same pattern was noticed for GBGL production but after 16 h of incubation, the repressive effect of thioglucose was partially reduced by salicin (Fig 5.8).

Figure 5.8: Effect of sophorose, thiocellobiose and salicin in thioglucose induced repression of GBGL production



In *Melanocarpus* sp. MTCC 3922, BGL production was high with avicel as carbon source. Compared to glucose, fructose showed a higher repression to BGL synthesis. Addition of glucose, fructose, cellobiose, sucrose, glycerol, sorbitol, mannitol and ethanol to fungi growing in 1 % CMC had repressive effect; maximum repression was noted with glucose, followed by ethanol, mannitol, and sorbitol (Kaur et al., 2006). In *Humicola grisea* var. *thermoidea*, avicel was found to be the best carbon source for β-glucosidase production. However, addition of 1 % glucose in to culture media containing avicel, CMC, sugar cane bagasse, wheat bran or cellobiose caused severe repression of BGL production (Nascimento et al., 2010).

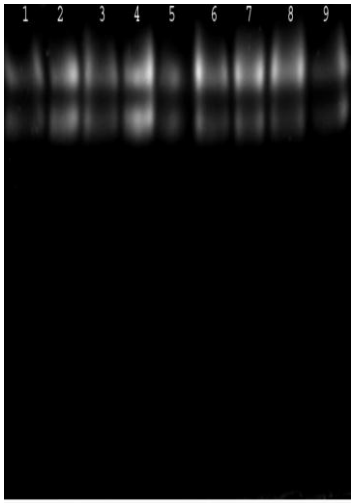
In the production medium containing sucrose as the carbon source, addition of glucose caused repression of β-glucosidase production in *Penicillium purpurogenum*. Addition of glucose did not stop growth of the organism but with increasing concentration of glucose, there was increase in the inhibition of enzyme production (Dhake and Patil, 2005). Growth of *Acremonium persicinum* in *Eisenia bicyclis* laminarin resulted in β-glucosidase production. When glucose was added to the laminarin, only negligible quantities of β-glucosidase were produced. Enzyme was detected only after complete exhaustion of glucose, supplementation of media with additional glucose further delayed the appearance of β-glucosidase. This corresponds to the strong repression β-glucosidase synthesis by glucose (Piston et al., 1999). In the present study, thioglucose was found to show more repressive effect than glucose possibly due to its 'thio' group that makes it difficult to get metabolized.

5.3.6. Native PAGE and zymogram analysis of β-glucosidase produced after 32 h of incubation

Only two bands of activity were noted in the zymogram analyses. The isoforms produced in the experiment corresponds to BGL 2 and BGL 3. Similar bands of activity were noted in all flasks including control (Fig 5.9: A, B, C, D).

Figure 5.9: Native PAGE and zymogram analysis of β-glucosidase

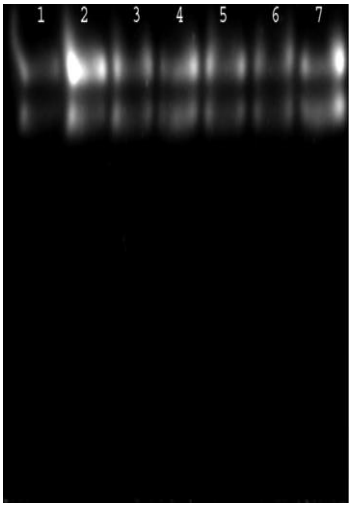
Gel A



Lane information

Lane 1 –Glucose
Lane 2 –Thioglucose
Lane 3 –Cellobiose
Lane 4 –Cellobiose-octaacetate
Lane5 –Thiocellobiose
Lane 6 –Lactose
Lane 7 –Galactose
Lane 8 –Sucrose
Lane 9 -Maltose

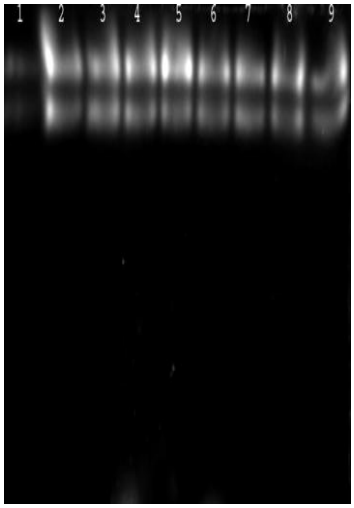
Gel B



Lane information

Lane 1 –Fructose
Lane 2 –Salicin
Lane 3 –Rhamnose
Lane 4 –Adonitol
Lane 5 –Arabinose
Lane 6 –Inositol
Lane 7 – Mannitol

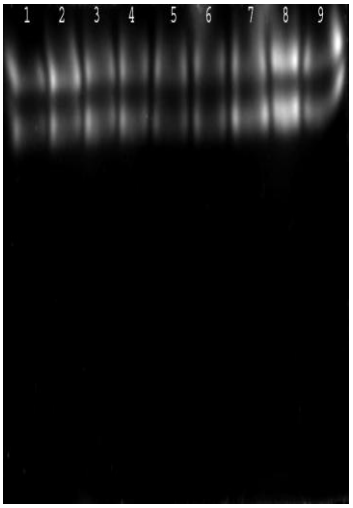
Gel C



Lane information

Lane 1 –Mannose
Lane 2 –Melibiose
Lane 3 –Sorbitol
Lane 4 –Rutin
Lane 5 –Quercetin
Lane 6 –Ribose
Lane 7 –Xylose
Lane 8 –Glycerol
Lane 9 -Trehalose

Gel D



Lane information

Lane 1 –Ascorbic acid
Lane 2 –pNPG
Lane 3 –MUG
Lane 4 –Sophorose
Lane 5 –Control
Lane 6 –Thioglucose + Sophorose
Lane 7 –Thioglucose + Thiocellobiose
Lane 8 –Thioglucose + Salicin
Lane 9 -Xylitol

In *Debaryomyces pseudopolymorphus*, cellobiose as sole carbon source in production media caused inductive effect but glucose was found to show repression towards β-glucosidase synthesis. One of the isoforms of β-glucosidase from this yeast was found to be constitutive and was not repressed by glucose (Arévalo Villena et al., 2006). *Monilia* sp. produced two intracellular (IG 1, IG 2) and one extracellular (EG 1)

β-glucosidase. IG 1 was a constitutive enzyme; cellobiose, cellulose and glucose seemed not to affect its production and only a basal level of production occurred compared to IG 2 and EG 1. IG 2 and EG 1 were inducible with highest level of activity when grown in cellulose. Cellobiose and glucose repressed the synthesis of IG 2 and EG1 and enzyme synthesis got de-repressed only when the carbohydrate level decreased in the medium. The repression of synthesis of IG 2 and EG 1 by cellobiose may be due to the accumulation of glucose in cells as a result of hydrolytic activity of IG 1 (Berry and Dekker, 1984).

In *Neurospora crassa*, aryl β-glucosidase and cellobiase were found to be inducible enzymes. Aryl β-glucosidase was found to be induced first, and after a one hour lag, cellobiase was induced. After 7 h of incubation, no further induction was noticed probably due to limitation of cellobiose which was supplied to a final concentration of 1 mM. Prior to conidia formation, the aryl β-glucosidase appeared to be semi constitutive (Eberhart and Beck, 1973). Beta-glucosidase from *Aspergillus nidulans* was found to be constitutive. In this case, the washed mycelia after starvation in saline were transferred to induction medium with various carbon sources at a final concentration of 0.05 % w/v. Beta-glucosidase production in presence of cellobiose, gentiobiose, lactose, sophorose, melibiose, trehalose, amygdalin, esculin, salicin and methyl β-glucoside was comparable to control that lacked any sugar. However, enzyme production showed catabolite repression. Glucose and 2-deoxy glucose (0.05 % w/v) fully inhibited enzyme production while glycerol (0.4 % v/v) reduced the enzyme production (Lee et al., 1996).

BGL production under optimized SmF conditions as outlined in section 4.3.3.2 resulted in the production of 7 isoforms (chapter 6, figure 6.4,). In the induction/repression studies, native PAGE and zymogram indicated only two bands of activity with comparable intensities in all flasks including control. This probably indicates the constitutive nature of the β-glucosidase isoforms BGL 2 and BGL 3 in *Byssochlamys fulva* while the other isoforms –BGL 1, BGL 4, BGL 5, BGL 6 and

BGL 7 could be inducible. It is likely that these isoforms are produced in low quantities and is hence difficult to detect under the conditions tried for induction studies.

Cellobiose is considered as a potential inducer of cellulase. However in the present study, cellobiose was not found to have any effect on β-glucosidase induction. A similar experiment in *Penicillium purpurogenum* also showed cellobiose to be ineffective in cellulase induction. This was explained to be due to the presence of constitutive extracellular β-glucosidase that cleaved cellobiose into glucose before cellobiose could enter into the cell. Crude intracellular enzyme when incubated with cellobiose showed transglycosylation activity, producing gentiobiose and laminaribose, while crude extracellular enzyme did not show transglycosylation property (Kurasawa et al., 1992). Thiogentiobiose is found to be a non-metabolizable inducer (Kurasawa et al., 1992). Similarly, thiocellobiose can be considered as a non-metabolizable sugar. Thiocellobiose is an inhibitor to β-glucosidase as evident from NMR studies using β-glucosidase from *Streptomyces* (Montero et al., 1998). In the present study, like cellobiose, thiocellobiose also had no inductive effect on β-glucosidase production. However in *Schizophyllum commune*, thiocellobiose was reported as an inducer (Rho et al., 1982).

The mechanism of induction is not fully understood. Once the inducer enters the cell, it activates certain proteins. These protein activators and activating elements (e.g. CAE in *Hypocrea jecorina*) triggers transcription of cellulase gene (Suto and Tomita, 2001). Some chemicals may have an inductive effect, but due to variation in the localization of the BGL produced, the inductive effect may not be evident. In *Trichoderma longibrachiatum*, cellobiose induced β-glucosidase was mostly localized in cytosol and cell debris, while use of cellulosic substrate resulted in a greater concentration of enzymes in culture filtrate (Sandhu and Sidhu, 1985). Most of the BGL in *Trichoderma pseudokoningii* was found to be cell wall associated when grown in lactose. However, when grown in lactose-sorbose medium, there occurred rapid release of enzyme into the culture fluid. This effect occurred only when sorbose was added in the beginning of cultivation and no late addition could initiate this phenomenon. The

mechanism is thought to be due to decreased availability of cell wall 1,3 glucan to which β-glucosidase binds. L-sorbose inhibit β-1,3-glucan synthase and hence the newly synthesized BGL doesn't have enough β-1,3–glucan to get attached and hence got released in to the culture fluid (Kubicek, 1983). Also appearance of increased levels of extracellular β-glucosidase in stationary phase may be due to autolysis of mycelium and not due to increased enzyme synthesis caused by the exhaustion of repressor chemicals (Piston et al., 1999).

Carbon catabolite repressor protein CRE1 could be produced constitutively in the fungal mycelium while the sub-cellular localization of it depends up on the extracellular carbon source. In *Sclerotinia sclerotiorum*, CRE1 was present in the nuclear fraction of mycelia grown in glucose containing media. When the mycelium was transferred to medium containing other carbon sources, CRE1 was detected in cytosol fractions. Thus the presence of glucose results in localization of CRE1 protein in the nucleus, which binds to the repressor binding region located upstream of cellulase genes and thus regulates transcription (Suto and Tomita, 2001).

Studies in human cell lines indicate differential mode of regulation of gene expression in β-glucosidase production. In epithelial, lymphoblast, histiocyte, glioblastoma and astrocytoma cell lines, a direct relationship was seen between mRNA levels and enzyme activity indicating transcriptional control of BGL expression. Fibroblast, promyelocyte and neuroglioma cell lines also showed a direct relation between mRNA and enzyme activity but compared to first group, enzyme activity was 6 fold than expected. In single monocyte cell line, high levels of mRNA were noted but only moderate level of enzyme activity could be detected. Also mRNA from normal cells was comparable to mRNA from patients with Gaucher's disease. This indicates that regulation of β-glucosidase activity is complex and may occur at multiple levels (Doll and Smith, 1993). In the present study, isoforms BGL 2 and BGL 3 are constitutive while BGL 1, BGL 4, BGL 5, BGL 6 and BGL 7may be inducible but the process of induction or secretion seems to be much complicated than the presence or absence of carbohydrates.

5.4. Conclusion

Beta-glucosidase production in *Byssochlamys fulva* NII 0930 was found to be regulated by complex factors than presence or absence of carbohydrates. BGL 2 and BGL 3 isoforms were found to be constitutively expressed and was secreted even in the absence of carbohydrates. However enhancement of production was noticed with xylose, inulin, mannitol, cellulose and glycerol. Thioglucose was found to repress BGL production. BGL 1, BGL 4, BGL 5, BGL 6 and BGL 7 may be inducible enzymes but more complex factors may be involved in induction and secretion.

Chapter 6

Purification and characterization of the β-glucosidase isoforms from *Byssochlamys fulva* NII 0930

Chapter 6

Purification and characterization of the β-glucosidase isoforms from *Byssochlamys fulva* NII 0930

6.1. Introduction

Purification helps to study enzymes in a simple system for understanding their properties, kinetics, structure, regulations and role in complex systems. Purification of enzymes is also important for their specific applications in medical and industrial processes. Level of purity required for the enzyme depends on the specific application. The strategies for purification of enzyme may be based on size or mass (centrifugation, gel filtration, dialysis, ultra filtration); polarity (ion-exchange chromatography, chromatofocusing, electrophoresis, isoelectric focusing, hydrophobic chromatography); solubility (change in pH, change in ionic strength, decrease in dielectric constant) and on specific binding sites or structural features (affinity chromatography, dye-ligand chromatography, immuno-adsorption, covalent chromatography, immobilized metal ion chromatography). The type of purification strategy employed depends on the structure and catalytic stability of the enzyme, localization of enzyme, specific application of purified enzyme and cost of purification.

Several methods are employed in the purification of β-glucosidase; the steps involved become more complicated with increase in the number of isoforms. Cell associated β-glucosidases in *Volvariella volvacea* were purified using sequential steps of chromatography through DEAE Sepharose CL-6B column, Sephacryl HR S-200, again through DEAE Sepharose CL-6B column and finally FPLC through Mono Q HR 5/5 column. FPLC on Mono P HR 5/5 column resulted in the separation of BGL 1 and BGL 2 isoforms (Cai et al., 1998). The culture filtrate of *Aspergillus tubingensis* was treated with DEAE-Sephadex, the bound proteins were eluted and passed through DEAE-Sepharose fast flow column. Two fractions of β-glucosidase activity were subjected to gel permeation using Sephacryl S-200 HR column. Further purification

using Mono P HR 2/20 anion exchange column resulted in the separation of total β-glucosidase into four isoforms (Decker et al., 2001).

Isoforms of β-glucosidase vary in their kinetic properties (Table 1.3). Cell wall bound β-glucosidases (BGL I and BGL II) in *Pichia etchellsii* were purified using a three step method involving ammonium sulphate precipitation, followed by ion exchange and hydroxyapatite chromatography. Both the isoforms showed similar temperature optima (50 °C), pH optima (6.0), pH stability and *Km* value (0.33 mM for pNPG), but varied in their molecular size and activity towards substrates. BGL I had a molecular mass of 156 kDa and showed specificity to aryl glucosides while BGL II with molecular mass 350 kDa showed more affinity to cellobiose (Wallecha and Mishra, 2003).

In the present study, the isoforms of BGL from *B. fulva* NII 0930 were purified to homogeneity using a combination of steps including acetone precipitation, cation exchange chromatography, anion exchange chromatography and gel permeation chromatography. The purified isoforms were characterized for their kinetic parameters.

6.2. Materials and Methods

6.2.1. Enzyme purification

6.2.1.1. Acetone precipitation of enzyme

Enzyme production was carried out by submerged fermentation using the optimized conditions of Box-Behnken design as described in Chapter 4 (Section 4.3.3.2) in a total volume of 1000 ml. For intracellular enzyme extraction, the fungus was grown in 1 % xylose as described in section 2.3.3. Extraction of the extracellular enzymes was performed as outlined under section 2.3.2 and intracellular enzyme as under section 2.3.3. For obtaining crude enzyme precipitate, 4 volume of chilled acetone (-20 °C) was added to cooled extract. The mixture was kept for 12 h at -20 °C, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The pellet was allowed to dry at room temperature to remove residual acetone. The pellets were then resuspended in 50 ml of 0.05 M citrate buffer (pH 4.8), and were used for further studies.

6.2.1.2. Acetone fractionation of crude enzyme preparation

Partial purification of the extracellular enzyme was carried out using acetone fractionation with increasing volume of solvent. Approximately 2000 ml of the crude enzyme preparation was used for acetone fractionation. To cooled crude enzyme extract, chilled acetone (-20 °C) was added at a ratio of 1: 0.5 v/v and the mixture was kept for 12 h at -20 °C, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The supernatant was recovered and acetone was added to further increase the ratio to 1:1. These steps were continued to obtain ratios of 1:1.5, and 1:2 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 50 ml of 0.05 M citrate buffer (pH 4.8), and were used for further studies.

6.2.1.3. Cation exchange chromatography

Half volume acetone precipitated BGL (1 v extract: 0.5 v acetone) and one volume acetone precipitated BGL (1 v extract: 1 v acetone) were purified by ion exchange chromatography on a cation exchange column (UNOsphere-S-matrix, Biorad, USA). Experiments were performed on a Biorad LP system. The column was equilibrated with citrate buffer (0.05 M, pH 3.0). Sample in citrate buffer (0.05 M, pH 3.0) was loaded by running it at 0.5 ml/min flow rate through the column and washed by running three column volumes of the loading buffer (0.05 M citrate buffer, pH 3.0) through the column at 0.5 ml/min. Elution was performed using a continuous gradient of NaCl from 0-1.0 M and 4 ml fractions were collected. Fractions giving the maximum activity were pooled together, dialyzed against 5 mM citrate buffer (pH 4.8) and were used for further purification.

6.2.1.4. Anion exchange chromatography

Two volume acetone precipitated BGL (1 v extract: 2 v acetone) was purified by ion exchange chromatography on an anion exchange column (High Q support, Biorad, USA). Experiments were performed on a Biorad LP system. The column was

equilibrated with Tris-HCl buffer (0.05 M, pH 7.5). Sample in Tris-HCl buffer (0.05 M, pH 7.5) was loaded by running it at 0.5 ml/min flow rate through the column and washed by running three column volumes of the loading buffer (0.05 M Tris-HCl buffer, pH 7.5) through the column at 0.5 ml/min. Elution was performed using a continuous gradient of NaCl from 0-1.0 M and 4 ml fractions were collected. Fractions giving the maximum activity were pooled together, dialyzed against 5 mM citrate buffer (pH 4.8) and were used for further purification.

6.2.1.5. Size exclusion chromatography

Sephadex G-200 (Sigma-Aldrich) was used as the matrix. The matrix was prepared as per the manufacturer's protocols, diluted with citrate buffer (0.05 M, pH 4.8) and loaded on to column. Experiments were performed on a Biorad LP system. The column was equilibrated with citrate buffer (0.05 M, pH 4.8) containing 0.14 M NaCl by running the buffer at 0.2 ml/min flow rate through the column for 16 h. Enzyme purified after anion exchange chromatography was used as the sample. 0.5 ml enzyme, concentrated by ultra-filtration using 10 kDa Amicon® centrifugal concentrator (Millipore, U.S.A), and filter sterilized through 0.22 µ syringe filter (Millipore, U.S.A) was loaded onto the column. Elution was done with citrate buffer (0.05 M, pH 4.8) containing 0.14 M NaCl at 0.2 ml/min flow rate. Fractions giving enzyme activity were collected and dialyzed against 5 mM citrate buffer (pH 4.8).

6.2.2. Characterization of the isoforms of β-glucosidase

Seven isoforms in extracellular fractions and four isoforms in intracellular fractions were characterized for their molecular weight by SDS PAGE. Three isoforms in the extracellular fractions were purified to homogeneity. The optimal temperature and pH of activity of the purified isoforms and their temperature stability were determined. Kinetics with pNPG as substrate and glucose inhibition kinetics of the three isoforms were also determined.

6.2.2.1. Determination of the molecular weight of isoforms of β-glucosidase

The 4 v acetone precipitated crude enzyme (intra and extracellular) and 1 v and 2 v acetone fractionated extracellular enzyme were run on SDS PAGE (Laemmli, 1970) along with standard protein markers (SM 1811 and SM 0661, Fermentas, USA). BGL activity bands were visualized by MUG staining and the zymogram was photographed as outlined in section 2.6. 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 overlapping the photographs of zymogram and the silver stained gel and the molecular weights were determined by comparison with the standard protein markers of known molecular weight.

6.2.2.2. Determination of the optimal temperature of activity for BGL 2, BGL 3, BGL 6

The purified isoforms were assayed at different temperatures using pNPG as substrate as outlined under section 2.5.1. Glucose tolerance under different temperature was also estimated as outlined under section 2.5.1. Optimal temperature was defined as the temperature at which maximum pNPG hydrolysis occurred, detected by increase in enzyme activity.

6.2.2.3. Determination of the optimal pH of activity for BGL 2, BGL 3, BGL 6

Optimal pH for the activity of isoforms was determined by assaying the enzymes at different pH. The differences in pH were attained by use of 0.05 M concentration of different buffers as given below (pH 1-2: HCl-KCl buffer; pH 3: Glycine-HCl buffer; pH 3-6: Citrate buffer; pH 7- 8: Na-Phosphate buffer; pH 7- 9: Tris-HCl buffer; pH 9-10: Glycine-NaOH buffer; pH 10: Na-Carbonate-bicarbonate buffer). Optimal pH was defined as the pH at which enzyme showed maximal activity.

6.2.2.4. Temperature stability of BGL 2, BGL 3, BGL 6

Temperature stability of isoforms was studied at 50 °C, which is the temperature at which cellulases are used for biomass hydrolysis. The enzyme preparation in citrate buffer (0.05 M) with optimum pH was incubated in a water bath maintained at 50 °C and samples withdrawn at different intervals were assayed (according to section 2.5.1.). Relative activity against 0 h incubation was calculated for each time interval and plotted against incubation time.

6.2.2.5. Kinetics of BGL 2, BGL 3, BGL 6 with pNPG as substrate

The *Vmax* and *Km* value of the purified isoforms with pNPG as substrate was determined graphically using a Lineweaver-Burk plot. BGL assays were performed (section 2.5.1.) at different time intervals (2.5 - 15.0 min) for multiple pNPG concentrations (1.0 - 10 mM for BGL 2, BGL 3; 10-100 mM for BGL 6) at the optimum temperature and pH for activity. The 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 intersect Y axis corresponds to V*max* and the value of [S] at which the line intersect X axis corresponds to *Km.*

6.2.2.6. Glucose inhibition kinetics of BGL 2, BGL 3, BGL 6

The glucose inhibition constant (*Ki*) of the purified isoforms were determined graphically using a Dixon Plot (Dixon, 1953). BGL assays were performed (section 2.5.1.) with different concentrations of glucose (0-500 mM for BGL 2; 0-250 mM for BGL 3; 0-2.5 M for BGL 6) for multiple pNPG concentrations (0.5-6.0 mM for BGL 2, BGL 3 and 4-20 mM for BGL 6) at 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 *Ki* for glucose inhibition. For BGL 6, inhibition kinetics at 55 °C was also done to determine the *Ki* value for glucose inhibition.

6.2.3. Transgylcosylation property of BGL

Transglycosylation property of BGL from *B. fulva* NII 0930 was studied. One ml of crude acetone precipitated BGL (100 U/ml) was mixed with 2.0 ml citrate buffer (0.05 M, pH 4.8) and 1.0 ml 20 mM cellobiose. The mixture was incubated at 50 °C for 30 minutes. The samples were heated at 80 °C for three minutes and was spotted on a TLC plate and ascending chromatography was performed using ethyl acetate, chloroform, water (3:2:1) as running solvent. The chromatogram was developed by spraying orcinol reagent (1 % orcinol dissolved in 10 % H_2SO_4 in ethanol) and incubating at 60 °C for 2 minutes.

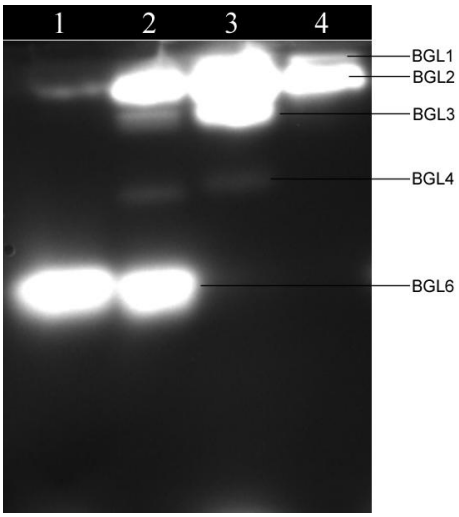
6.3. Results and discussion

6.3.1. *Acetone fractionation of BGL*

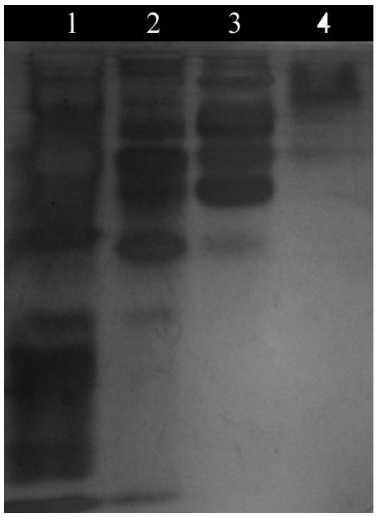
The solubility of protein depends on, among other things, the dielectric constant of the solution. Acetone added to aqueous enzyme preparation can reduce the dielectric constant and thereby help in precipitating the proteins (Bell et al., 1983). Acetone has the advantage of being relatively inexpensive and is available in a pure form with few contaminants that may inhibit or poison the enzyme. It can also be recovered and recycled. Molecular weight of the protein is an important determinant of the amount of acetone required for precipitation and in general, low molecular weight proteins are precipitated in a higher ratio of acetone (Simpson, 2004). In the present study, use of acetone was successful for precipitation of enzyme and fractionation of isoforms. With 4 v of acetone, all the isoforms were precipitated. High molecular weight isoforms like BGL 1 and BGL 2 were precipitated with 1:0.5 v/v acetone. An increase of acetone to 1 v resulted in the precipitation of BGL 2, BGL 3, and BGL 4 isoforms. With increase in acetone concentration, there was precipitation of low molecular weight isoforms. In 1:1.5 v/v acetone BGL 2, BGL 3, BGL 4 and BGL 6 isoforms were precipitated and with 1:2 v/v BGL 6 was precipitated (Fig 6.1).

Figure 6.1. Native PAGE analysis of acetone fractionated extract

A- Zymogram analysis



B – Silver staining of proteins



Lane information

Lane 1 – 1:2 v/v ppt.

Lane 2 – 1:1.5 v/v ppt.

Lane 3 – 1:1 v/v ppt.

Lane 4 – 1:0.5 v/v ppt.

Maximum specific activity was exhibited by the fraction obtained using 1:0.5 v/v acetone precipitation (Table 6.1). However maximum activity retention in 1.0 M glucose was seen in 1:2 v/v acetone precipitated fraction. Beta-glucosidase in these fractions showed very high levels of specific activity.

Table 6.1: Fractionation of BGL by acetone precipitation

Fraction	Enzyme activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Glucose tolerance % activity retention	
				0.5 M glucose	1.0 M glucose
Crude extract	1560.42	0.40	3876.46	25.68	17.22
1:0.5 v/v acetone precipitate	10758.33	0.65	16625.42	4.37	1.88
1:1 v/v acetone precipitate	5865.97	0.88	6629.85	6.65	2.92
1:1.5 v/v acetone precipitate	1376.39	0.69	1998.33	19.78	12.97
1:2 v/v acetone precipitate	6855.56	1.72	3991.84	40.84	24.13

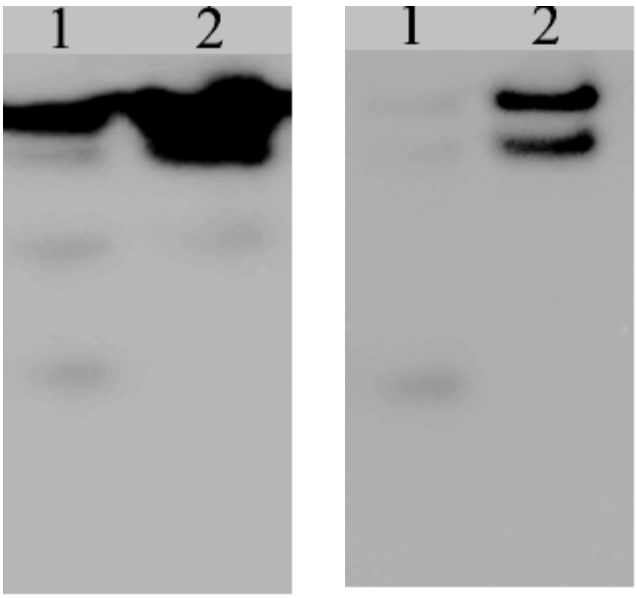
In *Podophyllum peltatum*, a β-glucosidase with specific activity of 75800 U/mg protein was reported (Dayan et al., 2003). *Thermoascus aurantiacus* IMI 216529 was found to secrete an extracellular β-glucosidase. After growing the fungus in fermentor with 300 L of medium containing paper waste, wheat bran and yeast extract for 6 days, the culture supernatant was processed for BGL purification. After DEAE Sepharose, Ultrogel AcA 44 and Mono-P column chromatography, a single band of protein with β-glucosidase activity was obtained. The total protein concentration of purified β-glucosidase was only 1 mg and the enzyme showed a high specific activity of 190000 U/mg protein (Parry et al., 2001). In the present study, even the 1:0.5 v/v acetone fraction consisting of several other proteins showed a specific activity of 16625 U/mg protein. This indicates the efficiency of β-glucosidase secreted by *B. fulva* NII 0930. Filamentous fungi like *Trichoderma* have highly efficient protein secretion machinery where by strains could produce 40 g/L cellulase (Iwashita, 2002). Thus cloning and expression of *bgl* gene under a strong secretion machinery could increase protein secretion and there by total β-glucosidase production.

Byssochlamys belongs to the family Trichocomaceae. In a phylogenetic study, the taxa Trichocomaceae is found to be of three lineages and the second lineage was further divided in to two clades, one containing *Thermoascus* and the other containing *Paecilomyces* and *Byssochlamys*. Results indicate a strong relation (posterior probability value of 0.99) between these two clades. Both *Thermoascus* and *Paecilomyces* are thermo-tolerant or thermophilic genera and also phylogenetically, members of *Thermoascus* like *Thermoascus crustaceus* share a *Paecilomyces* anamorph in *Byssochlamys/Paecilomyces* clade of Trichocomonaceae (Houbraken and Samson, 2011). Thus *Thermoascus aurantiacus* IMI 216529 and *B. fulva* NII 0930 are phylogentically related taxa and this may explain the similarities seen with the high specific activities of BGL secreted by the strains.

Zymogram analyses of crude and 1:1 v/v acetone fractions were carried out in absence and presence of 1.0 M glucose. Among the isoforms, BGL 6 was found to show high levels of activity even in 1.0 M glucose. Other isoforms like BGL 2, BGL 3 and BGL 4 were found to show decreased activity in presence of glucose (Fig 6.2).

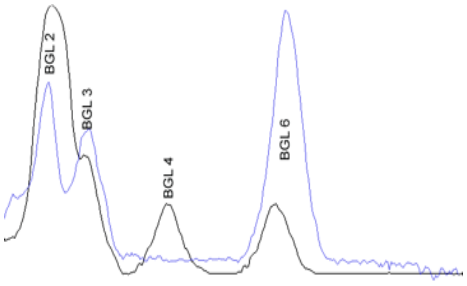
Figure 6.2. Zymogram analysis of crude and acetone precipitates (inverted images)

A- Zymogram (No Glucose) B – Zymogram (1.0 M Glucose)



Lane information

Lane 1 – Crude acetone ppt.
Lane 2 - 1:1 v/v acetone ppt.



C- Plot showing the signal intensities of methyl umbelliferol fluorescence due to BGL activity for Lane 1 (Black line – No glucose , Blue line – 1.0 M glucose)

In many instances, glucose tolerant isoforms were low molecular weight proteins. Of the four isoforms of β-glucosidase in *Aspergillus tubingensis*, BGL I and BGL II were high molecular weight proteins with a mass of 131 kDa and 126 kDa, showing low tolerance to glucose. BGL III and BGL IV had a similar mass of 54 kDa but were highly glucose tolerant (Decker et al., 2001). A similar result was obtained in the present study with BGL 6 of 35 kDa showing high levels of glucose tolerance. Among the two isoforms of β-glucosidase from *Aspergillus oryzae*, BGL I had a molecular mass above 130 kDa and was strongly inhibited by glucose. BGL II had a molecular weight of 30 kDa but was glucose tolerant with a high *Ki* value of 953 mM (Günata and Vallier, 1999). In *Aspergillus oryzae*, major isoform had a molecular weight of 130 kDa and was highly inhibited by glucose. The other isoform, produced in lower quantities with a molecular mass of 43 kDa was highly resistant to glucose inhibition (Riou et al., 1998). Beta-glucosidase from *Candida peltata* had a glucose tolerance with *Ki* value 1.4 M, having a molecular weight of 43 kDa (Saha and Bothast, 1996). In pixel density analysis (Fig 6.2), BGL 6 showed more signal intensity in presence of 1.0 M glucose than in the absence of glucose. This may be due to the stimulatory effect of glucose on the activity

of BGL 6. Such stimulatory effect of glucose on low molecular weight BGL from *Scytalidium thermophilum* has been reported (Zanoelo et al., 2004).

6.3.2. SDS PAGE and zymogram analysis for determining molecular weight

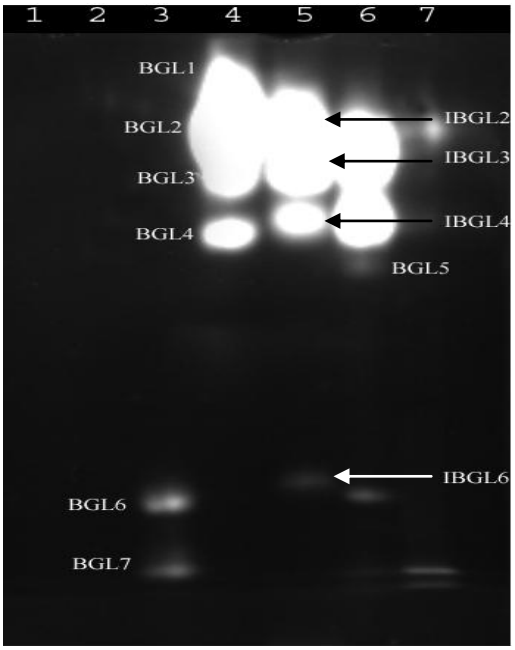
The acetone precipitated fractions of extracellular enzyme showed seven bands of activity in SDS PAGE (Fig 6.3- A, B). They correspond to BGL 1, BGL 2, BGL 3, BGL 4, BGL 5, BGL 6 and BGL 7 isoforms of β-glucosidase from *B. fulva* NII 0930. The acetone precipitated intracellular crude enzyme showed four distinct bands on zymogram corresponding to IBGL 2, IBGL 3, IBGL 4 and IBGL 6 isoforms (Fig 6.3- A, B). Interestingly, the position of these intracellular isoforms was comparable to BGL 2, BGL 3, BGL 4 and BGL 6 isoforms, but obviously having a higher molecular weight than the corresponding extracellular isoforms. The molecular weight determined by non-reductive SDS PAGE was as follows (Table 6.2).

Table 6.2: Molecular weights of secreted and intracellular BGLs of *B. fulva* NII 0930 as determined by SDS PAGE

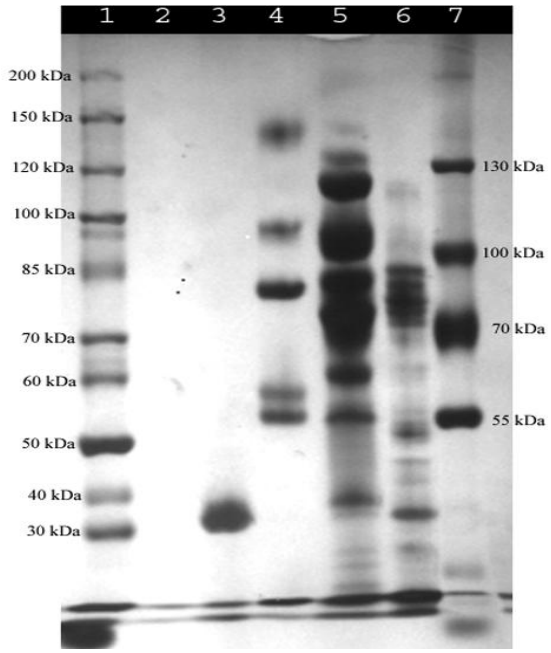
Secreted BGL		**Intracellular BGL**	
Designation/ Name	Molecular Weight (kDa)	Designation/ Name	Molecular Weight (kDa)
BGL 1	195	-	-
BGL 2	135	IBGL 2	140
BGL 3	120	IBGL 3	125
BGL 4	94	IBGL 4	98
BGL 5	70	-	-
BGL 6	35	IBGL 6	41
BGL 7	27	-	-

Figure 6.3: Zymogram and electrophoretic analysis of crude and acetone precipitates fractions

A. Zymogram analysis



B. Silver staining of proteins



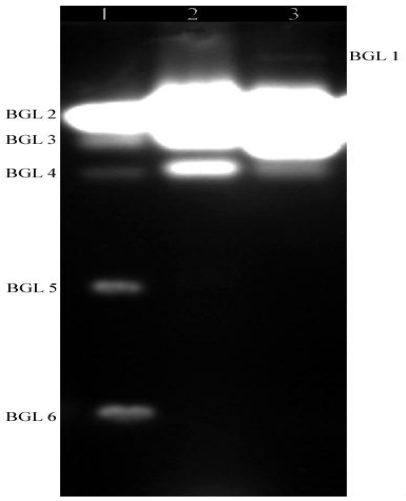
Lane information

Lane 1 – Marker-SM 0661
Lane 3 –1:2 v/v acetone ppt (extracellular)
Lane 4 - 1:1 v/v acetone ppt (extracellular)
Lane 5 - Crude acetone precipitated intracellular enzyme
Lane 6 - Crude acetone precipitated extracellular enzyme
Lane 7- Marker-SM1811

In a similar work with *Aspergillus* sp., we had reported the presence of two extracellular and two intracellular BGL. The electrophoretic position of intracellular enzymes were comparable to the extracellular ones but intracellular enzymes had higher molecular weight than the extracellular isoforms (Elyas et al., 2010). Isoforms of β-1,3-glucanases were noted in intracellular and apoplastic regions in leaves of *Avena sativa*. The intracellular and extracellular isoforms had similar isoelectric points but significant differences were noted in molecular weight with extracellular isoforms having higher molecular weight. They attribute this to the presence of neutral carbohydrates in extracellular enzymes (Fink et al., 1988). The difference in molecular weight in intra and extracellular isoforms may be due to the presence of additional peptides like signal peptide in intracellular forms or partial de-glycosylation of extracellular isoforms.

All the isoforms from *B. fulva* were found to be monomeric proteins, with similar activity bands in native PAGE (Fig 6.4) and SDS PAGE.

Figure 6.4: Zymogram and Native PAGE analysis of acetone precipitates fractions



Lane Information

Lane 1 - 1:1.5 v/v acetone ppt

Lane 2 - 1:1.0 v/v acetone ppt

Lane 3 - 1:0.5 v/v acetone ppt

Both monomeric and multimeric β-glucosidases are reported in fungi. The multimeric forms may be a homo-dimer with subunit molecular mass (SDS PAGE) of 94 kDa in *Candida wickerhamii* (Skory et al., 1996), 120 kDa in *Aspergillus niger* (Seidle et al., 2004), 28 kDa in *Penicillium decumbens* (Mamma et al., 2004), 71 kDa in *Penicillium aurantiogriseum* (Petruccioli et al., 1999), 118 kDa in *Aspergillus pulverulentus* (Mase et al., 2004) or hetero-dimer of 80 kDa and 60 kDa in *Alternaria alternata* (Martínez et al., 1988) or trimer with sub unit molecular mass (SDS PAGE) of 120 kDa in *Thermoascus aurantiacus* (Parry et al., 2001), 102 kDa in *Paecilomyces* (Yan et al., 2008), 110 kDa in *Aspergillus niger* (Rashid and Siddiqui, 1997) or tetramer with sub unit molecular mass (SDS PAGE) of 60 kDa in *Candida sake* (Gueguen et al., 2001), 61.1 kDa in *Penicillium aurantiogriseum* (Petruccioli et al., 1999), 115 kDa in *Penicillium decumbens* (Mamma et al., 2004), 95 kDa in *Aspergillus fumigatus* (Kitpreechavanich et al., 1986).

Monomeric 81 kDa (by SDS PAGE) BGL is reported in *Trichoderma reesei* (Chirico and Brown, 1987), 110 kDa in *Penicillium purpurogenum* (Jeya et al., 2010) and *Fusarium oxysporum* (Christakopoulos et al., 1994), 92 kDa in *Melanocarpus* (Kaur et

al., 2007), 50 kDa in *Talaromyces thermophilus* (Nakkharat and Haltrich, 2006), 53 kDa in *Phanerochaete chrysosporium* (Nijikken et al., 2007), 43 kDa in *Chaetomium thermophilum* var. *coprophilum* (Venturi et al., 2002), *Aspergillus oryzae* (Riou et al., 1998) and *Candida peltata* (Saha and Bothast, 1996), 167 kDa in *Chalara paradoxa* (Lucas et al., 2000), 77 kDa in *Aspergillus oryzae* (Zhang et al., 2007), 128 kDa in *Acremonium persicinum* (Piston et al., 1999) and 85 kDa in *Thermoascus aurantiacus* (Shepherd et al., 1988). In SDS PAGE, the four isoforms of β-glucosidase (I-IV) from *Aspergillus tubingensis* behaved as monomeric proteins with molecular mass of 131, 126, 54 and 54 kDa (Decker et al., 2001).

6.3.3. Purification of BGL 2

BGL 2 in 1:0.5 v/v acetone precipitated protein fraction was further purified by cation exchange chromatography. BGL 2 was bound to the column at pH 3.0. On elution with a gradient of NaCl, BGL 2 eluted as a single peak of activity corresponding to fractions 24-27 (Fig 6.5). Five other peaks of protein were detected but no BGL activity was seen in those fractions. The fractions showing BGL activities were pooled and dialyzed against 5 mM citrate buffer (pH 4.8). Zymogram analysis of pooled fractions showed a single activity band while no band of protein was seen in silver staining (Fig 6.6). Presence of activity band and lack of protein band in gel may be attributed to the high specific activity of BGL 2.

Figure 6.5: Ion exchange chromatography profile of BGL 2

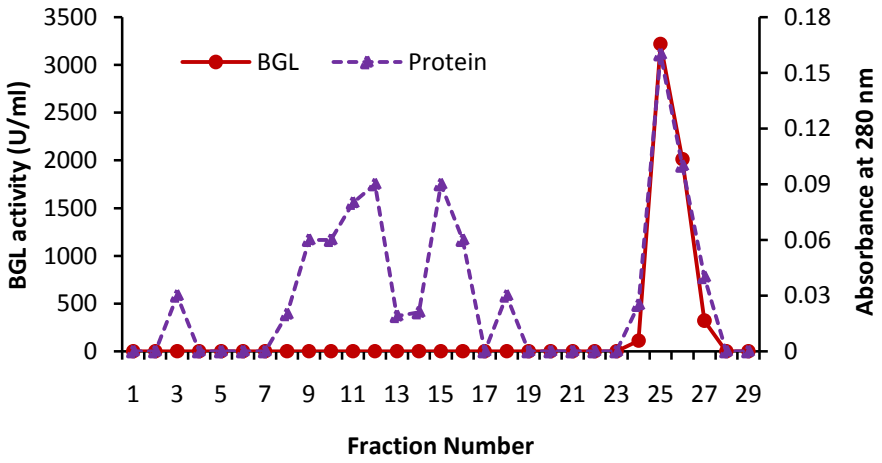
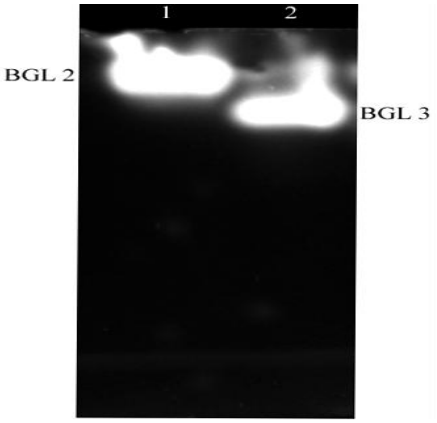


Figure 6.6: Zymogram and Native PAGE analysis of cation exchange fractions



Lane Information

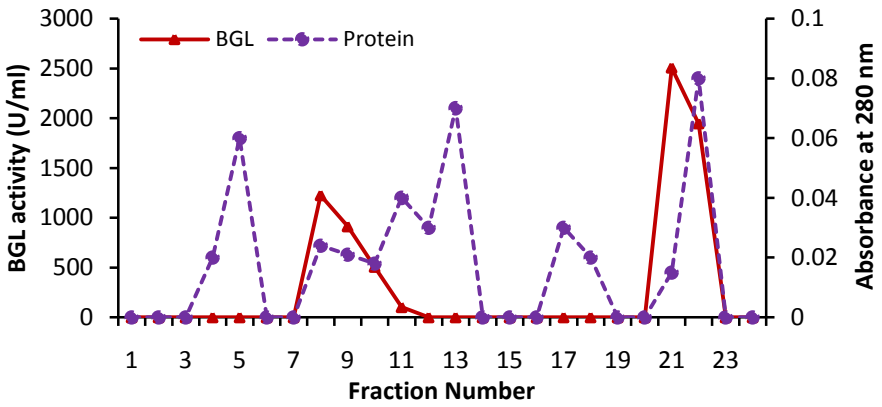
Lane 1- Fractions 24-27
(cation exchange 1:0.5 v/v ppt)

Lane 2- Fractions 8-12
(cation exchange 1:1.0 v/v ppt)

6.3.4. Purification of BGL 3

Beta-glucosidases in 1:1 v/v acetone precipitated protein fraction were further purified by cation exchange chromatography. Binding of BGL to UNOsphere-S ® - matrix was highest at pH 3.0. On a gradient of NaCl, BGL eluted as two peaks of activities corresponding to fractions 8-11 and fractions 23-24 (Fig 6.7). The fractions 8-12, were pooled together and dialyzed against 5 mM citrate buffer (pH 4.8). Similarly the fractions 23-24 were pooled together and dialyzed against 5 mM citrate buffer (pH 4.8). Zymogram showed a single activity band corresponding to BGL 3 in pooled fractions of 8-12 (Fig 6.6) while two bands of activity were seen in pooled fractions of 23-24, corresponding to BGL 2 and BGL 4. Purity of BGL 3 was confirmed by silver staining. Similar to BGL 2, no protein bands were detected.

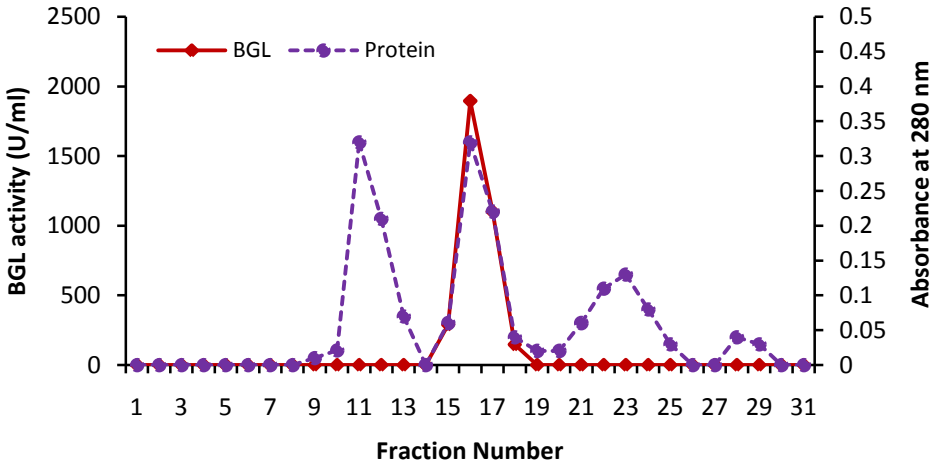
Figure 6.7: Ion exchange chromatography profile of BGL 2, BGL 3 and BGL 4



6.3.5. Purification of BGL 6

Beta-glucosidase in 1:2 v/v acetone precipitated protein fraction was further purified by anion exchange chromatography. BGL was bound to the column at pH 7.5 and on a gradient of NaCl; it was eluted as a single peak of activity corresponding to fractions 15-18 (Fig 6.8). There were 4 major protein peaks spanning fractions 9-13, 15-19, 20-25 and 28-29 respectively of which the BGL activity corresponded to the peak spanning fractions 15-19. The fractions with BGL activity were pooled together and dialyzed against 5 mM citrate buffer (pH 4.8). Zymogram showed a single band of activity but multiple bands of protein were detected in silver staining.

Figure 6.8: Ion exchange chromatography profile of BGL 6



The enzyme was concentrated by ultra filtration using a 10 kDa cutoff membrane and size exclusion chromatography was performed as described under section 6.2.1.5. Fractions 18-20 showing the highest protein peak also showed BGL activity (Fig 6.9). Zymogram showed a single activity band corresponding to BGL 6. Purity of BGL 6 was confirmed by silver staining where a single protein band corresponding to BGL 6 was detected (Fig 6.10).

Figure 6.9: Size exclusion chromatography profile of BGL 6

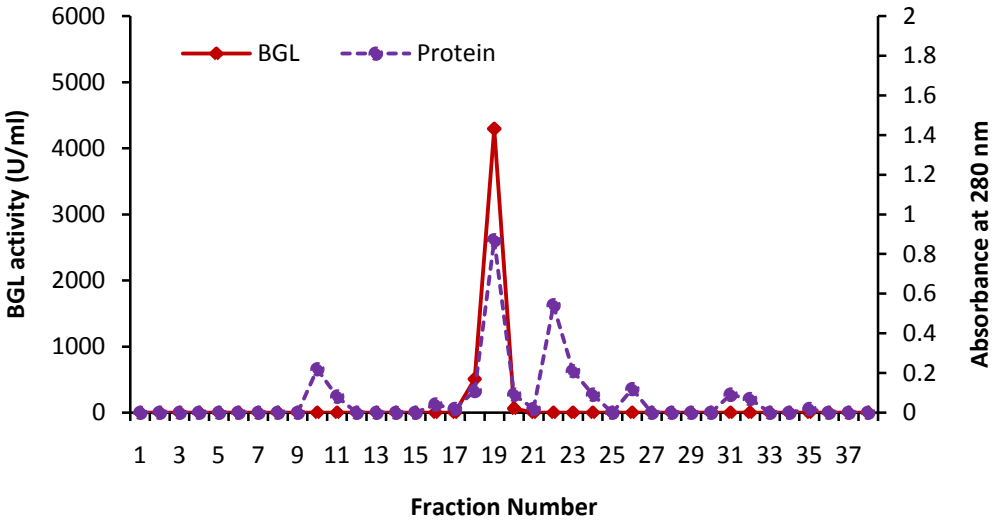
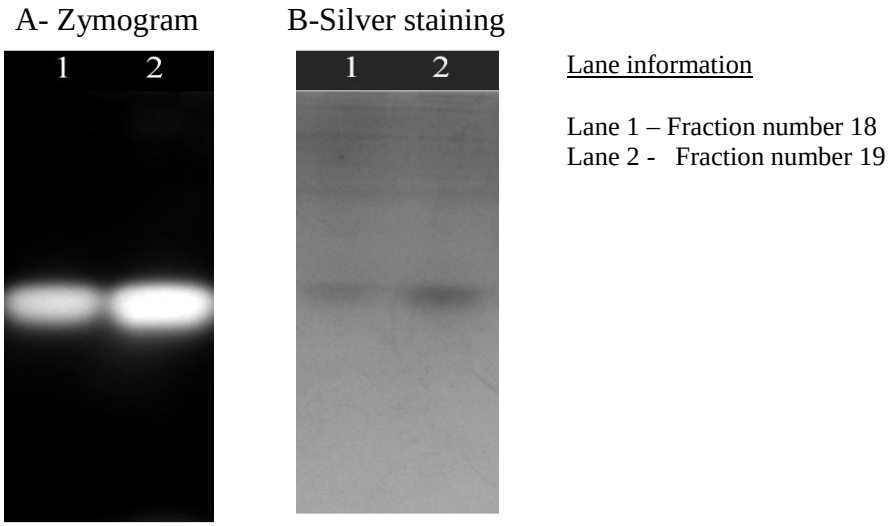


Figure 6.10: Zymogram analysis of fractions from size exclusion chromatography

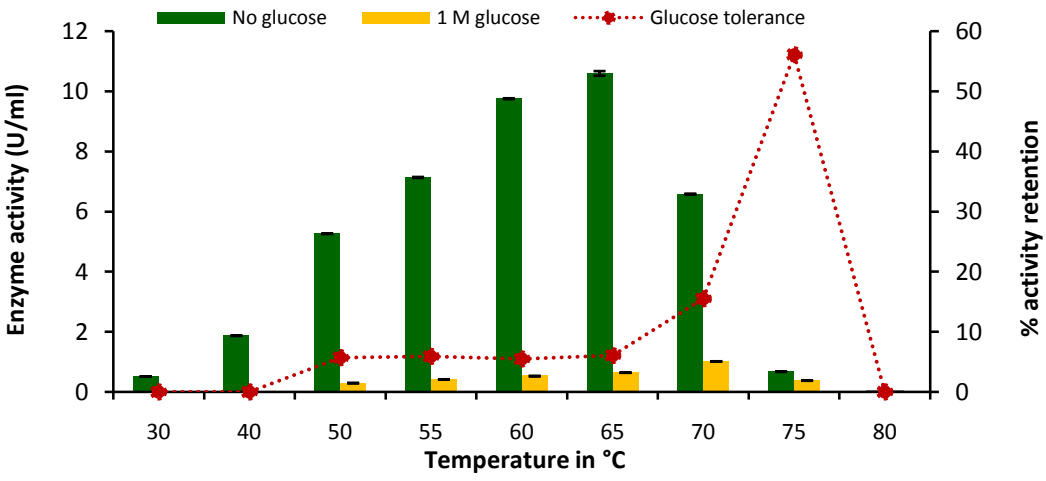


6.3.6. Optimum temperature for activity

BGL 2 showed an optimum activity at 65 °C among the temperatures tested and thereafter an increase in temperature resulted in a decreased enzyme activity (Fig 6.11). At 80 °C the enzyme showed no hydrolytic activity. The enzyme was found to get inhibited by glucose. Among the tested temperatures, maximum activity in presence of 1.0 M glucose was seen at 70 °C. Activity retention was found to be highest at 75 °C

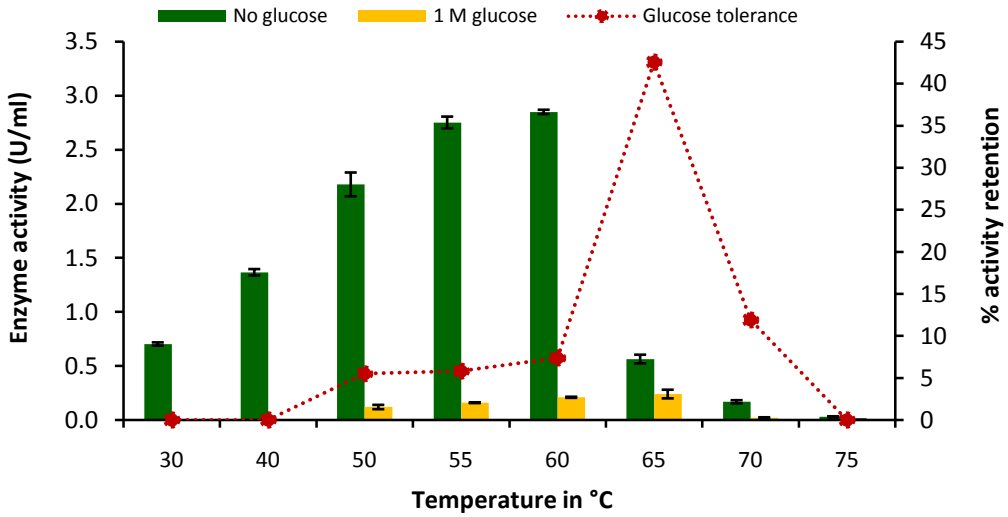
indicating a positive effect of glucose on enzyme activity at higher temperature. Optimum temperature of activity varies with fungal taxa from 20 °C in *Saccharomyces cerevisiae*, *Pichia anomala* (Barbagallo et al., 2004) to 80 °C as in *Thermoascus aurantiacus* (Parry et al., 2001). Fungi like *Penicillium purpurogenum* (Jeya et al., 2010), *Trichoderma reesei* (Chen et al., 1992), *Penicillium decumbens* (Mamma et al., 2004), *Chaetomium thermophilum* (Venturi et al., 2002), *Aspergillus japonicus* (Decker et al., 2000; Korotkova et al., 2009), *Aspergillus aculeatus*, *Aspergillus foetidus*, *Aspergillus niger*, *Termitomyces clypeatus* (Sengupta et al., 1991) and *Thermomyces lanuginosus* (Lin et al., 1999) are found to secrete β-glucosidase with an optimum temperature at 65 °C. Two isoforms of β-glucosidases, BGL I and BGL II from *Aspergillus tubingensis* had an optimum temperature of activity at 65 °C (Decker et al., 2001). High temperature of reaction helps in solubilizing substrates and prevents microbial contamination. Cellulase from *Thermoascus aurantiacus* (Shepherd et al., 1988; Voutilainen et al., 2008), *Chrysosporium lucknowense* (Bukhtojarov et al., 2004) and *Chaetomium thermophilum* (Voutilainen et al., 2008) have a temperature optima of 65 °C. We have also observed that the highly efficient cellulases from *Trichoderma reesei* and *Penicillium janthinellum* though has sustained optimal activity around 50-55 °C, are highly active with an enhanced reaction rate at 60 -65 °C (unpublished results). With a high temperature optimum, BGL 2 can be supplemented to these cellulase enzymes for efficient hydrolysis of biomass.

Figure 6.11: Determination of the optimal temperature for BGL 2 activity



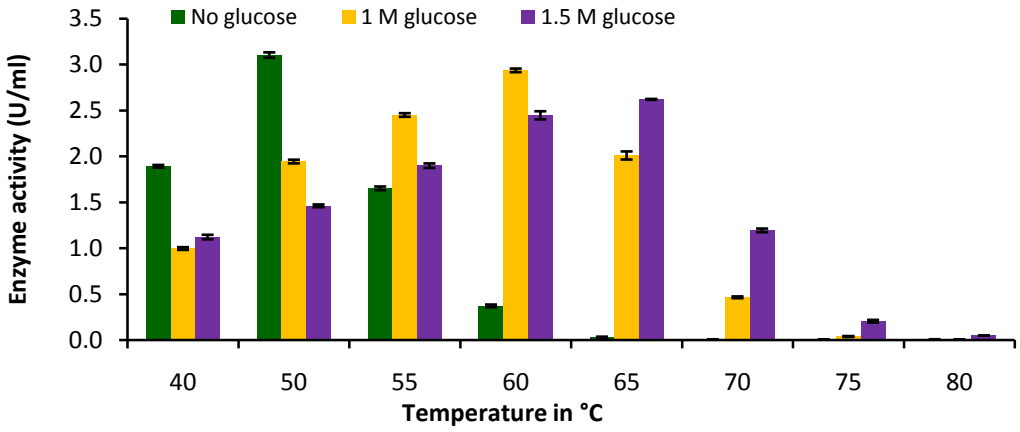
BGL 3 showed an optimum temperature of activity at 60 °C, an increase in temperature beyond the optimum caused decreased enzyme activity (Fig 6.12). A similar enhancement of activity in presence of 1.0 M glucose beyond optimum temperature, as noted in BGL 2 was also seen in BGL 3. In presence of 1.0 M glucose, the optimum temperature of activity was at 65 °C and the same temperature showed maximum activity retention. 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* (Kimura 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). Cellulases from many fungi have an optimum temperature for activity at 60 °C. Such fungal cellulases as from *Acremonium thermophilum*, *Hypocrea jecorina* (Voutilainen et al., 2008), *Trichoderma viride* (Beldman et al., 1985) and *Aspergillus terreus* (Garg and Neelakantan, 1982) may be supplemented with BGL 3 for efficient biomass hydrolysis.

Figure 6.12: Determination of the optimal temperature for BGL 3 activity



Among the temperatures tested, BGL 6 was most active at 50 °C. Beyond 60 °C, the activity was minimal and no activity was seen at 70 °C (Fig 6.13). Similar pattern of glucose enhancing the optimum temperature for activity as in BGL 2 and BGL 3 was also noticed in this isoform. With 1.0 M glucose, optimum temperature of activity was at 60 °C and with 1.5 M glucose, maximal activity was obtained at 65 °C. A peculiar feature was noted in this glucose tolerant isoform. Though glucose is an inhibitor, at 55 °C enzyme was more active in 1.0 M glucose than in the absence of glucose. At 60 °C the phenomenon was more evident and at 65 °C, maximum enzyme activity was noted at 1.5 M glucose. At 70 °C and 75 °C, though no enzyme activity was noted in the absence of glucose, the enzyme became active at this in presence of the "inhibitor", the more activity being recorded at higher glucose concentrations. A number of fungi produce BGL with optimum activity at 50 °C. This include BGL from *Thermoascus aurantiacus* (Hong et al., 2006), *Talaromyces thermophilus* (Nakkharat and Haltrich, 2006), *Monascus purpureus* (Daroit et al., 2008), and *Humicola grisea* (Nascimento et al., 2010). Beta glucosidase from *Candida peltata* (Saha and Bothast, 1996) and *Aspergillus oryzae* (Riou et al., 1998), well known for their high glucose tolerance, had a similar temperature optima as with the glucose tolerant isoform BGL 6.

Figure 6.13: Determination of the optimal temperature for BGL 6 activity



Beta-glucosidase gene (*bgl3*) isolated from a thermophilic actinomycete *Microbispora bispora* was cloned and expressed as intracellular protein BGL B in *E. coli*. At optimum temperature of activity (60 °C), BGL B was more active in presence of

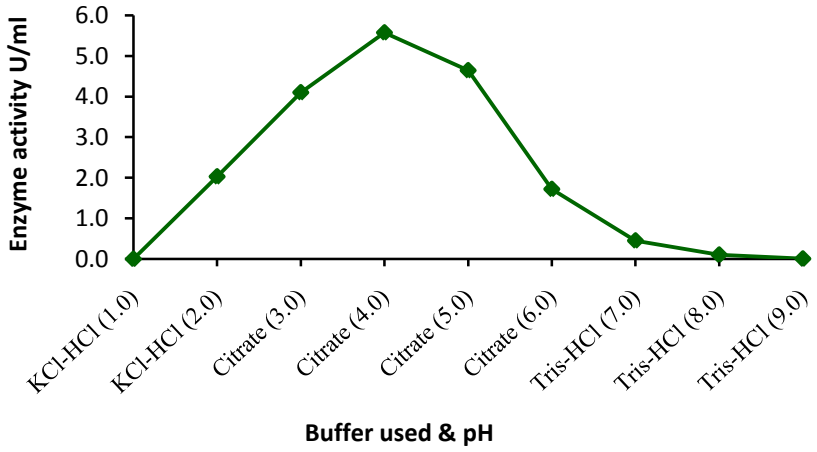
glucose. At a glucose concentration of 0.1-0.3 M, there was a 2 to 3 fold enhancement in activity. The enzyme activity became lower than the control (no glucose) only when concentration of glucose was raised to 2.2 M (Wright et al., 1992). In *Humicola grisea* var. *thermoidea,* enzyme activity was enhanced 2.2 fold with glucose concentration of 100-175 mM at 50 °C. The activity decreased thereafter with increase in glucose concentration; however enzyme activity was more compared to control till 500 mM glucose. A similar stimulation of activity was also noted with xylose. The stimulatory effect of glucose on *H. grisea* var. *thermoidea* was attributed to its possible binding to a monosaccharide-specific site which may induce a conformational change at the active site that results in increased hydrolysis rate. With increase in glucose concentration, competition between glucose and the substrate for the active site may occur, leading to a gradual decrease in enzyme activity. At higher glucose concentrations, the competition for the active site becomes more severe, resulting in an activity lower than that observed in the absence of the monosaccharide (Nascimento et al., 2010). Interestingly, BGL from *Humicola grisea* and BGL 6 in the current study had similar temperature and pH optimum.

In *Aspergillus terreus,* monosaccharides like glucose, xylose, arabinose, galactose and fructose as well as disaccharides like salicin, cellobiose and sucrose significantly enhanced activity of βGI isoform. Glucose reduced the activity of βGII and βGIII, while other monosaccharides had no effect. Also cellobiose and salicin decreased activity of both βGII and βGIII (Nazir et al., 2009). In *Aspergillus niger*, β-glucosidase incubated with cellobiose or pNPG resulted in the extension of shelf life by about 5 fold. It was explained as the possible protective effect of substrates on the catalytic site of the enzyme (Yan and Lin, 1997). Beta-glucosidase from *Saccharomyces cerevisiae* was more active in presence of glucose. At 30 °C, maximum enhancement was noticed with 15 % glucose (Spagna et al., 2002). In *Scytalidium thermophilum*, increase in BGL activity was noted with glucose and xylose, causing a 2.6 and 2.4 fold increase at 150 mM. Only a small decrease in enhancement was noted at 200 mM. At 500 and 700 mM, the activity was comparable to that in the absence of glucose or xylose. Mannose, fructose, ribose, galactose, L-sorbose, arabinose, lactose, cellobiose, maltose, sucrose at 50 mM level had no enhancement effect (Zanoelo et al., 2004).

6.3.7. Optimum pH for activity

Among the pH tested for β-glucosidase activity, BGL 2 showed an optimum pH 4.0 for activity. BGL 2 was active at acidic pH from 1.5-6.0 (Fig 6.14). Many fungi like *Aureobasidium pullulans* (Leite et al., 2007), *Saccharomyces cerevisiae* (Barbagallo et al., 2004), *Talaromyces emersonii* (Murray et al., 2004), *Trichoderma reesei* (Chen et al., 1992), *Aspergillus pulverulentus* (Mase et al., 2004), *Aspergillus niger* (Abdel-Naby et al., 1999; Seidle et al., 2004), *Aspergillus tubingensis* (Decker et al., 2001) and *Thermoascus aurantiacus* (De Palma-Fernandez et al., 2002) are also reported to secrete β-glucosidase with an optimum activity at pH 4.0. Cellulase from fungi like *Hypocrea jecorina* with temperature optimum 65 °C and pH optimum 4.0 (Voutilainen et al., 2008) can be used for cocktail design with BGL 2 for biomass hydrolysis.

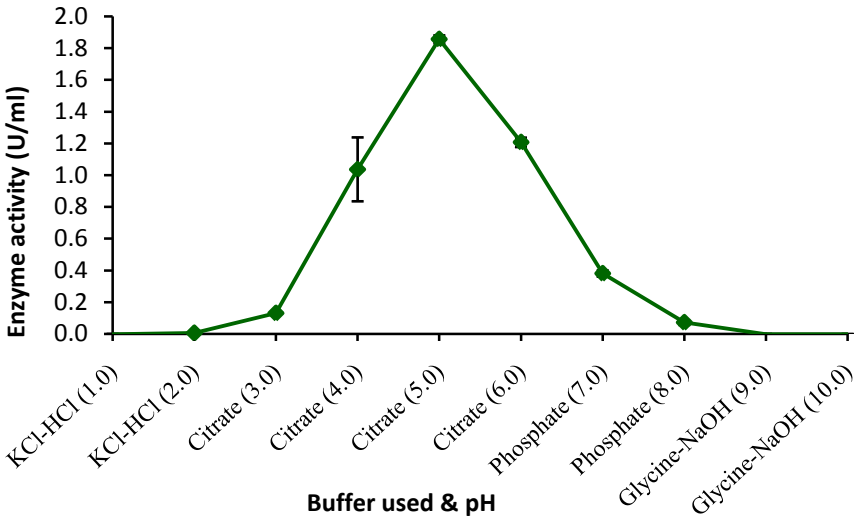
Figure 6.14: Determination of the optimum pH for BGL 2



BGL 3 showed an optimum pH 5.0 for activity. BGL 3 was active from pH 3.5 to 7.5 (Fig 6.15). Beta-glucosidase with an optimum activity at pH 5.0 is reported to be secreted by *Termitomyces clypeatus* (Sengupta et al., 1991), *Thermoascus aurantiacus* (Hong et al., 2006), Peni*cillium verruculosum* (Korotkova et al., 2009), *Penicillium purpurogenum* (Jeya et al., 2010) *Candida peltata* (Saha and Bothast, 1996), *Aspergillus oryzae* (Zhang et al., 2007), *Aspergillus sojae* (Kimura et al., 1999), *Aspergillus niger* (Yan and Lin, 1997), *Alternaria alternata* (Martínez et al., 1988), *Aspergillus aculeatus*

(Decker et al., 2000) and *Pyrococcus furiosus* (Kaper et al., 2000). Beta-glucosidase III and IV from *Aspergillus tubingensis* had a broad optimum between pH 4.0 and 6.0 with maximum activity at 5.0 (Decker et al., 2001). BGL 3 can be used to enhance hydrolytic efficiency of cellulase from fungi like *Thermoascus aurantiacus* (Voutilainen et al., 2008) having temperature optimum of 60 °C and pH optimum of 5.0 for activity.

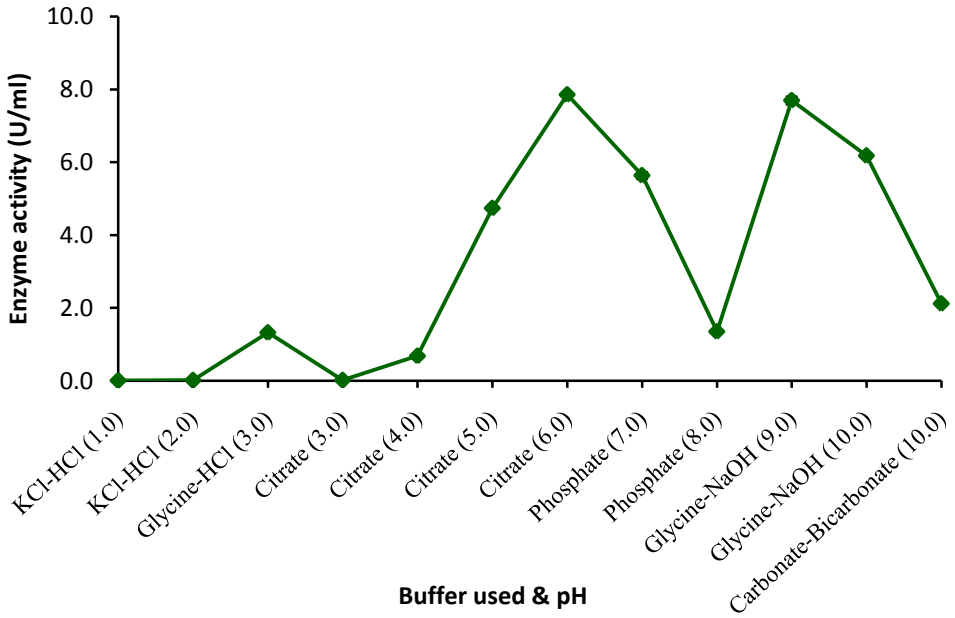
Figure 6.15: Determination of the optimum pH for BGL 3 activity



BGL 6 showed two peaks of activity, one at an acidic pH 6.0 and the other at an alkaline pH 9.0 (Fig 6.16). Fungi like *Paecilomyces* (Yang et al., 2008) *Candida wickerhamii* (Skory et al., 1996), *Thermomyces lanuginosus* (Lin et al., 1999), *Pichia etchellsii* (Wallecha and Mishra, 2003), *Humicola grisea* var. *thermoidea* (Nascimento et al., 2010; Peralta et al., 1997), *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 found to secrete β-glucosidase with an optimum pH 6.0. Fungal β-glucosidase with optimum activity at alkaline pH seems to be rare. However in *Mucor miehei* a β-glucosidase with optimum activity at pH 8.0 was reported (Yoshioka and Hayashida, 1980). In *Volvariella volvacea*, BGL I had an optimum activity at pH 7.0 and BGLII at pH 6.2, both the isoforms showed 35% relative activity at pH 8.0 (Cai et al., 1998). βG1 isoform from *Aspergillus terreus* showed a broad pH for

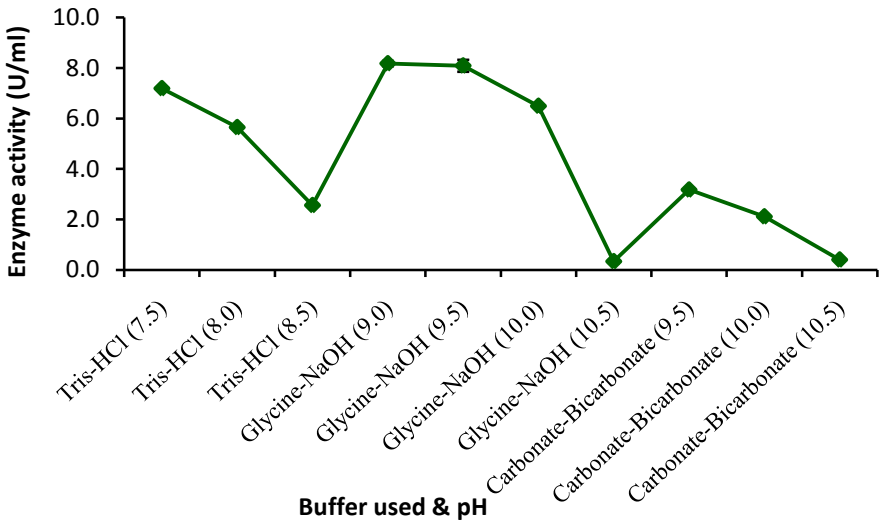
activity from 2-10. A relative activity of 82% was noted even at pH 10 (Nazir et al., 2009).

Figure 6.16: Determination of the optimal pH for BGL 6 activity



It seemed that glycine somehow enhanced the BGL 6 activity. In glycine-HCl buffer, at pH 3.0 there was activity but in citrate buffer with pH 3.0, no activity was noted. Similarly at alkaline pH, Glycine-NaOH buffer was found to be more effective compared to carbonate-bicarbonate buffer of same pH (Fig 6.17). In *Botrytis cinerea*, optimum pH for β-glucosidase activity was found to depend on the buffer used. In phosphate buffer, activity was optimum at pH 6.5, but in citrate phosphate buffer, optimum activity was at pH 7.0 (Gueguen et al., 1995). BGL A and BGL B from *Aspergillus* SA58 showed a temperature optimum of 60 °C, BGL A was active at pH 4.0, BGL B at pH 3.0 but both the isoforms showed a second peak of activity at pH 9.0 (Elyas et al., 2010). Among other enzymes, phytase from *Aspergillus niger* showed optimum activity at pH 2.0 and 5.0 (Xiong et al., 2004). Beta-glucosidase from *Paecilomyces thermophila* had an optimum pH 6.2 but showed a broad range of activity from 3.0 to 11.0 (Yang et al., 2008). BGL 6 can be supplemented to cellulase of *Melanocarpus albomyces* (Szijarto et al., 2008), that have similar temperature and pH optima for activity, for enhancing biomass hydrolysis.

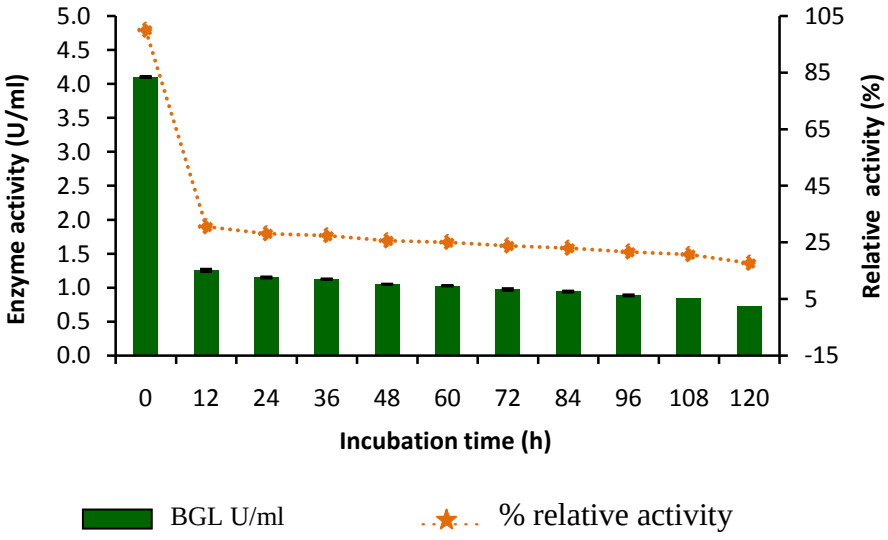
Figure 6.17: Effect of buffer on BGL 6 activity



6.3.8. Temperature stability

All the three isoforms of β-glucosidase had high temperature stability at 50 °C. Compared to the other two isoforms, BGL 2 showed a rapid decline in relative activity to 31 % after 12 h of incubation (Fig 6.18). An incubation to 120 h resulted in the loss of 82 % activity. BGL 2 had a half life of 9 h at 50 °C.

Figure 6.18: Temperature stability of BGL 2 at 50 °C



BGL 3 and BGL 6 showed high levels of stability at 50 °C. The loss of activity was gradual with BGL 3 retaining 92 % and BGL 6 retaining 87 % activity after 12 h of

incubation. Even after 72 h of incubation, BGL 3 retained 50 % activity and after 120 h, BGL 3 had a relative activity of 32 % (Fig 6.19). Half life period of BGL 3 was calculated to be 75 h. BGL 6 had a half life of 66 h and retained 35 % activity even after incubating for 120 h (Fig 6.20). Such a high temperature stability of β-glucosidase can be exploited in biomass hydrolysis which is mostly carried out at 50 °C. Moreover, immobilization of β-glucosidase can be highly economical because of this high thermal stability and many batches of hydrolysis can be performed using a single addition of immobilized BGL.

Figure 6.19: Temperature stability of BGL 3 at 50 °C

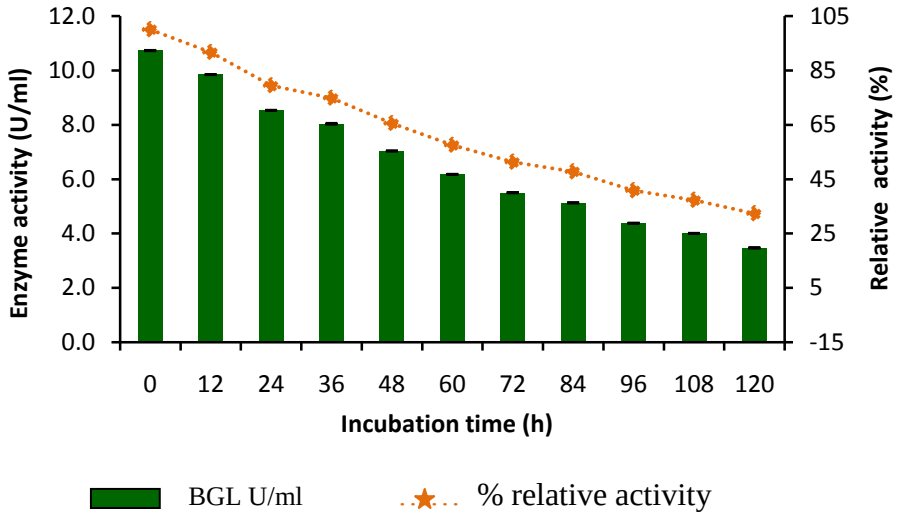
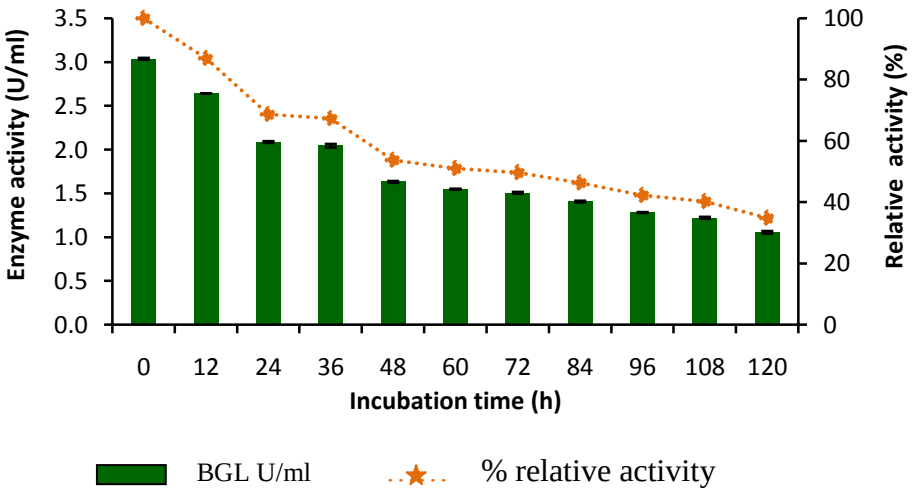


Figure 6.20: Temperature stability of BGL 6 at 50 °C



Thermal stability of β-glucosidase varies with fungal taxa. In *Monascus purpureus*, extracellular β-glucosidase showed a $t_{1/2}$ value of 315 min at 50 °C (Daroit et al., 2008). Beta-glucosidase from *Fusarium proliferatum* showed 75 % activity retention at 50 °C when incubated for 1 h (Fawzi, 2003). In *Penicillium purpurogenum,* β-glucosidase showed a $t_{1/2}$ of 10 h at 65 °C (Jeya et al., 2010). Beta-glucosidase from *Thermoascus aurantiacus* had an optimum activity at 80 °C and showed a half life of 18 minutes and 24 h at 80 °C and 70 °C respectively (Kalogeris et al., 2003). In *Melanocarpus*, at 50 °C, pH 5.0 and 6.0, β-glucosidase retained 100 % activity for 200 minutes but a relative activity of only 64 % was seen with 360 min of incubation. At 60 °C, pH 5.0, enzyme lost 80 % of activity after 30 min of incubation and at pH 6.0 under same temperature, 55 % activity was lost (Kaur et al., 2007). Recombinant β-glucosidase from *Penicillium brasilianum* expressed in *Aspergillus oryzae* showed a $t_{1/2}$ of 24 h when incubated at 65 °C, pH 5 and 6.0. At 65 °C, pH 4.0, $t_{1/2}$ of 10 h was noted (Krogh et al., 2010). Enzyme from *Debaromyces pseudopolymorphus* showed only 30 % relative activity after 3 h of incubation at the optimum temperature of activity of 40 °C (Villena et al., 2006). In *Scytalidium thermophilum,* BGL was stable at 50 °C for 60 min but the stability at 55 °C for the same time period was below 20 % (Zanoelo et al., 2004).

Thermal stability also varies with isoforms from same fungal strain. In *Aspergillus tubingensis*, BGL III and BGL IV were completely inactivated after 20 h of incubation at 60 °C, while BGL I and BGL II retained 23 % and 86 % of their initial activity. BGL II was found to be highly thermo-stable with a 30 % activity retention even after incubating at 60 °C for 118 h (Decker et al., 2001). In *Aspergillus terreus*, isoforms βGI, βGII and βGIII varied in their thermal stability. βGI was stable within a temperature of 50 -70 °C for 4 h between pH 5.0-7.0. βGII retained 100 % activity at pH 3.0-5.0 for 3 h at 50 °C but lost activity at temperatures of 60-70 °C after 30 min of incubation. βGIII showed complete activity retention when incubated at 50 °C for 4 h (Nazir et al., 2009).

In *Paecilomyces*, β-glucosidase was found to have an optimum temperature of activity at 55 °C. But the enzyme showed very low thermal stability and retained only 18 % activity at 55 °C after 4 h incubation. At 50 °C, 89.6 % activity was retained for the

same duration of incubation (Yan et al., 2008). Beta-glucosidase from *Paecilomyces thermophila* at 30 min incubation was fully stable at 50 °C but only 40 % relative activity was noted at its optimum temperature of activity of 70 °C (Yang et al., 2008). Beta-glucosidase from *B. fulva* NII 0930 showed high thermal stability compared to other fungal β-glucosidases reported in the literature. Such a high temperature stability of β-glucosidase can be exploited in biomass hydrolysis which is mostly carried out at 50 °C with a standard enzyme retention time below 48 h. Moreover, immobilization of β-glucosidase can be highly economical because of this high thermal stability and many batches of hydrolysis can be performed using a single addition of immobilized BGL.

6.3.9. Enzyme Kinetics

The reaction kinetics of the purified isoforms was determined using Lineweaver-Burk plot. With pNPG as substrate, BGL 2 showed a *Vmax* value of 0.45 U/ml and an apparent *Km* value of 0.43 mM (Fig 6.21). BGL 3 showed a *Vmax* value of 1.85 U/ml and a *Km* value of 0.63 mM (Fig 6.22). Among the three isoforms, a very high *Km* value was shown by BGL 6. From Lineweaver-Burk plot, a *Vmax* value of 2.78 U/ml and a *Km* value of 33.33 mM were obtained for BGL6 (Fig 6.23). This clearly indicates the low affinity of BGL 6 towards pNPG.

Figure 6.21: Lineweaver-Burk plot of BGL 2

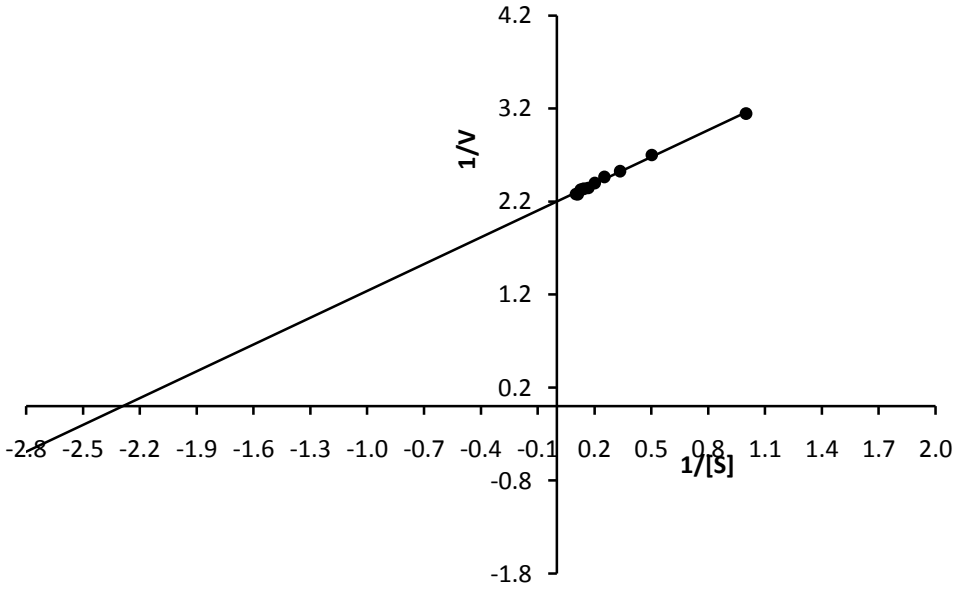


Figure 6.22: Lineweaver-Burk plot of BGL 3

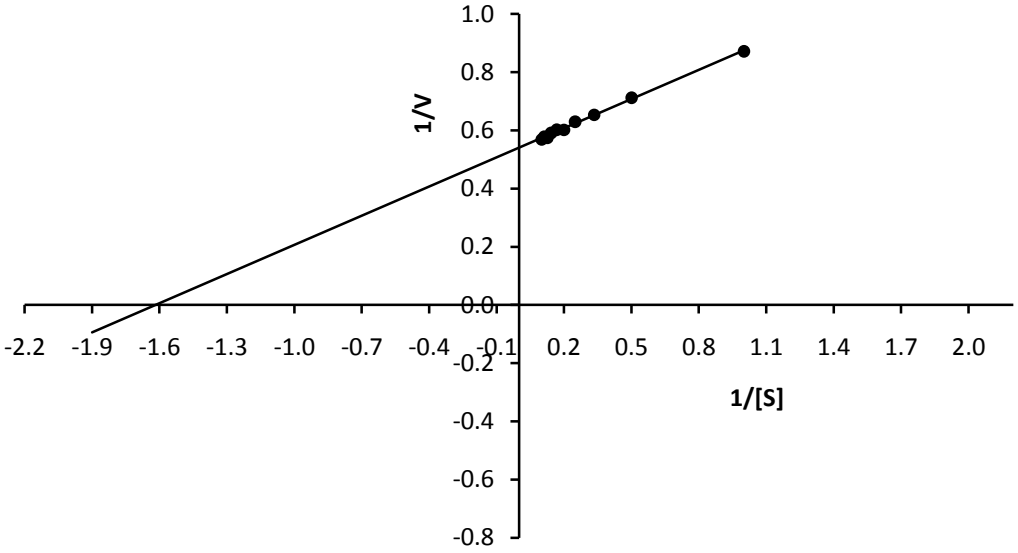
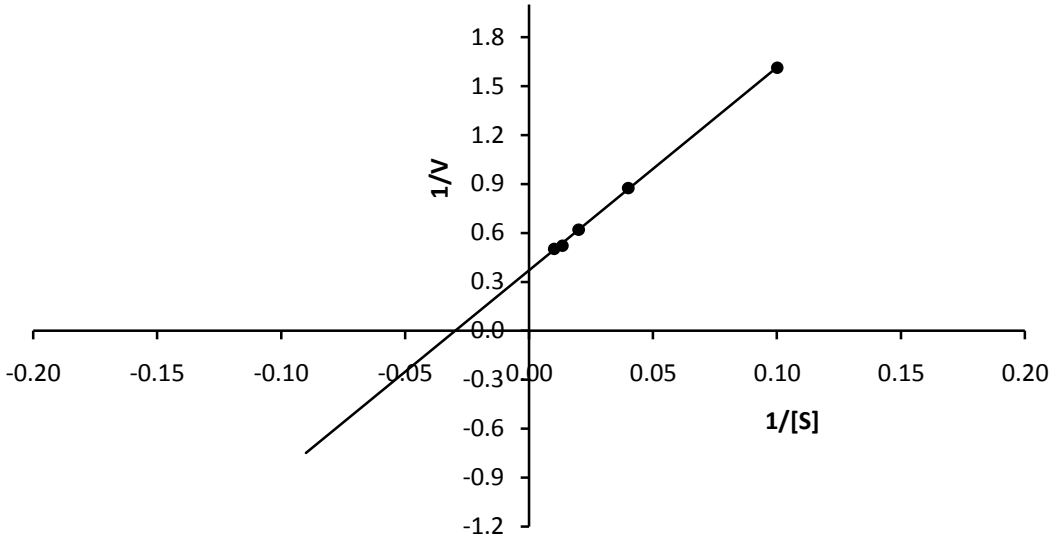


Figure 6.23: Lineweaver-Burk plot of BGL 6



The Michaelis constant (*Km*) is a means of characterizing an enzyme's affinity for a substrate. The *Km* is defined as the substrate concentration at which the reaction rate is half its maximum velocity. Thus, a low *Km* value means that the enzyme has a high

affinity for the substrate. Beta-glucosidase from fungal taxa shows variation in *Km* value for pNPG hydrolysis. It varies from 0.001 mM as in *Penicillium funiculosum* (Kantham and Jagannathan, 1985) to 21.7 mM in *Aspergillus niger* (Yan and Lin, 1997). *Km* for pNPG hydrolysis by β-glucosidase from *Paecilomyces* was found to be 0.11 mM (Yan et al., 2008) and that from *Paecilomyces thermophila* was 0.27 mM (Yang et al., 2008).

Many β-glucosidases have a K*m* value similar to that of BGL 2 and BGL 3 of *B. fulva*. The K*m* for pNPG hydrolysis was calculated to be 0.4 mM in *Aspergillus terreus* (Workman and Day, 1982), 0.41 mM for intracellular enzyme of *Humicola* (Araujo et al., 1983), 0.44 mM for intracellular enzyme of *Candida cacaoi* (Drider et al., 1993) and extracellular enzyme of *Penicillium verruculosum* (Korotkova et al., 2009), 0.45 mM for *Sclerotinia sclerotiorum* (Kalifa et al., 2007) and 0.62 mM in *Phanerochaete chrysosporium* (Tsukada et al., 2006). There are only few reports of β- glucosidase with high *Km* value for pNPG hydrolysis. Three isoforms βGI, βGII, βGIII purified and characterized from *Aspergillus terreus*, had a molecular weight of 29 kDa, 43 kDa and 98 kDa. *Km* value for pNPG hydrolysis for βGI was 14.2 mM, 4.37 mM for βGII and 11.1 mM for βGIII (Nazir et al., 2009). Beta-glucosidase from *Aspergillus niger* had a high *Km* of 21.7 mM to pNPG hydrolysis and a *Ki* value of 543 mM to glucose (Yan and Lin, 1997). In this respect, BGL from *Aspergillus niger* was similar to BGL 6 of *B. fulva* NII 0930. The *Km* value of 33.33 mM in BGL 6 seems to be the highest among fungal β-glucosidase.

6.3.10. Inhibition Kinetics

The inhibition constant, *Ki* is the dissociation constant for the enzyme-inhibitor complex. It indicates how potent a molecule is to create inhibition to enzyme activity. It is defined as the concentration required for half maximum inhibition. In all the three isoforms, glucose behaved as a competitive inhibitor. The *Ki* value of glucose for BGL 2 was 12.0 mM (Fig 6.24), while a negative *Ki* value of glucose was obtained for BGL 3 (Fig 6.25). BGL 6 was highly glucose tolerant with a *Ki* value of 0.6 M at 50 °C (Fig 6.26) which was enhanced to 1.03 M at 55 °C (Fig 6.27).

Figure 6.24: Dixon plot for determination of glucose inhibition constant (*Ki*) of BGL 2

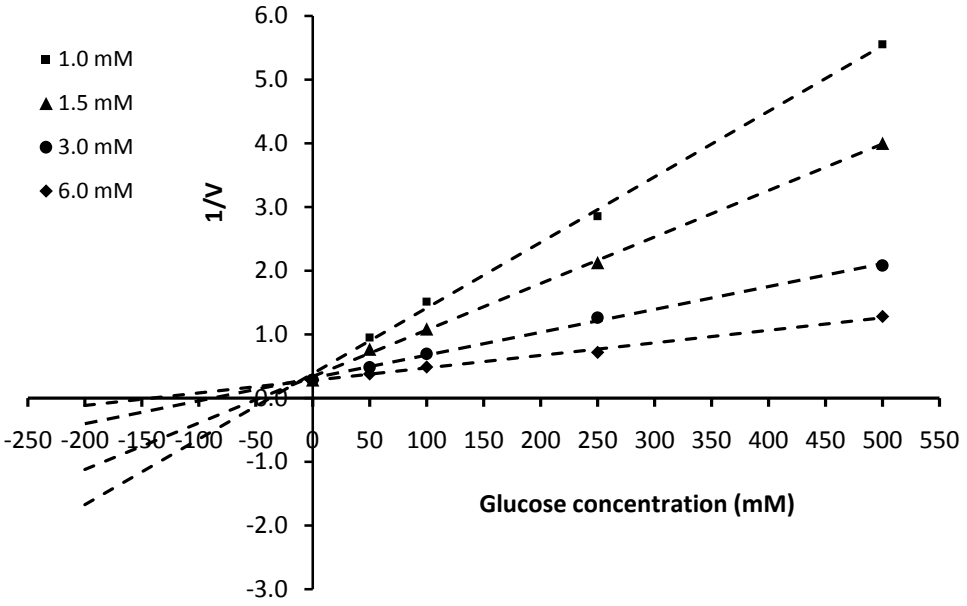


Figure 6.25: Dixon plot for determination of glucose inhibition constant (*Ki*) of BGL 3

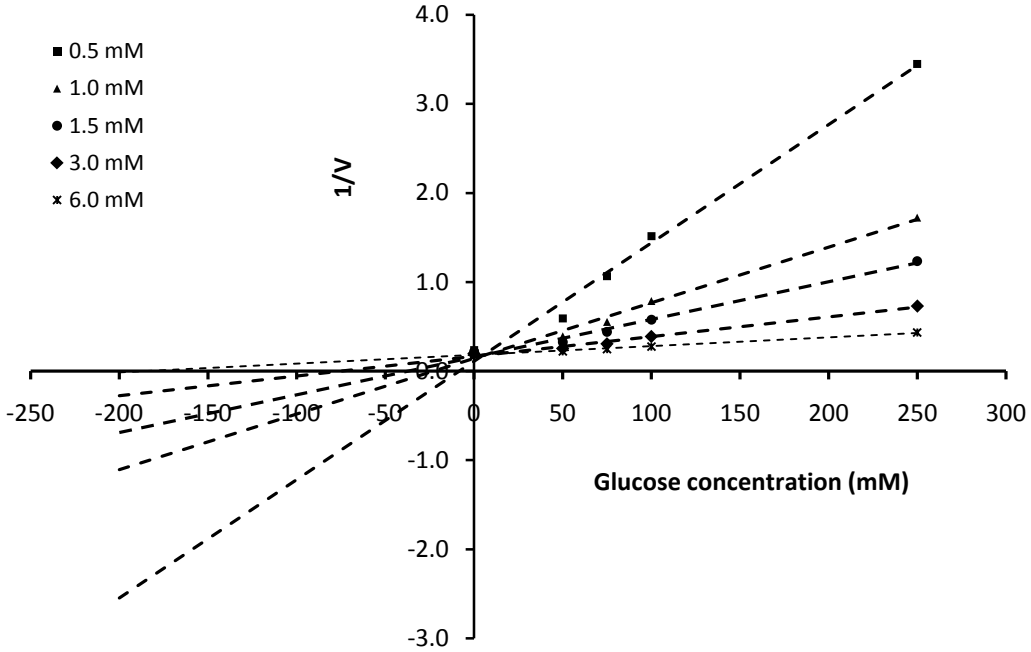


Figure 6.26: Dixon plot for determination of glucose inhibition constant (*Ki*) of BGL 6 at 50 °C

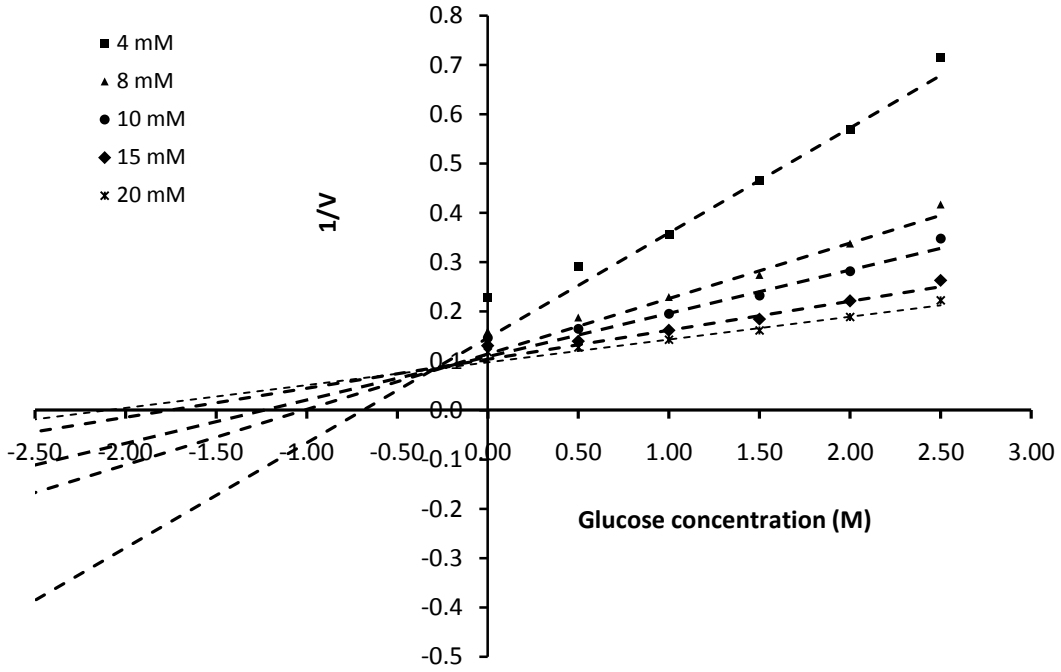
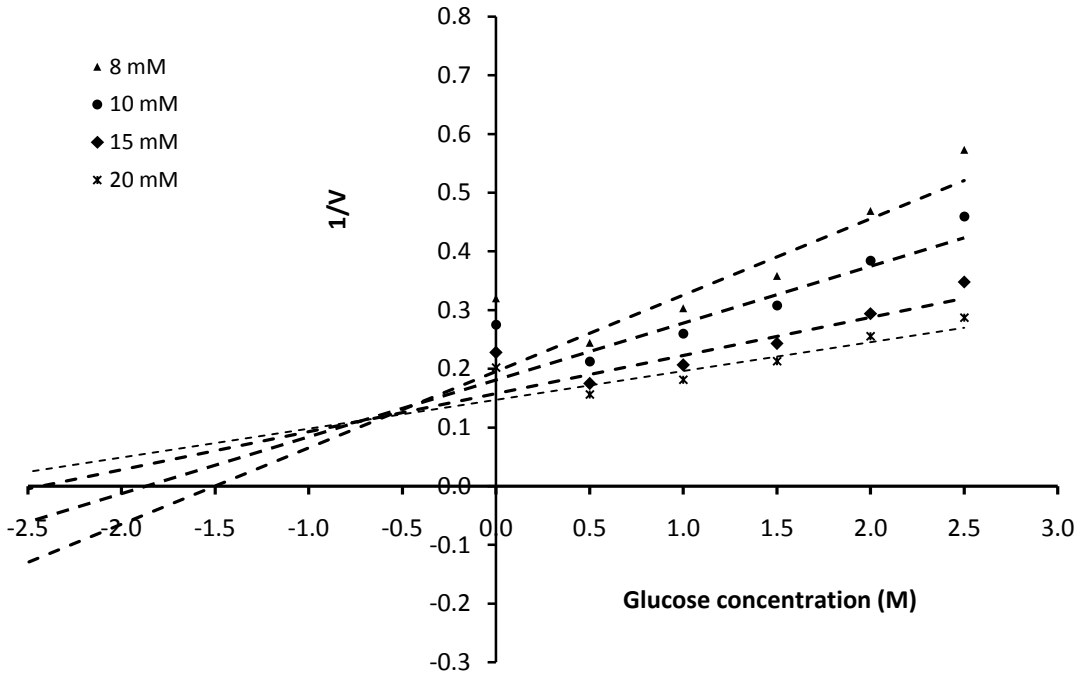


Figure 6.27: Dixon plot for determination of glucose inhibition constant (*Ki*) of BGL 6 at 55 °C



Fungal β-glucosidases varies in sensitivity to glucose. The *Ki* value of glucose may be as low as 700 µM in *Trichoderma reesei* (Chirico and Brown, 1987), 0.24 mM and 0.29 mM in *Penicillium decumbens* (Mamma et al., 2004), 0.25 mM in *Talaromyces emersonii* (Murray et al., 2004), 0.29 mM in *Thermoascus aurantiacus* (Parry et al., 2001), 0.51 mM in *Hypocrea jecorina*, 0.93 mM in *Penicillium verruculosum*, 2.73 mM in *Aspergillus japonicus* (Korotkova et al., 2009), 2.3 mM in *Penicillium brasilianum* (Krogh et al., 2010), 2.9 mM in *Aspergillus oryzae* (Langston et al., 2006), 9.2 mM in *Aspergillus japonicus* (Decker et al., 2000), 11.03 mM in *Chalara paradoxa* (Lucas et al., 2000), 21.5 mM in *Penicillium purpurogenum* (Jeya et al., 2010), 66 mM in *Talaromyces thermophilus* (Nakkharat and Haltrich, 2006) and 73 mM in *Paecilomyces thermophila* (Yang et al., 2008).

In *Aspergillus tubingensis*, BGL III and BGL IV isoforms had a low molecular weight (54 kDa). Compared to BGL I and BGL II, BGL III had high *Km* value of 3.2 mM (pNPG) and *Ki* value of 470 mM (glucose). Also BGL IV had a *Km* value of 6.2 mM (pNPG) and glucose inhibition constant (*Ki* value) of 600 mM (Decker et al., 2001). Beta-glucosidase isoform BGL II purified from *Aspergillus oryzae* with molecular weight 30 kDa had a *Km* of 6.4 mM to pNPG hydrolysis. But this isoform showed high level of tolerance to glucose with a *Ki* value of 953 mM (Günata and Vallier, 1999). This correlates to the present study that BGL 6 with low molecular weight had high *Km* value to pNPG and have high *Ki* value to glucose. Beta-glucosidase from *Aspergillus oryzae* had a high *Km* of 21.7 mM to pNPG and also high glucose tolerance with *Ki* 543 mM for glucose (Yan and Lin, 1997). In *Aspergillus oryzae*, major isoform had a molecular weight of 130 kDa and was highly inhibited by glucose. The other isoform, produced in lower quantities with a molecular mass of 43 kDa was highly resistant to glucose inhibition. The *Ki* value for the glucose tolerant isoform was 1.36 M but had a *Km* of 0.55 mM for pNPG hydrolysis at temperature 50 °C and pH 5.0 (Riou et al., 1998). Beta-glucosidase of *Candida peltata* had a molecular mass of 43 kDa with optimum temperature of 50 °C and optimum pH 5.0. The enzyme showed high glucose tolerance with a *Ki* value of 1.4 M glucose with *Km* value 2.3 mM for pNPG. However the enzyme showed low thermal stability with only 57 % relative activity at 50 °C, pH 5.0 with

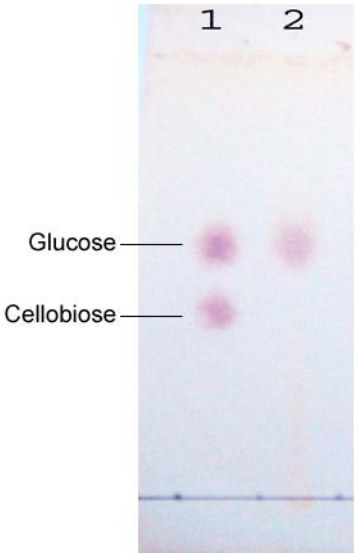
30 min of incubation (Saha and Bothast, 1996). Intracellular β-glucosidase from *Candida sake* had a *Km* value of 6.9 mM for pNPG and *Ki* value of 200 mM to glucose (Gueguen et al., 2001). *Thermoascus aurantiacus* had a low *Km* of 0.02 mM and *Ki* of 300 mM (Hong et al., 2006) and BGL from *Debaryomyces vanrijiae* had *Km* 0.77 mM and *Ki* of 439 mM glucose (Belancic et al., 2003).

Industrial processes, aims at high concentrations of glucose through biomass hydrolysis and hence it is highly desirable to have β-glucosidases that are tolerant to high concentrations of glucose. The sensitivity of BGL to glucose otherwise will cause accumulation of cellobiose resulting in inhibition of endo and exoglucanase. The high glucose tolerance shown by BGL 6 indicates its potential use in industrial production of glucose from cellulosic biomass.

6.3.11. Transgylcosylation property of BGL

No transglycosylation was noticed by the crude enzyme consisting of all the seven isoforms with cellobiose as donor and acceptor (Fig 6.28).

Fig 6.28: TLC analysis of cellobiose hydrolysis



Lane information

Lane1 – glucose and cellobiose

Lane 2- cellobiose incubated with crude BGL

The experiment conditions provided were similar to that employed in hydrolysis. Within 30 minutes of incubation, the cellobiose provided was completely hydrolyzed in to glucose. Thus under the conditions commonly employed for biomass hydrolysis, β-glucosidase from *B. fulva* NII 0930 showed no transglycosylation activity, which otherwise would decrease the hydrolytic efficiency.

In transglycosylation, the substrate is generally a glycoside (disaccharide or aryl linked glucoside). The enzyme-substrate intermediate makes the substrate an activated donor and the complex may be trapped by a nucleophile like alkyl, aryl alcohol, monosaccharide or disaccharide rather than water. This results in the formation of a new glycoside. Transglycosylation is a kinetically controlled reaction and can be enhanced by lowering thermodynamic activity of water by using high temperatures, high substrate concentration and organic solvents (Bhatia et al., 2002; Bruins et al., 2003; Hancock et al., 2005).

Transglycosylation reactions are generally demonstrated by use of alcohol as nucleophile (Gargouri et al., 2004; Ito et al., 2007; Yan and Liau, 1998). However, transglycosylation can occur when substrate act as both donor and acceptor. Beta-glucosidase from *Phanerochaete chrysosporium* showed transglycosylation when incubated with laminaribose. Steady state kinetic analysis indicate that transglycosylation can mimic substrate inhibition leading to decrease in hydrolysis (Kawai et al., 2004). BGL II isoform from *Trichoderma reesei* was found to show transglycosylation with cellobiose as substrate. When incubated with 10 % cellobiose, both glucose and cellotriose were formed in equal concentrations. However with increase of cellobiose concentration to 20 %, cellotriose was found to be higher than glucose and also small amounts of cellotetraose and sophorose were also detected. Incubation with 40 % glucose resulted in the synthesis of sophorse and cellobiose (Saloheimo et al., 2002). Beta-glucosidase from *Paecilomyces thermophila* showed high levels of transglycosylation when incubated in cellobiose. Even at 15 min incubation, no glucose was formed but cellotriose was detected. Transglycosylation resulting in cellotriose formation was more compared to glucose release at various time intervals of 6 h of incubation (Yang et al., 2008). There are also reports of β-glucosidase lacking

transglycosylation activity. Beta-glucosidase of *Candida peltata* had no transglycosylation property and converted all the cellobiose (10 %) in to glucose in absence and presence of 6 % glucose. A conversion of 90 % was achieved using 1.5 U/ml enzyme in 144 h at pH 5.0 and 50 °C (Saha and Bothast, 1996).

6.4. Conclusion

B. fulva NII 0930 produced extracellular BGL consisting of seven isoforms. Among these, three isoforms were purified to homogeneity and their properties were studied revealing their peculiar kinetic characters. BGL 2 with optimum temperature of activity at 65 °C, pH 4.0 had a good thermal stability at 50 °C. BGL 3 with optimum temperature of activity at 60 °C and optimum pH 5.0 showed a remarkably high $t_{1/2}$ of 75 h. BGL 6 had maximum activity at pH 6. 0 while temperature optimum was 50 °C. At this temperature, this isoform exhibited a t1/2 of 66 h. Even the β-glucosidase from *Candida peltata* reported to have a *Ki* value of 1.4 M glucose had a low thermal stability with only 57 % relative activity at 50 °C, pH 5.0 with 30 min of incubation. This high temperature stability of isoforms of *B. fulva* NII 0930 is of particular importance in biomass hydrolysis and there is a greater potential to use this enzyme in immobilized form for multiple cycles for hydrolysis. BGL 6 also showed a peak of activity at pH 9.0, indicating its potential in diverse applications including biomass hydrolysis using alkaline cellulases. Glucose tolerance of BGL 6 was remarkably high than many of the β-glucosidase reported in literature and its activity enhancement in presence of glucose makes it a potential candidate in biomass hydrolysis. Absence of transglycosylation also adds to the superiority of BGL from *B. fulva* for hydrolytic reactions. The high specific activity shown by the enzyme makes *bgl* an interesting gene for cloning and over-expression studies. Successful over-expression may reduce the cost of glucose tolerant β-glucosidase production.

Chapter 7

Partial amplification of *bgl* gene from *B. fulva* NII 0930 and analysis of the C terminal peptide sequences

Chapter 7

Partial amplification of *bgl* gene from *B. fulva* NII 0930 and analysis of the C terminal peptide sequences

7.1. Introduction

Microbial β-glucosidases have been the subject of extensive research due to its biological role and potential applications. The hydrolytic and transglycosylation properties of the enzyme are widely employed in industrial process for generating products of interest. Though ubiquitous in nature, β-glucosidase from different taxa differs in their kinetic properties, including specificity of substrate molecules. Based on sequence and folding similarities, glycosidases are classified in to 88 families. The families are grouped in to clans based on similarity of tertiary structure, catalytic residues and mechanism of action (Bhatia et al., 2002; Henrissat, 1991; Henrissat and Bairoch, 1996). According to data base CAZy, glycoside hydrolases are classified in 115 families (www.cazypedia.org).

Fungi produce large number of extracellular enzymes. About 453 characterized fungal glycoside hydrolases have been cataloged, spanning over 131 fungal species. They represent over 46 different GH activities and cover 44 of the115 CAZy database GH families (Murphy et al., 2011). BGLs have been classified into glycosyl hydrolase (GH) GH1, GH3, GH5, GH9 and GH30 families based on their amino acid sequences (Cantarel et al., 2009; Henrissat, 1991; Henrissat and Bairoch, 1996; Opassiri et al., 2007). Family 3 BGL are generally bacterial, yeast and mold origin while family 1 glycosyl hydrolases are mostly β-glucosidases from archaebacteria, plants and mammals (Bhatia et al., 2002). However β-glucosidase belonging to family 1 are reported from fungal species like *Candida wickerhamii* (Skory and Freer, 1994), *Phanerochaete chrysosporium* (Tsukada et al., 2006) and *Trichoderma reesei* (Saloheimo et al., 2002).

Several researchers have been successful in sequencing *bgl* gene. Beta-glucosidase from *Agrobacterium tumefaciens* was cloned and sequenced. A single open reading frame of 2457 nucleotides were present corresponding to 818 amino acid residues in the translated sequence, with a predicted molecular mass of 88,289 Da (Castle et al., 1992). cDNA's encoding two intracellular enzymes from *Phanerochaete chrysosporium* belonging to glycosyl hydrolase family 1 were cloned and sequenced. The cDNA sequence contained open reading frames of 1389 base pairs for *bgl1A* and 1623 base pairs for *bgl1B* encoding 462 and 530 amino acids (Tsukada et al., 2006).

From the studies conducted, it was noted that *B. fulva* produce at least seven extracellular isoforms. These seven isoforms may be produced by several genes or may be the post translational modification of a single gene. In *Aspergillus kawachii*, two extracellular and one intracellular β-glucosidase were coded by *bglA* gene. The isoforms shared same properties but differed in molecular weight and localization. Differences in the molecular weight were brought about by glycosylation of the protein (Iwashita et al., 1999). Minor amount of extracellular BGL activity was reported in *Trichoderma reesei* even after the deletion of *bgl1* gene indicating the presence of multiple genes encoding BGL (Saloheimo et al., 2002). Hence to check for multiple *bgl* genes that may be responsible for the different isoforms, PCR amplification were done using degenerate primers. Since BGLs belong to different GH families, degenerate PCR probes specific for amplification of GH1, GH3 and GH5 family β-glucosidases were designed and the PCR products were sequenced. The sequences were analyzed by BLAST (Altschul et al., 1990) to check for the existence of *bgl* genes belonging to same or different GH families.

7.2. Materials and Methods

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

7.2.1. Chromosomal DNA Isolation from B. fulva

Chromosomal DNA from *B. fulva* NII 0930 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 resuspnded in 10 mM Tris-EDTA buffer (pH 8.0).

7.2.2. Primer designing

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

Degenerate primers for three major GH families i.e., – family 1 (GH1), family 3 (GH3) and family 5 (GH5) were designed and were tried for PCR amplification of BGL genes from the chromosomal DNA of the fungus. Based on the results from the first set of degenerate primers, another set of degenerate primers which could amplify larger stretch of *bgl* gene were designed using either of the software – PerlPrimer (Marshall, 2004) or FastPCR (Kalendar et al., 2009). The degenerate primers designed for amplification of GH1, GH3 and GH5 family BGLs are listed in Table 7.1, and the degenerate primers designed for amplification of larger stretch of GH3 family BGL of *B. fulva* is given in table 7.2.

Table 7.1: List of Degenerate primers designed for PCR amplification of GH1, GH3 and GH5 family β-glucosidases

SL #	SEQ CODE	SEQUENCE
		FAMILY 1 GLYCOSYL HYDROLASE
1	BGL1_AF1	5'-GCA ACG GCG GCT TAT CAR RTN GAR GG -3'
2	BGL1_AF2	5'-TGG TAA AGG ACA ATC TAT CTG GGA YAC NTT -3'
3	BGL1_DR1	5'-TGT AAC ACC ATG GCT CGT TGA RNG TNA YCC A -3'
4	BGL1_DR2	5'-CGT GAT GCC CGA ATA TAG TGT AAC ACY MNG GYT CRT T -3'
5	BGL1_FF1	5′-CAC TCT ACA CGG TCA CTG GTA TGA RCC NTG GRA -3'
6	BGL1_FR1	5'-TGT TGA TCC GCC GCG TYC CAN GGY TC -3'
7	BGL1_JR1	5'-CTC CCT TAC ACG TGC AGC CRT TYT CNG T -3'
8	BGL1_IR1	5'-CGG TTC CAT ACC CAA TTC AGG WRY TTN CKR AA -3'
		FAMILY 5 GLYCOSYL HYDROLASE
9	BGL5_BF1	5'- TGG GTG GCT GGT GCT AGA RCC NTG GHT -3'
10	BGL5_BF2	5'- TGC GGG GCG TTA ATC TAG GNG GNT GGY T-3'
11	BGL5_DR1	5'- GGT ACT CCA GTT GTC CGT CGA CRT ANG GNT C-3'
12	BGL5_DR2	5'- CGA TCA TTA CCT TCA GAC CAG CAK CNC BNG CCC A-3'
13	BGL5_ER1	5'- GCC CTT TCG CCC AGA ATT RTC RAA NCC-3'
14	BGL5_FF1	5'- ACG GCG ATC GAG GCC NTN AAY GAR CC-3'
15	BGL5_FF2	5'- GGA CAC TAC CGT ATT CTT CCA TGA YGG NTT YNT-3'
16	BGL5_HR1	5'- ATC GGT CAT TGC CCC GSW CCA YTC NCC-3'
17	BGL5_IR1	5'- CCC AGG CGT CTA GCT GTG CYT CDA TRA A-3'
18	BGL5_JR1	5'- CCC CAT TTT CTA GTA GAT CTT GCA TRT CCC ANC C-3'
		FAMILY 3 GLYCOSYL HYDROLASE
19	BGL3a_DF1	5'-TGC GGT ATA AAC GTA GGG GCN ACN TKB GA -3'
20	BGL3a_EF1	5'-TGC GCA AAG CAT TAT ATT TGC WAY GAN MWR GA -3'
21	BGL3a_GR1	5'-GGA ATG GTG TGC GAA CCA RTC NSW CAT -3'
22	BGL3a_GR2	5'-ACC GGA ATG GTG TGC GAN CCA RTC NSW CA -3'
23	BGL3_GF1	5′-CGG TTT CCA AGG CTT CGT AAT GWS NGA YTG G-3'
24	BGL3_GF2	5'-CCA ACG CTG GGC TGG AYA TGW SNA TG -3′
25	BGL3_KR1	5'-GCT TCG TCC GGC GTA ACA ARR TAN GGR WA -3'
26	BGL3_KR2	5'-CAA AAT AGG GAA AGT TAG CAG TTC CAS WNC CCC ANC C -3'

Table 7.2. List of specific degenerate primers designed for PCR amplification of long stretch of GH3 family β-glucosidase of *B. fulva*

SL #	SEQ CODE	SEQUENCE
		Degenerate Primers
1	BF_1F	5′-GAG AAG GTC AAC MTS ACG ACC GGT GYY G -3'
2	BF_2F	5′-CAC GAG CTG TAC YTK TGG CCS TTT G -3'
3	BF_3F	5′-GCC TAC TCK CCS CCY TTC TAY CCK TC -3'
4	BF_1R	5′-GRK GCA GYT TSC GCG AMG AGY T -3'
5	BF_2R	5′-AAC TYA CCA GCT GRG GAA CCT CRT CAC C -3'
6	BF_3R	5′-CAW ASA RAC CMG GGT TGC CAC CRG RAG -3'

7.2.3. PCR Amplification of partial gene sequences of BGL

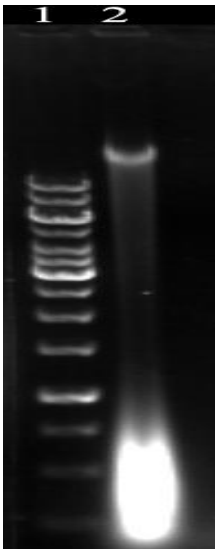
Amplification of partial gene sequences of β-glucosidases were performed by polymerase chain reaction using different sets of the degenerate primers designed for each family. PCR reactions contained 0.5 unit of Fermentas (USA) *Taq* DNA polymerase, 1X buffer, 200 μM of each deoxynucleotide triphosphate, 2.0 μM $MgSO_4$ 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, 50-65 °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. 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.

7.3. Results and Discussion

7.3.1. Chromosomal DNA isolation

DNA preparation from *B. fulva* was successful and high molecular weight chromosomal DNA was obtained (Figure 7.1). The DNA isolated was suspended in 10 mM Tris EDTA buffer (pH 8.0) and was used as template for PCR reactions.

Figure 7.1: Chromosomal DNA isolation from *B. fulva*



Lane Information

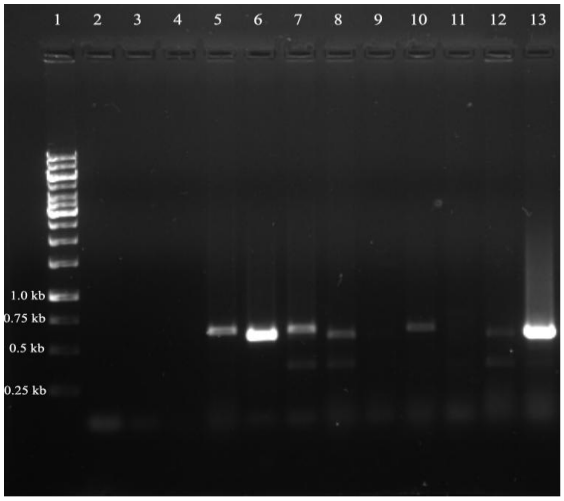
1 -Generuler® -1kb ladder (Fermentas)

2 -Chromosomal DNA from *B. fulva*

7.3.2. PCR amplification using degenerate primers for detecting glycosyl hydrolase family specific bgl.

PCR Amplification of *bgl* genes were performed using combination of degenerate primers designed for glycosyl hydrolase families GH1, GH3 and GH5. Figures 7.2 shows the amplicons obtained with primer pairs belonging to GH1, GH3 and GH5. The PCR products between 0.25-1.0 kb were sequenced using forward primer.

Figure 7.2: PCR amplification of *bgl* genes of *B. fulva*



Lane Information

1	GeneRuler ® 1kb ladder (Fermentas)
5	Primer pair BGL3_GF1-KR2
6	Primer pair BGL3_GF2-KR1
7	Primer pair BGL3a_DF1-GR1
8	Primer pair BGL3a_DF1-GR2
9	Primer pair BGL3a_EF1-GR1
10	Primer pair BGL3a_DF1-KR2
11	Primer pair BGL5_FF1-HR1
12	Primer pair BGL1_AF1-FR1
13	Primer pair BGL3_GF2-KR2

7.3.2.1. Sequence of PCR amplicons and BLAST results

Sequences of amplicons generated using degenerate primers for glycosyl hydrolase families GH1, GH3 and GH5 were subjected to BLAST analysis. Only sequence of amplicons generated by degenerate primers for GH3 showed similarities to *bgl* gene. Sequences showing significant similarities to *bgl* gene and the BLAST analysis are given below

A. Primer- BGL3a_DF1 and BGL3a_GR1

The nucleotide sequence obtained was 270 bp as given below

```
> BGL3a_DF1- GR1
TCATGTTAATGCATTTCACGTGGCTTCAGCTTGCCGAGGATTTCGCGAAAAGGGATCCCAGGTC
ATCCTTGGCCCAGTAGCTGGTCCTCTCGGCCGCTCTGCGATGGGAGGAAGAAACTGGGAGGGCT
TTTCGCCCGATCCCTATTTGACTGGTGACCTCTTCACGTCATCGATAAATGGCATTCAACGTGC
TGGAGTTCAAGCATGCGCGAAGCATTTTATTGGAAATGAACAGGAAACCCACCGACAATCTACC
ACTGATGACGAATT
```

BLAST analysis indicated that the sequence has 76 % identity with 50 % coverage to *Aspergillus flavus* NRRL 3357 (XM 002384812.1) hypothetical protein which has the domain signatures of glycosyl hydrolase family 3 N terminal domain. Also the sequence had close similarity to the following

Penicillium marneffei ATCC 18224 beta-glucosidase, putative (XM 002153563.1)

Aspergillus flavus NRRL 3357 BGL2 precursor, putative (XM 002381830.1)

Aspergillus oryzae RIB 40 BGL-G mRNA (XM 001825121.1)

Sclerotinia sclerotiorum 1980 hypothetical protein (XM 001593890.1)

B. Primer- BGL3a_DF1 and BGL3a_GR2

The nucleotide sequence obtained was 229 bp as given below

```
> BGL3a_DF1- GR2
ATCTTTTTTTAATTAGCATTTCACGTGGCTTCAGCTTGCCGAGGATTTCGCGAAAAGGGATCCC
AGGTCATCCTTGGCCCAGTAGCTGGTCCTCTCGGCCGCTCTGCGATGGGAGGAAGAAACTGGGA
GGGCTTTTCGCCCGATCCCTATTTGACTGGTGACCTCTTCACGTCATCGATAAATGGCATTCAA
CGTGCTGGAGTTCATGCTTGCGCTAAGTATTTTATTA
```

The sequence has 76 % identity with 58 % coverage to *Aspergillus flavus* NRRL 3357 (XM 002384812.1) hypothetical protein which has the domain signatures of glycosyl hydrolase family 3 N terminal domain. The sequence also had close similarity to the following

Penicillium marneffei ATCC 18224 beta-glucosidase, putative (XM 002153563.1)

Sclerotinia sclerotiorum 1980 hypothetical protein (XM 001593890.1)

Aspergillus terreus NIH 2624 hypothetical protein (XM 001211891.1)

Botryotinia fuckeliana B 05.10 hypothetical protein (XM 001554472.1)

Aspergillus flavus NRRL 3357 BGL 2 precursor, putative (XM 002381830.1)

C. Primer- BGL3a_DF1 and BGL3_KR2

The nucleotide sequence obtained was 192 bp as given below

```
> BGL3a_DF1- KR2
TTAAGTTAAATAACTGGTTTTACTTCGCGGCCAGGCATGGGCCAGGAGCACAATGGAAAGGGTG
TTGACGTCCCTTAGAAACCTGTCGCTGGTCCCTGGGACGTTCCCCTAACGGTGGTCGTAACTGG
GAATGCTTCTCTCCGGATCTCGTTCTTACGGGTGTGTTGATGGCGAATACCATCTTTGCTATTC
```

BLAST analysis indicated a 76 % identity with 75% coverage to *Penicillium chrysogenum* Wisconsin 54-1255 hypothetical protein (XM 002561992.1) which has the domain signatures of glycosyl hydrolase family 3 N and C terminal domain and fibronectin III like domain. The sequence also had close similarity to the following

Thielavia terrestris NRRL8126 glycoside hydrolase family 3 protein (X003655340.1)

Penicillium decumbens strain CICC 40361 *bgl1* mRNA (GU 320212.1)

Myceliophthora thermophila ATCC 42464 GH family 3 protein (XM 003663372.1)

D. Primer- BGL3_GF1 and BGL3_KR2

The nucleotide sequence obtained was 400 bp as given below

```
> BGL3_GF1- KR2
TAAAACTCTAGTTATTCGTCTCTTCCTGCGGTGTTGGCGATTCATTGGCTGGCCTTGATATGTC
TATGCCGGGTGACGCTGTCATGGGCAATGGCCAGACATTCTGGGGCACTAACTTGACCATTGCT
GTTGTCAACGGTACCATTCCAGAATGGCGCCTGGATGACATGGCTGTTCGCATTATGGCCGCAT
ACTACAAGGTCGGCCGTGACCGCCACCAGGTCCCGATTAACTTCGACTCCTGGACCACAGACAC
ATATGGTTACAAGCATGCTCTTGTATGCCAGAACTACGTCAAGGTAAATGAACATGTGGACTTG
CTTGGCAACCATGCTGAATTCATTCTGAAGATCTTATCCTTTTTCATTGTTCTGTTGAATATTT
AATTAATAGCTTATTT
```

BLAST analysis indicated that the sequence has 79 % identity with 76 % coverage to *Thermoascus aurantiacus* thermostable *bgl1* mRNA (DQ 114397.1), which has the domain signatures of glycosyl hydrolase family 3 N and C terminal domain and fibronectin III like domain. Also the sequence had close similarity to the following

Thermoascus aurantiacus var. *levisporus bgl1* (EU 269025.1)

Arthroderma otae CBS 113480 *bgl1* mRNA (XM 002850778.1)

Trichophyton rubrum CBS 118892 beta glucosidase mRNA (XM 003238444.1)

Aspergillus aculeatus bgl1 mRNA (JN 121997.1)

E. Primer- BGL3_GF2 and BGL3_KR1

The nucleotide sequence obtained was 317 bp as given below

```
> BGL3_GF2- KR1
CAATATAATTTTGCTGTCTGTGCATGGACAGACATTCTGGGGCACTAACTTGACCATTGCTGTT
GTCAACGGTACCATTCCAGAATGGCGCCTGGATGACATGGCTGTTCGCATTATGGCCGCATACT
ACAAGGTCGGCCGTGACCGCCACCAGGTCCCGATTAACTTCGACTCCTGGACCACAGACACATA
TGGTTACGAGCATGCTCTTGTAGGCCAGAACTACGTCAAGGTAAATGAACATGTGGACGTGCGT
GGCAACCATGCGGAAATCATTCGGAAGATCGGATCCTCAAGCATTGTCCTGCTGAATATCT
```

BLAST analysis revealed a 87 % identity with 78 % coverage to *Thermoascus aurantiacus* thermostable *bgl1* mRNA (DQ 114397.1), which has the domain signatures of glycosyl hydrolase family 3 N and C terminal domain and fibronectin III like domain. Besides this, the sequence had close similarity to the following

Thermoascus aurantiacus var. *levisporus bgl1* (EU 269025.1)

Talaromyces emersonii beta-glucosidase gene, complete cds (AY 072918.4)

Aspergillus aculeatus bgl1 mRNA (JN 121997.1)

Arthroderma otae CBS 113480 *bgl1* mRNA (XM 002850778.1)

F. Primer- BGL3_GF2 and BGL3_KR2

The nucleotide sequence obtained was 360 bp as given below

```
> BGL3_GF2- KR2
CAAATATTATCGCTGTAATGGGCATGGCCAGACATTCTGGGGCACTAACTTGACCATTGCTGTT
GTCAACGGTACCATTCCAGAATGGCGCCTGGATGACATGGCTGTTCGCATTATGGCCGCATACT
ACAAGGTCGGCCGTGACCGCCACCAGGTCCCGATTAACTTCGACTCCTGGACCACAGACACATA
TGGTTACGAGCATGCTCTTGTAGGCCAGAACTACGTCAAGGTAAATGAACATGTGGACGTGCGT
GGCAACCATGCGGAAATCATTCGGAAGATCGGATCCTCGAGCATTGTCCTGCTGAATAACGAGG
AAATGTTTCCTTTTACTTTCTGTTTTTTTTTTTTTTTTT
```

BLAST analysis indicated that the sequence has 78 % identity with 79 % coverage to *Thermoascus aurantiacus* thermostable *bgl1* mRNA (DQ 114397.1), which has the domain signatures of glycosyl hydrolase family 3 N and C terminal domain and fibronectin III like domain. Also the sequence had close similarity to the following

Thermoascus aurantiacus var. *levisporus* bgl1 (EU 269025.1)

Talaromyces emersonii beta-glucosidase gene, complete cds (AY 072918.4)

Penicillium decumbens strain CICC 40361 *bgl1* mRNA (GU 320212.1)

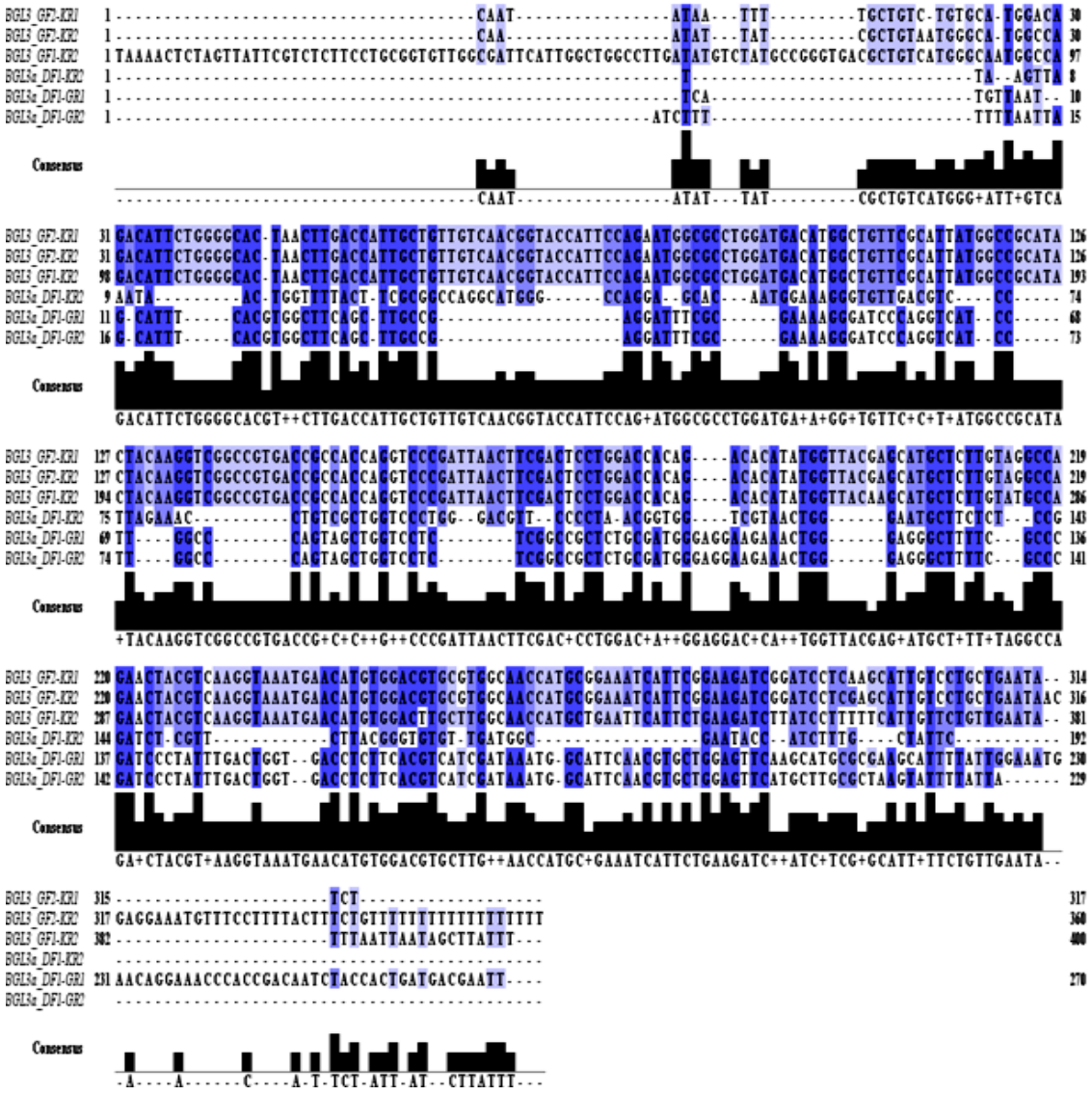
Aspergillus aculeatus bgl1 mRNA (JN 121997.1)

Arthroderma otae CBS 113480 bgl1 mRNA (XM 002850778.1)

7.3.2.2. Multiple sequence alignment of sequenced fragments

Multiple sequence alignment revealed the diversity of the amplicons. Sequences from primers BGL3_GF2-KR1, BGL3_GF2-KR2, BGL3_GF1-KR2 were similar (Fig 7.3). Also sequences from primers BGL3a_DF1-GR1, BGL3_DF1-GR2, were identical. Thus three distinct groups of sequences were obtained, with similar BLAST results within the group, but different between the groups, indicating multiple genes involved in β-glucosidase transcription. In *Aspergillus kawachii*, three isoforms of BGL were reported, all the three being encoded by a single gene *bglA*. However, these isoforms had similar enzymatic properties but differed only in molecular weight and localization. On deglycosylation, all the three isoforms had same molecular weight (Iwashita et al., 1999; Iwashita et al., 1998). But in the present study all the seven isoforms had different molecular weights and the three purified isoforms varied considerably in their kinetic properties. This, along with the sequence results suggests the possible involvement of multiple genes in β-glucosidase transcription.

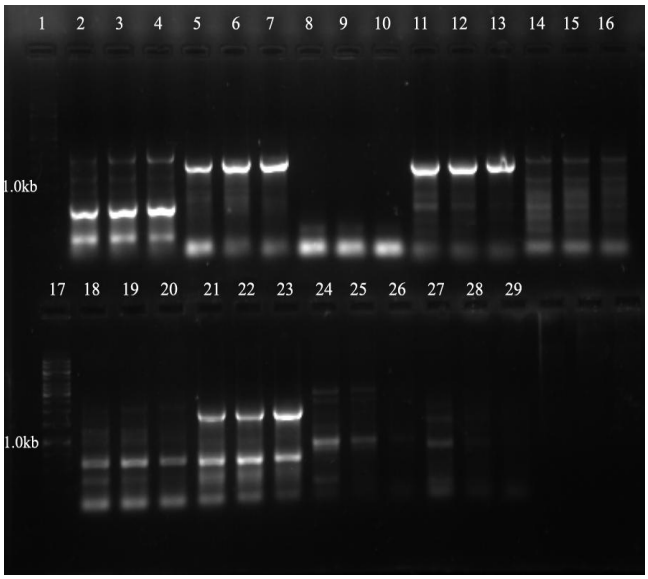
Figure 7.3: Multiple sequence alignment of amplicons



7.3.3. Sequencing and blast analysis of amplicons from specific degenerate primers

Specific degenerate primers, designed based on the result of sequences generated using GH3 degenerate primers, were used to amplify larger stretch of *bgl* gene. Gradient PCR with combination of primers produced amplicons as shown in figure 7.4. PCR products having amplicons above 0.6 kb were sequenced.

Figure 7.4: PCR amplification of bgl gene using specific degenerate primers



Lane information

1	GeneRuler ® 1kb ladder (Fermentas)
2-4	Primer pair BF_1F-1R
5-7	Primer pair BF_1F-2R
8-10	Primer pair BF_1F-3R
11-13	Primer pair BF_2F-2R
14-16	Primer pair BF_2F-3R
17	GeneRuler ® 1kb ladder (Fermentas)
18-20	Primer pair BF_2F-1R
21-23	Primer pair BF_3F-2R
24-26	Primer pair BF_3F-3R
27-29	Primer pair BF_3F-1R

7.3.3.1. Sequence of PCR amplicons and BLAST analysis

Sequences of amplicons generated using specific degenerate primers were subjected to BLAST analysis. Only sequence of amplicons generated by degenerate primers combinations having reverse primer BF_2R showed similarities to *bgl*. Sequences showing significant similarities to *bgl* gene are given below

A. Primer- BF_1F and BF_2R

The nucleotide sequence obtained was 1024 bp as given below

```
> BF_1F-2R
CCACTTCCTTTGTTTGCCTGCTGGTTGTGATGAGGCGCTGACACGGTACACCTCATCGTAAGCCTGGGTTA
CCACCAGGGCCGCCACCATCAGGGAGGACGGGCTGGGGAGAACCGTCAGTGGCACCCGCGGGGACGTAGTA
TCCTCGGTCTTCAGTCCATAGTGGGGGTCGGCGGAAGCCTTCTTGGGATCCGTCGAGTTCAGCCACGGGTA
GATGTACAATGGAATCTTGTCAATGTTGTCGGGGTAAAGATAGTCGGAGTAGTCGGTGCTGTAGTTGCCCA
AAGCAGGGGCTTCTTTGGTCTGACCGGACGCAGGTCCATACGGCGCCACGGGGATGGGCTGCACATAGAGG
TTAGAGTAGCCAAAGGTGGTATAACTAAGTCCATAGCCGAACTCGTAAACAGGGGTGTCATTCGCTTTGTC
GAAGTGTCTGTAGTCAATGAAGACGCCTTCGGTGAAGTCAACCTGCGGCGCACCCTTGCCATTGTTGGGTT
CCATGAGCAGGGGAGCACCATAGGAGTCGAGATCCTTACCCCAGGTGAAGGGCGTCTTTCCACCGGGGTTG
ACACGGCCATACAGGACATCGACCAAAGAGTTGCCGCTCTCCTGGCCAGGGAGACCGGCCCAGACGATGGC
AGTGATGTTAGGGTTGTCGTTCCAATCTCCGGCCTCGACCGCACCAACACCGTGGATGACCAGGATGGTGT
TGTTGCAGTTAGCCGTGACAGTCTTGACGACTTGGTCTGCTCCTTGCCAGACAGTAATGTTGTTCCAGTCT
CCCATGTTACCGGCGACGTTGATATTATCCTTCACCAGAGTCTGAGTTGACGAACACGATGGAAGACAGTA
GCTGCAAGCATACTGTTCTATCACATCCACATTACCGTTGTCATGACGGATAAACTGCACCCTTGGCCCTT
GGAAAAAAATCCCGTTCTGATTCCGCCTTGCTCAGGTACCAAGGTAAAGAAAAGTTATATCAGTAACCCCT
TCCCCCAAGCAAATGACCCAGTGTACCGTG
```

B. Primer- BF_2F and BF_2R

The nucleotide sequence obtained was 1168 bp as given below

```
> BF_2F-2R
AAAAAATATTAATGACTATGTTGGTGAGTGGCGCTGACACGGTACACCTCATCGTATAAGCCTGGGTTACC
ACCAGGGCCGCCACCAGCAGGGAGGACGGGCTGGGGAGAACCGTCAGTGGCACCCGCGGGGACGTAGTCCT
CGGTCTTCAGTCCATAGTGGGGGTCGGCGGAAGCCTTCTTGGGATCCGTCGAGTTCAGCCACGGGTAGATG
TACAATGGAATCTTGTCAATGTTGTCGGGGTAAAGATAGTCGGAGTAGTCGGTGCTGTAGTTGCCCAAAGC
AGGGGCTTCTTTGGTCTGACCGGACGCAGGTCCATACGGCGCCACGGGGATGGGCTGCACATAGAGGTTAG
AGTAGCCAAAGGTGGTATAACTAAGTCCATAGCCGAACTCGTAAACAGGGGTGTCATTCGCTTTGTCGAAG
TGTCTGTAGTCAATGAAGACGCCTTCGGTGAAGTCAACCTGCGGCGCACCCTTGCCATTGTTGGGTTCCAT
GAGCAGGGGAGCACCATAGGAGTCGAGATCCTTACACCAGGTGAAGGGCGTCTTTCCACCGGGGTTGACAC
GGCCATACAGGACATCGACCAAAGAGTTGCCGCTCTCCTGGCCAGGGAGACCGGCCCAGACGATGGCAGTG
ATGTTAGGGTTGTCGTTCCAATCTCCGGCCTCGACCGCACCAACACCGTGGATGACCAGGATGGTGTTGTT
GCAGTTAGCCGTGGCAGTCTTGACGACTTGGTCTGCTCCTTGCCAGACAGTAATGTTGTTCCATCTCCCAT
GTTACCGGCGACGTTGATATAACCTTCACAAAAGTCCGAGTTGACGAACACGATGGAAGACGTAGCCTGCG
AGGCAATACTGTTTCTAATCACATCCACATTAACCGTTGTCAATTGACGAAAAAAACTGCACCTTGCCTTT
GGAAGAGGATCCTCGTCTGTATCCGCTGCCCGAGTTAACAAAGTAAAGGAAAAGTTAATCAGATACCACTT
TCCCAGCCAATGACCAGTGTAAACGTGTCACAAGCCCGTTCAATGACACGTTGGCGCCCTCTTGGGAAAAG
CAGAACTCTTCACCGAAGAATGCAATGAACTTCTCAAGCCGTCACGGAAGCCTTCCTTGGCTCCCAAGACA
GTGCTCAAGGAATTCGAAACTTCTCCAGAAGT
```

C. Primer- BF_3F and BF_2R

The nucleotide sequence obtained was 838 bp as given below

```
> BF_3F-2R
TAACGACTTCCGTTGTCCGTGTTTGGTTGAGATGGCGCTGACACGGTACACCTCATCGTATAAGCCTGGGT
TACCACCAGGGCCGCCACCAGCAGGGAGGACGGGCTGGGGAGAACCGTCAGTGGCACCCGCGGGGACGTAG
TCCTCGGTCTTCAGTCCATAGTGGGGGTCGGCGGAAGCCTTCTTGGGATCCGTCGAGTTCAGCCACGGGTA
GATGTACAATGGAATCTTGTCAATGTTGTCGGGGTAAAGATAGTCGGAGTAGTCGGTGCTGTAGTTGCCCA
AAGCAGGGGCTTCTTTGGTCTGACCGGACGCAGGTCCATACGGCGCCACGGGGATGGGCTGCACATAGAGG
TTAGAGTAGCCAAAGGTGGTATAACTAAGTCCATAGCCGAACTCGTAAACAGGGGTGTCATTCGCTTTGTC
GAAGTGTCTGTAGTCAATGAAGACGCCTTCGGTGAAGTCAACCTGCGGCGCACCCTTGCCATTGTTGGGTT
CCATGAGCAGGGGAGCACCATAGGAGTCGAGATCCTTACCCCAGGTGAAGGGCGTCTTTCCACCGGGGTTG
ACACGGCCATACAGGACATCGACCAAAGAGTTGCCGCTCTCCTGGCCAGGGACACCGGCCCAGACGATGGC
AGTGATGTTAGGGTTGTCGTTCCAATCTCCCGGCCTCGACCGCACCAACACCGTGGATGACCATGATGGTG
TTGTTGCAGTTAGCCGTGACAGTCTTGACGACTTGGTCTGCTCCTTGCCAGACAGTAATGTGTCCCGGTCT
CCATGTTACCGGCGACGTTTGATATAACCTTTCACAAATTCGAGTTGAACAACACGA
```

7.3.3.2. Multiple sequence alignment of sequenced fragments

Multiple sequence alignment of amplicons revealed high level of similarity and hence a consensus sequence based on sequence from primer BF_2F-2R was created as below

>Consensus sequence

```
ACTTCCTTTGTATGACT-ATGGTTGTGATGTGGCGCTGACACGGTACACCTCATCGTATAAGCCTGGGTTA
CCACCAGGGCCGCCACCAGCAGGGAGGACGGGCTGGGGAGAACCGTCAGTGGCACCCGCGGGGACGTAGTC
CTCGGTCTTCAGTCCATAGTGGGGGTCGGCGGAAGCCTTCTTGGGATCCGTCGAGTTCAGCCACGGGTAGA
TGTACAATGGAATCTTGTCAATGTTGTCGGGGTAAAGATAGTCGGAGTAGTCGGTGCTGTAGTTGCCCAAA
GCAGGGGCTTCTTTGGTCTGACCGGACGCAGGTCCATACGGCGCCACGGGGATGGGCTGCACATAGAGGTT
AGAGTAGCCAAAGGTGGTATAACTAAGTCCATAGCCGAACTCGTAAACAGGGGTGTCATTCGCTTTGTCGA
AGTGTCTGTAGTCAATGAAGACGCCTTCGGTGAAGTCAACCTGCGGCGCACCCTTGCCATTGTTGGGTTCC
ATGAGCAGGGGAGCACCATAGGAGTCGAGATCCTTACCCCAGGTGAAGGGCGTCTTTCCACCGGGGTTGAC
ACGGCCATACAGGACATCGACCAAAGAGTTGCCGCTCTCCTGGCCAGGGAGACCGGCCCAGACGATGGCAG
TGATGTTAGGGTTGTCGTTCCAATCTCC-GGCCTCGACCGCACCAACACCGTGGATGACCAGGATGGTGTT
GTTGCAGTTAGCCGTGACAGTCTTGACGACTTGGTCTGCTCCTTGCCAGACAGTAATGTTGTTCCAGTCTC
CCATGTTACCGGCGACGTT-GATATAA-CCTTCACAAAAGTCCGAGTTGACGAACACGATGGAAGAC-GTAG
CCTGCGAGGCAATACTGTTTCTAATCACATCCACATTAACCGTTGTCAATTGACGAAAAAAACTGCACCTT
—GCCTTTGGAAGAGGATCCTCGTCTGTATCCGC—TGCCCGAGTTAACAAAGTAAAGGAAAAGTTA-ATCAG
ATACCACTTTCCC—AGCCAATGACC-AGTGTAAACGTGTCACAAGCCCGTTCAATGACACGTTGGCGCCCT
CTTGGGAAAAGCAGAACTCTTCACCGAAGAATGCAATGAACTTCTCAAGCCGTCACGGAAGCCTTCCTTGG
CTCCCAGACAGTGCTCAAGGAATTCGAAACTTCTCCAGAAGT
```

7.3.3.3. BLAST analysis of the consensus sequence

BLAST analysis indicated that the sequence has 75 % identity with 69 % coverage to *Thermoascus aurantiacus* var. *levisporus* β-1,4-glucosidase (*bgl1*) mRNA, complete cds (EU 269024.1) which has the domain signatures of glycosyl hydrolase family 3 N and C terminal domain and fibronectin III like domain. Also the sequence had 75 % identity with 68 % coverage to the following

Thermoascus aurantiacus thermostable *bgl1* mRNA, complete cds (DQ 114397.1)

Thermoascus aurantiacus var. *levisporus bgl1* gene, complete cds (EU 269025.1)

Thermoascus aurantiacus thermostable *bgl1* gene, complete cds (DQ 114396.1)

7.3.3.4. Six-frame translation of consensus sequence

The consensus sequence was translated in to the corresponding amino acids using Transeq (EMBOSS) software. The six-frame translated amino acid sequences are given below

>consensus sequence 1-1170_ frame1
TSFV*LWL*CGADTVHLIV*AWVTTRAATSREDGLGRTVSGTRGDVVLGLQSIVGVGGSLLG
IRRVQPRVDVQWNLVNVVGVKIVGVVGAVVAQSRGFFGLTGRRSIRRHGDGLHIEVRVAKGG
ITKSIAELVNRGVIRFVEVSVVNEDAFGEVNLRRTLAIVGFHEQGSTIGVEILTPGEGRLST
GVDTAIQDIDQRVAALLARETGPDDGSDVRVVVPISGLDRTNTVDDQDGVVAVSRDSLDDLV
CSLPDSNVVPVSHVTGDVDITFTKVRVDEHDGRRSLRGNTVSNHIHINRCQLTKKTAPCLWK
RILVCIRCPS*QSKGKVNQIPLSQPMTSVNVSQARSMTRWRPLGKSRTLHRRMQ*TSQAVTE
AFLGSQDSAQGIRNFSR

>consensus sequence 1-1170_ frame2
LPLYDYGCDVALTRYTSSYKPGLPPGPPPAGRTGWGEPSVAPAGT*SSVFSP*WGSAEAFLG
SVEFSHG*MYNGILSMLSG*R*SE*SVL*LPKAGASLV*PDAGPYGATGMGCT*RLE*PKVV
*LSP*PNS*TGVSFALSKCL*SMKTPSVKSTCGAPLPLLGSMSRGAP*ESRSLPQVKGVFPP
GLTRPYRTSTKELPLSWPGRPAQTMAVMLGLSFQSPASTAPTPWMTRMVLLQLAVTVLTTWS
APCQTVMLFQSPMLPATLI*PSQKSELTNTMEDVACEAILFLITSTLTVVN*RKKLHLAFGR
GSSSVSAARVNKVKEKLIRYHFPSQ*PV*TCHKPVQ*HVGALLGKAELFTEECNELLKPSRK
PSLAPKTVLKEFETSPEV

>consensus sequence 1-1170_ frame3
FLCMTMVVMWR*HGTPHRISLGYHQGRHQQGGRAGENRQWHPRGRSPRSSVHSGGRRKPSWD
PSSSATGRCTMESCQCCRGKDSRSSRCCSCPKQGLLWSDRTQVHTAPRGWAAHRG*SSQRWY
N*VHSRTRKQGCHSLCRSVCSQ*RRLR*SQPAAHPCHCWVP*AGEHHRSRDPYPR*RASFHR
G*HGHTGHRPKSCRSPGQGDRPRRWQ*C*GCRSNLRPRPHQHRG*PGWCCCS*P*QS*RLGL
LLARQ*CCSSLPCYRRR*YNLHKSPS*RTRWKT*PARQYCF*SHPH*PLSIDEKNCTLPLEE
DPRLYPLPELTK*RKS*SDTTFPANDQCKRVTSPFNDTLAPSWEKQNSSPKNAMNFSSRHGS
LPWLPRQCSRNSKLLQKX

>consensus sequence 1-1170_ frame4
TSGEVSNSLSTVLGAKEGFRDGLRSSLHSSVKSSAFPKRAPTCH*TGL*HVYTGHWLGKWYL
INFSFTLLTRAADTDEDPLPKARCSFFRQLTTVNVDVIRNSIASQATSSIVFVNSDFCEGYI
NVAGNMGDWNNITVWQGADQVVKTVTANCNNTILVIHGVGAVEAGDWNDNPNITAIVWAGLP
GQESGNSLVDVLYGRVNPGGKTPFTWGKDLDSYGAPLLMEPNNGKGAPQVDFTEGVFIDYRH
FDKANDTPVYEFGYGLSYTTFGYSNLYVQPIPVAPYGPASGQTKEAPALGNYSTDYSDYLYP
DNIDKIPLYIYPWLNSTDPKKASADPHYGLKTEDYVPAGATDGSPQPVLPAGGGPGGNPGLY
DEVYRVSATSQP*SYKGS

>consensus sequence 1-1170_ frame5
FWRSFEFLEHCLGSQGRLP*RLEKFIAFFGEEFCFSQEGANVSLNGLVTRLHWSLAGKVVSD
*LFLYFVNSGSGYRRGSSSKGKVQFFSSIDNG*CGCD*KQYCLAGYVFHRVRQLGLL*RLYQ
RRR*HGRLEQHYCLARSRPSRQDCHG*LQQHHPGHPRCWCGRGRRLERQP*HHCHRLGRSPW
PGERQLFGRCPVWPCQPRWKDALHLG*GSRLLWCSPAHGTQQWQGCAAG*LHRRRLH*LQTL
RQSE*HPCLRVRLWT*LYHLWLL*PLCAAHPRGAVWTCVRSDQRSPCFGQLQHRLLRLSLPR
QH*QDSIVHLPVAELDGSQEGFRRPPLWTEDRGLRPRGCH*RFSPARPPCWWRPWW*PRLIR
*GVPCQRHITTIVIQRKX

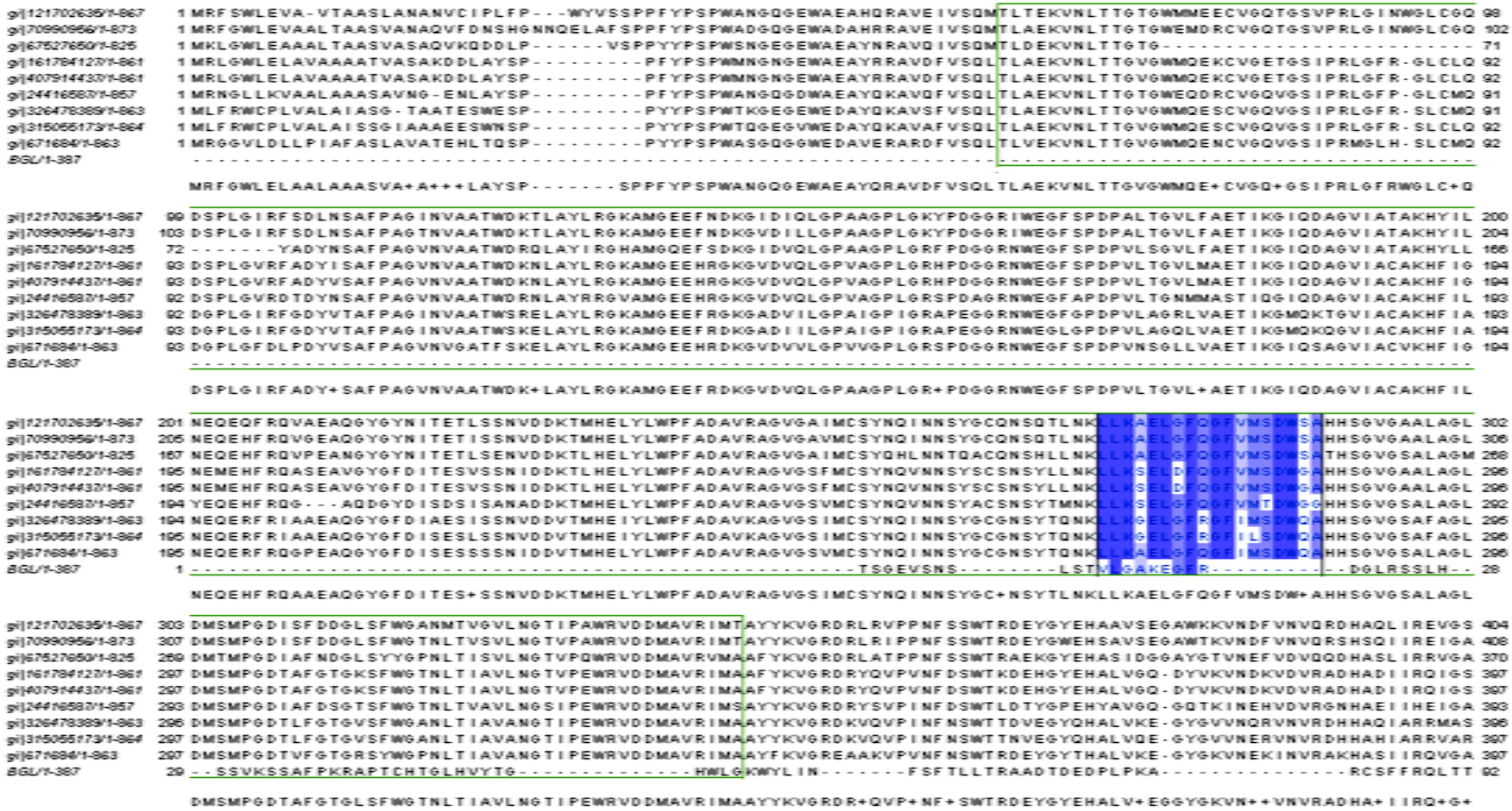
>consensus sequence 1-1170_ frame6
LLEKFRIP*ALSWEPRKASVTA*EVHCILR*RVLLFPRGRQRVIERACDTFTLVIGWESGI*
LTFPLLC*LGQRIQTRILFQRQGAVFFVN*QRLMWM*LETVLPRRLRLPSCSSTRTFVKVIS
TSPVTWETGTTLLSGKEQTKSSRLSRLTATTPSWSSTVLVRSRPEIGTTTLTSLPSSGPVSL
ARRAATLWSMSCMAVSTPVERRPSPGVRISTPMVLPCSWNPTMARVRRRLTSPKASSLTTDT
STKRMTPLFTSSAMDLVIPPLATLTSMCSPSPWRRMDLRPVRPKKPLLWATTAPTTPTIFTP
TTLTRFHCTSTRG*TRRIPRRLPPTPTMD*RPRTTSPRVPLTVLPSPSSLLVAALVVTQAYT
MRCTVSAPHHNHSHTKEV

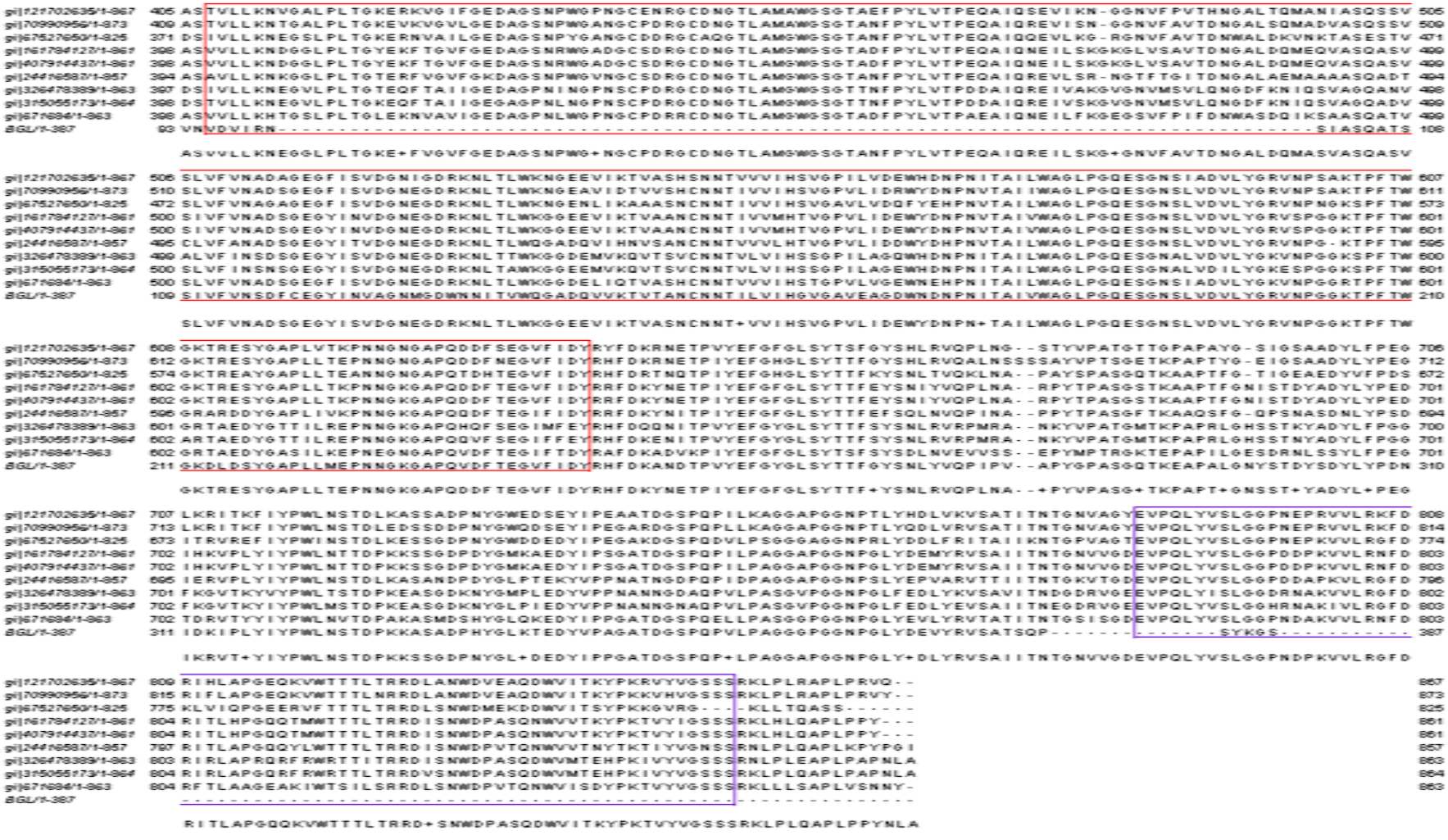
7.3.3.5. Protein –protein BLAST analysis of translated sequences

Protein –protein BLAST (blastp) of peptide sequences, generated by translation of the consensus sequence of nucleotide, was performed. Frame 4 translation (390 amino acids) had significant similarities with β-1,4-glucosidase of *Thermoascus aurantiacus* var. *levisporus* (ABX79552) with 71 % query coverage and 75 % identity. Also the sequence had close similarity (71 % query coverage and 75 % identity) to *Thermoascus aurantiacus* β-glucosidase (AAZ95587), *Thermoascus auranticus* thermostable β-glucosidase (AAZ 95587.1).

The translated peptide sequence had the glycosyl hydrolases family 3 C terminal domain conserved (Pfam 00933). Since only the C terminal amino-acid residues could be obtained, a multiple sequence alignment with known BGLs belonging to glycosyl hydrolase family 3, including the *Thermoascus aurantiacus* BGLs were performed to understand the similarities better. Multiple sequence alignment was done with ClustalW (Larkin et al., 2007) and was visualized using Jalview (Waterhouse et al., 2009). Comparison of the sequence with *T. aurantiacus* and other BGLs of the GH3 family indicated a close similarity with these proteins for the C terminal region for which sequence was available for *B. fulva* BGL. BGLs belonging to the family 3 glycosyl hydrolases typically contain the Glycosyl hydrolase Family 3 N terminal domain, Glycosyl hydrolase Family 3 C, both of which are considered to be contributing to the catalytic site architecture and a C terminal Fibronectin III like domain with proposed function in binding to the substrate (Pozzo et al., 2010). The protein sequence of *B. fulva* BGL matched the C terminal region of the consensus of BGLs spanning the regions of the glycosyl hydrolases family 3 C terminal domain and the fibronectin-like domain (Fig 7.5).

Fig 7.5: Multiple sequence alignment of the *B. fulva* BGL with family 3 glycosyl hydrolase protein sequences





Legends for the multiple sequence alignment (Fig 7.5)

>BGL: *Byssochlamys fulva* BGL - This study
>gi|121702635|ref|XP_001269582.1| β-glucosidase, putative [*Aspergillus clavatus* NRRL 1]
>gi|70990956|ref|XP_750327.1| β-glucosidase [*Aspergillus fumigatus* Af293]
>gi|67527650|ref|XP_661706.1| hypothetical protein AN4102.2 [*Aspergillus nidulans* FGSC A4]
>gi|161784127|gb|ABX79552.1| β-1,4-glucosidase [*Thermoascus aurantiacus* var.*levisporus*]
>gi|407914437|gb|AFU51372.1| β-glucosidase [*Thermoascus aurantiacus*]
>gi|24416587|gb|AAL69548.3| β-glucosidase [*Rasamsonia emersonii*]
>gi|326470827|gb|EGD94836.1| β-glucosidase [*Trichophyton tonsurans* CBS 112818]
>gi|315055173|ref|XP_003176961.1| β-glucosidase 1 [*Arthroderma gypseum* CBS 118893]
>gi|74054462|gb|AAZ95587.1| thermostable β-glucosidase [*Thermoascus aurantiacus*]
>gi|671684|gb|AAA86880.1| H antigen precursor [*Ajellomyces capsulatus*]

Green boxed region –Glycosyl hydrolase family 3 N terminal domain
Red boxed region: Glycosyl hydrolase family 3 C terminal domain
Violet color: Fibronectin III type domain
Blue shaded region with blue colored letter: Active site signature within the N terminal domain

Since only the C terminal region was available, the active site signature [LIVM](2) – [KR] – X – [EQKRD] – X(4) – G – [LIVMFTC] – [LIVT] – [LIVMF] – [ST] – D – X(2) – [SGADNIT] could not be detected in the *B. fulva* BGL. BGLs belonging to family 3 of glycosyl hydrolyses are known to have a three domain structure composed of an (α/β)8 TIM –barrel domain similar, a six-stranded α/β sandwich domain (both of which are important for active-site organization), and a C-terminal fibronectin type III domain of unknown function (Varghese et al., 1999; Pozzo et al., 2010). Substrate binding pocket is at the interface of the first two domains (Fig 7.6).

Figure 7.6: Crystal structure of Glycosyl hydrolase Family 3 β-glucosidase showing 3 domain structure

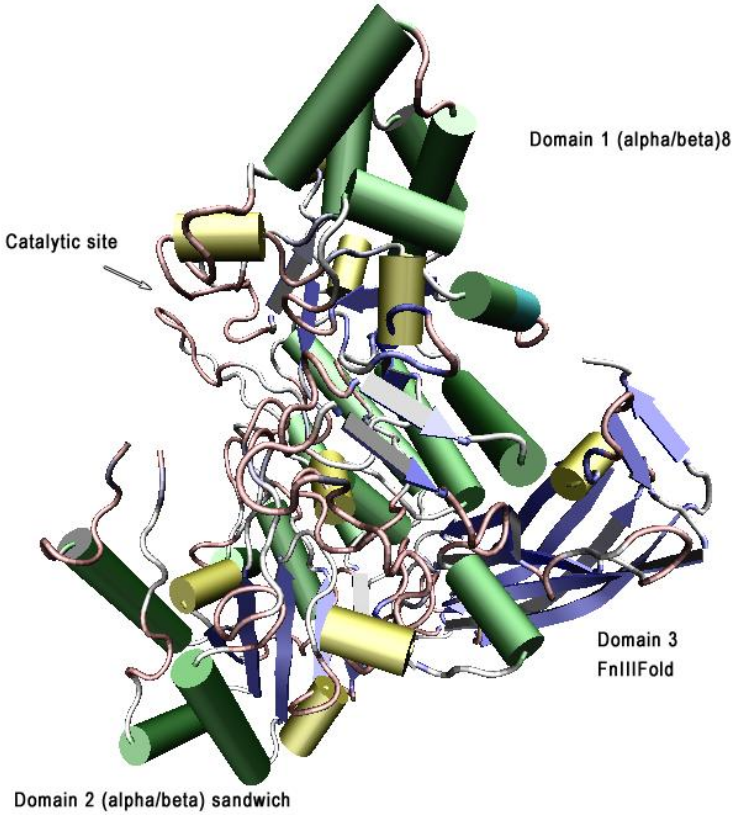
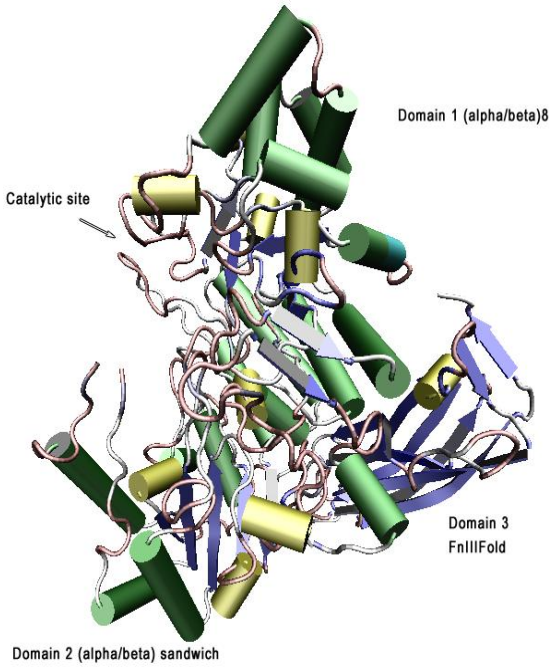


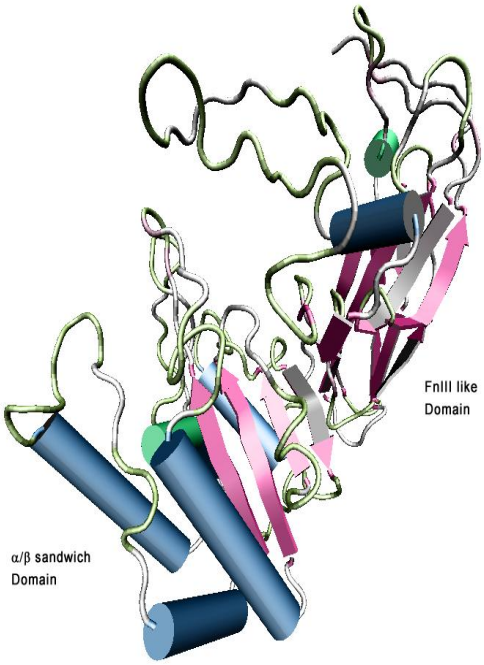
Image generated from crystal structure of β-glucosidase 3B from *Thermotoga neapolitiana* (Pozzo et al., 2010, PDB file-2X41) using VMD software (Humphrey et al., 1996).

The available sequence of *B. fulva* β-glucosidase was modeled to have an idea about its domain structure by homology modeling using the Swiss Model server through Deepview software (Guex and Peitsch, 1997). While the model is not perfect due to the lack of residues pertaining to the N terminal, it still provided an overview about the structure of the C terminal domain which was very similar to the BGL 3B from *Thermotoga neapolitiana* whose structure was used as the template to model the C terminal of *B. fulva* BGL (Fig 7.7).

Figure 7.7: 3D structure of the C terminal domain of *B. fulva* BGL compared to that of *T. neapolitaia*



Crystal structure of *T neapolitiana* BGLB rendered using VMD



3D structure of the C terminal domains of *B. fulva* generated through homology modeling

The 3D structure generated by homology modeling resembled the structure of the C terminal of the *T. neapolitiana* BGL B. Very few crystal structures of family 3 β- glucosidases have been resolved till date and homology modeling efforts have been taken by some researchers to deduce the structure of such BGLs which will aid in understanding their structure function relationships, reaction mechanisms including residues that form their catalytic site (Jeya et al., 2010, Sorensen, 2010, Del-Pozo et al., 2012) This in turn will help in tailoring the enzyme for its destined functions – i.e., to improve biomass hydrolysis or catalyzing specific transglycosylation mechanisms

While the homology model generated of the *B. fulva* BGL cannot be regarded as a good structure or accurate representation due the lack of sufficient residues, it still serves

as a good starting point to look at the *B. fulva* enzyme's structure. Generation of the full length sequence of protein is the next logical step obtaining an accurate model. However, even looking at this preliminary structure, it is apparent that the limited number of C terminal residues itself could provide sufficient information about the α/β sandwich domain and the fibronectin III (Fn II) like domain which seems to be a feature shared by fungal β-glucosidases.

7.4. **Conclusion**

Beta-glucosidases are peculiar enzymes with diversity in kinetic parameters. Even in strains of same species, diversity in number and characters of enzyme can be noticed, eventually helping in the survival of the strain. Occurrence of isoforms is common in most of the BGL secreting fungal taxa. This multiplicity may be due to the presence of different *bgl* genes or post translational modifications like differential processing of mRNA or glycosylation of protein. *B. fulva* was found to secrete seven isoforms. Amplification and sequence analysis indicate that β-glucosidase from *B. fulva* belongs to glycosyl hydrolase family 3. The translated sequence from 1170 bp nucleotide revealed the presence of a glycosyl hydrolase family 3 C terminal domain. Multiple sequence alignments with similar fungal β-glucosidases indicated that the obtained protein sequence of *B. fulva* shared homology with the C terminal sequences of these fungi. Homology modeling revealed the presence of the typical α/β sandwich motiff and a fibronectin III like motif further towards the C terminal. The information is important, since structure information is available only for a very few fungal GH family 3 BGLs and knowledge about their structures can aid in design of better enzymes for biomass hydrolysis or for transglycosylation reactions.

Chapter 8

Immobilization of β-glucosidase on magnetic nanoparticles

Chapter 8

Immobilization of β-glucosidase on magnetic nanoparticles

8.1. Introduction

Safe and sustainable methodologies for hydrolysis rely up on biocatalysis which offer mild reaction conditions, environmental safety, high efficiency and selectivity. Reuse of enzymes reduces the cost of production and thus researches aim at developing suitable technologies to immobilize enzymes. Enzyme immobilization provides many advantages over free enzymes namely enzyme reuse, continuous operation, controlled product formation, simple processing and enhanced stability under both storage and operational conditions (Gargouri et al., 2004; Sheldon, 2007). Easy separation of enzyme from the product simplifies enzyme applications and supports a reliable and efficient reaction technology. Also, reuse of enzymes provides a cost advantage which is a prerequisite for establishing enzyme–catalyzed process (Tischer and Wedekind, 1999).

The technique of immobilization may be by binding to a support (carrier), entrapment (encapsulation) and cross linking. The choice of immobilization method depends on the enzyme, support, chemical reagents and reactor (Gargouri et al., 2004). Support binding can be physical by means of hydrophobic or van der Waals interactions, ionic or covalent in nature. Physical means of immobilization is not industrially applicable owing to possible detachment of enzymes under change in ionic strength and pH. Covalent binding prevents leaching of enzymes, but chances of enzyme deactivation during immobilization occur at a high rate. Entrapment of enzyme in polymer network or microcapsules is another method of immobilization employed for industrial purposes like in a hollow fiber reactor. Cross linking of enzymes using bi-functional reagents offer highly concentrated enzyme activity in the catalyst, high stability and low production cost owing to exclusion of carriers (Sheldon, 2007).

Particle size of carriers plays an important role in the success of immobilization. Nanoparticles offer high surface area to which large number of enzyme molecules can

be immobilized. Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm (Mohanraj and Chen, 2006). A number of inorganic carriers can be used, including alumina, silica and zeolites. However, recovery of nanoparticles becomes difficult especially when biomass is used as substrate for catalysis. Magnetic nanoparticles are a new technology whereby immobilized enzymes can easily be recovered under strong magnetic fields, even from viscous reactants/products. Magnetic nanoparticles have been synthesized with different compositions and phases, including iron oxides, such as Fe_3O_4 and γ-Fe_2O_3, pure metals, such as Fe and Co, spinel-type ferromagnets, such as $MgFe_2O_4$, $MnFe_2O_4$ and $CoFe_2O_4$, as well as alloys, such as $CoPt_3$ and FePt. The common methods of synthesis employed include co-precipitation, thermal decomposition and/or reduction, micelle synthesis, hydrothermal synthesis, and laser pyrolysis techniques (Lu et al., 2007). In the present study, an attempt was made to prepare magnetic nanoparticles and to improve its stability by silica coating. Purified β-glucosidases were successfully immobilized onto magnetic nanoparticles and the immobilized enzymes were recovered using a strong magnet.

8.2. Materials and Methods

8.2.1. Synthesis of magnetic nanoparticles

Magnetic nanoparticles were synthesized by a modified protocol of Berger et al (1999). Four milliliters of 2.0 M $FeCl_2$ was mixed with 20 ml of 2.0 M $FeCl_3$ in a 500 ml Erlenmeyer flask. Using a burette, 250 ml of 0.7 M ammonium hydroxide was added to this mixture drop by drop under vigorous agitation. Stirring was stopped once a black precipitate of magnetite (Fe_3O_4) was formed, and the precipitate was allowed to settle. The supernatant was decanted and the rest of the suspension was centrifuged at 2000 rpm for 5 min to obtain the dark sludge like magnetite. The magnetite was washed thrice with distilled water and recovered as pellet after centrifugation (2000 rpm, 5 min). The pellet was dried at 50 °C overnight.

8.2.2. Silica coating of magnetic nanoparticles

For enhancing stability of magnetic nanoparticles, silica coating was performed as per the procedure of Bo et al (2008). 10.98 g of sodium silicate was dissolved in deionized water. The pH of the solution was adjusted to 12.5 by addition of 2.0 M HCl. 500 mg of magnetic nanoparticles, prepared as outlined in section 8.2.1 were added to this solution and was ultra-sonicated for 30 min. The suspension was then kept in a shaking water bath at 80 °C. Using a burette, 2.0 M HCl was added drop wise into the solution so as to attain a pH of 6-7 in a time span of 3 h. The precipitate was washed several times with deionized water and recovered as pellet by centrifugation at 2000 rpm for 5 min. The brown yellow precipitate was dried at 50 °C overnight.

8.2.3. Silica coating of magnetic nanoparticles under N_2 atmosphere

Silica coated nanoparticles produced as outlined in section 8.2.2 showed oxidation as evident from brown coloured supernatant. Hence a modification of the procedure was performed. In this method, silica coating using sodium silicate was done at 60° C with stirring by a magnetic stirrer under nitrogen containing atmosphere. The precipitate was recovered as outlined under section 8.2.2.

8.2.4. Functionalization of magnetic nanoparticles

Functionalization of magnetic nanoparticles were performed using APTES (3-amino propyl-triethoxysilane) as per the protocol of Can et al (2009). Four gram of silica coated magnetic nanoparticles was sonicated in 150 ml 50 % ethanol for 30 min. To this suspension, 16.16 g of APTES was added and the solution was stirred at 40 °C under N_2 atmosphere for 2 h. The suspension was cooled to room temperature and the APTES modified nanoparticles were collected by centrifugation at 2000 rpm for 5 min. The pellet was washed thrice with deionized water and dried at 70 °C.

8.2.5. Immobilization of β-glucosidase

Immobilization protocol as outlined by Can et al (2009) was used with minor modifications. 1.53 g of silica coated nanoparticles was dispersed in 60 ml 5 % glutaraldehyde. The suspension was kept overnight with stirring at 4 °C. The nanoparticles were recovered using a magnet and washed thrice with deionized water. It was dispensed in 20 ml citrate buffer (pH 4.8, 0.05 M). To this, purified β-glucosidase equivalent to 2000 U in citrate buffer (pH 4.8, 0.05 M) was added and incubated at 4 °C for 5 h with mild agitation. Purified β-glucosidase isoforms- BGL 2, BGL 3 and BGL 6 were immobilized individually onto nanoparticles. After incubation, the nanoparticles were separated by a magnet and washed thrice with citrate buffer (pH 4.8, 0.05 M). The immobilized nanoparticles were stored at 4 °C in citrate buffer (pH 4.8, 0.05 M). The particles were diluted and β-glucosidase activity was determined as outlined in section 2.5.1.

8.2.6. Polyelectrolyte coating of enzyme immobilized magnetic nanoparticles

The enzyme immobilized magnetic nanoparticles were coated with polyelectrolyte. PAH –Poly (allylamine hydrochloride) was taken as the +ve polyelectrolyte and PS (Polystyrene) was used as the negative polyelectrolyte. 10 mg/ml stock solution of each polyelectrolyte was prepared in deionized water. The enzyme immobilized nanoparticles, dispersed in 10 ml citrate buffer (pH 4.8, 0.05 M) corresponding to total activity of 211 U of BGL 2, 469 U of BGL 3 and 1150 U of BGL 6 were used for polyelectrolyte coating. To the immobilized enzyme, 100 µl stock solution of PAH was added and was shaken at 100 rpm for 10 minutes. The suspension was centrifuged at 5000 rpm, 4 °C for 5 min and washed once with citrate buffer (pH 4.8, 0.05 M), recovered by centrifugation (5000 rpm, 4 °C for 5 min) and reconstituted to 10 ml using the same buffer. To this 100 µl stock solution of PS was added and was shaken at 100 rpm for 10 min. The suspension was centrifuged at 5000 rpm, 4 °C for 5 min and was washed once with citrate buffer (pH 4.8, 0.05 M), recovered by

centrifugation (5000 rpm and 4 °C for 5 min) and reconstituted to 10 ml using the same buffer. The process was repeated twice with alternating the polyelectrolyte and the immobilized enzyme was recovered, the activity was assayed as outlined in section 2.5.1 and stored at 4 °C in citrate buffer (pH 4.8, 0.05 M).

8.2.7. Analysis of magnetic nanoparticles by Scanning Electron Microscopy (SEM)

The nanoparticles and silica coated nanoparticles were spread on clean cover slips and dried at 50 °C for 16 h. The cover slips were coated with gold particles and were visualized under scanning electron microscope (NIIST SEM).

8.2.8. Analysis of magnetic nanoparticles by Dynamic Light Scattering Spectrophotometry (DLS)

The nanoparticles, silica coated nanoparticles, enzyme immobilized nanoparticles and polyelectrolyte coated enzyme immobilized nanoparticles were filtered through 0.22 µ filter. The size of nanoparticles and their behavior in aqueous suspension in presence and absence of ionic detergent polysorbate 80 (0.01 % v/v) was analyzed using Zeta Potential Analyzer (Malvern Instruments). The suspension was poured in to a plastic cuvette and the light scattering of nanoparticles were measured against distilled water as blank.

8.2.9. Analysis of magnetic nanoparticles by Atomic Force Microscopy (AFM)

To study the size and surface morphology of nanoparticles, atomic force microscopy was employed. The nanoparticles were filtered through 0.22 µ filter and the filtrate was serially diluted to 10^{-6} dilutions. 100 µl of the filtrate as well as 10^{-6} dilution were spread across clean cover slips and were dried at 50 °C for 16 h. The samples were then analyzed using atomic force microscope.

8.3. Results and Discussion

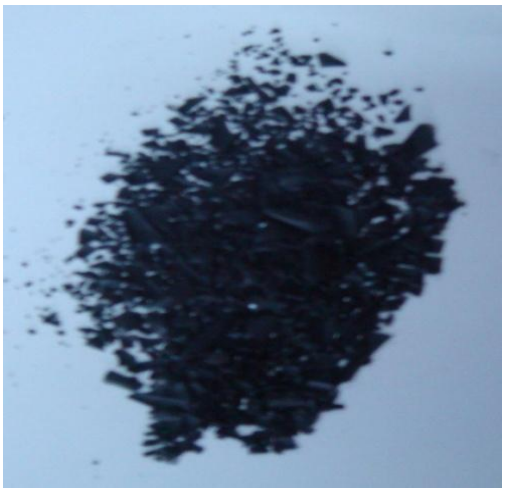
8.3.1. Synthesis of magnetic nanoparticles

Magnetic nanoparticles (magnetite-Fe_3O_4) were successfully synthesized by co-precipitating $FeCl_2$ and $FeCl_3$ in presence of ammonia.

$$2FeCl_3 + FeCl_2 + 8NH_3 + 4H_2O \rightarrow Fe_3O_4 + 8NH_4Cl$$

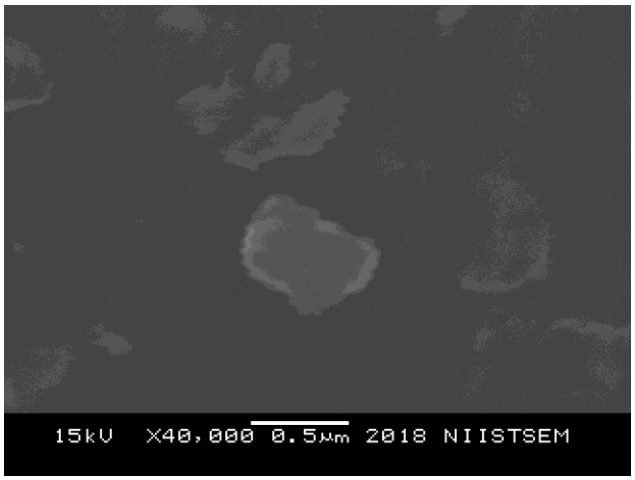
The nanoparticles were washed with deionized water and dried at 50 °C and stored in an air tight container. The magnetite so obtained was black in colour (Fig 8.1). The magnetic nature of the particles was ascertained by their movement under an external magnetic field.

Figure 8.1: Magnetic nanoparticles



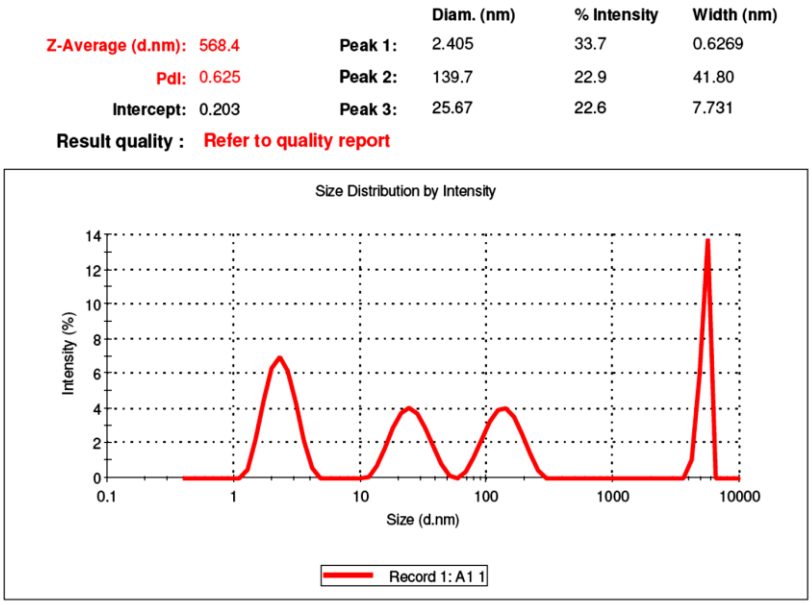
SEM analysis of magnetic nanoparticles revealed irregular shaped particles with uneven margins (Fig 8.2). The size, shape and composition of magnetic nanoparticles depend on the Fe^{2+}/Fe^{3+} ratio, type of salt, reaction temperature, pH and ionic strength of the media (Lu et al., 2007).

Figure 8.2: SEM image of magnetic nanoparticles



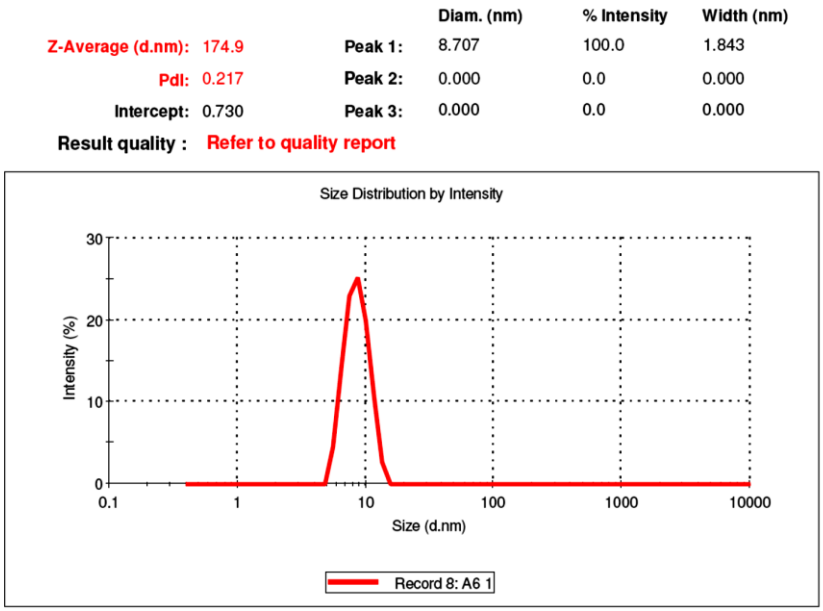
Dynamic light scattering analysis showed four peaks of size intensity corresponding to 2.4 nm, 25.7 nm, 140 nm and 8000 nm (Fig 8.3). Nanoparticles owing to their small size and large surface area can lead to particle-particle aggregation (Mohanraj and Chen, 2006). This problem aggravates when nanoparticles is magnetic due to magnetic attraction between the particles.

Figure 8.3: DLS analysis of magnetic nanoparticles



Addition of tween 80 (0.01 % v/v) to the nanoparticles reduced the number of peaks to one with an average size intensity of 8.7 nm (Fig 8.4). Tween 80 (Polysorbate 80) is a common surfactant added during hydrolysis experiment. It reduces the enzyme adsorption to the lignocellulosic substrate (Eriksson et al., 2002; Tu et al., 2007). Addition of polysorbate 80 to nanoparticles were found to reduce aggregation of silver nanoparticles (Li et al., 2012). Addition of tween 80 increased the stability of Fe_3O_4 magnetic nanoparticles by the hydrophilic block of tween 80 that stabilizes the nanoparticles in water (Wang et al., 2011).

Figure 8.4: DLS analysis of magnetic nanoparticles in presence of 0.01 % v/v tween 80



The AFM images of magnetic particles synthesized in the present study confirmed their nanoscale size (Fig 8.5). AFM analyses indicated that the undiluted nanoparticles showed a tendency to aggregate (Fig 8.6). The aggregated particles appeared in the form of branched flakes with irregular or serrated margins. Similar self aggregation has been reported with lipase immobilized magnetic nanoparticles (Dyal et al., 2003). However, in diluted samples (10^{-6} dilution) the aggregation was highly

decreased as evident from the size distribution graph (Fig 8.7). The particle size along the reference line was below 10 nm.

Figure 8.5: Surface morphology of magnetic nanoparticles with corresponding 3D size range

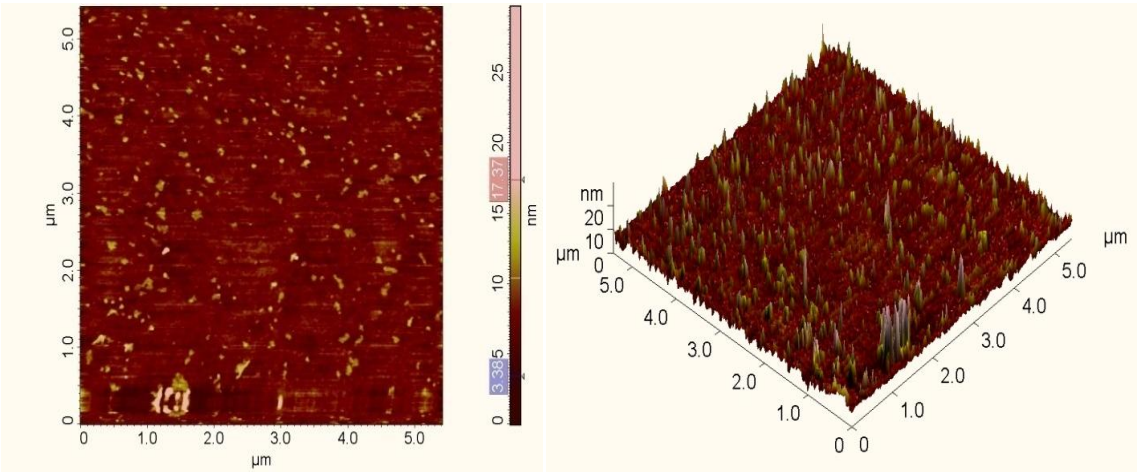


Figure 8.6: Aggregation of magnetic nanoparticles and size distribution graph of particles along the reference line

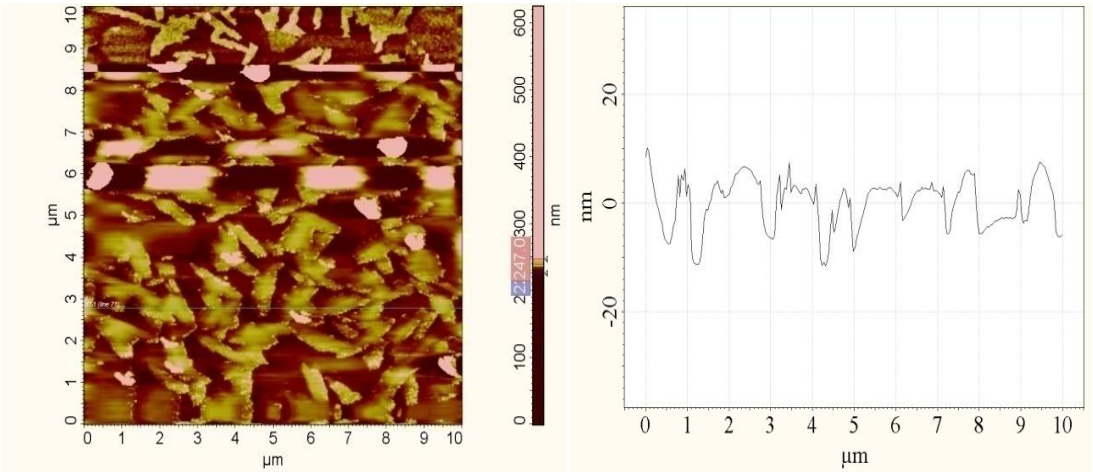
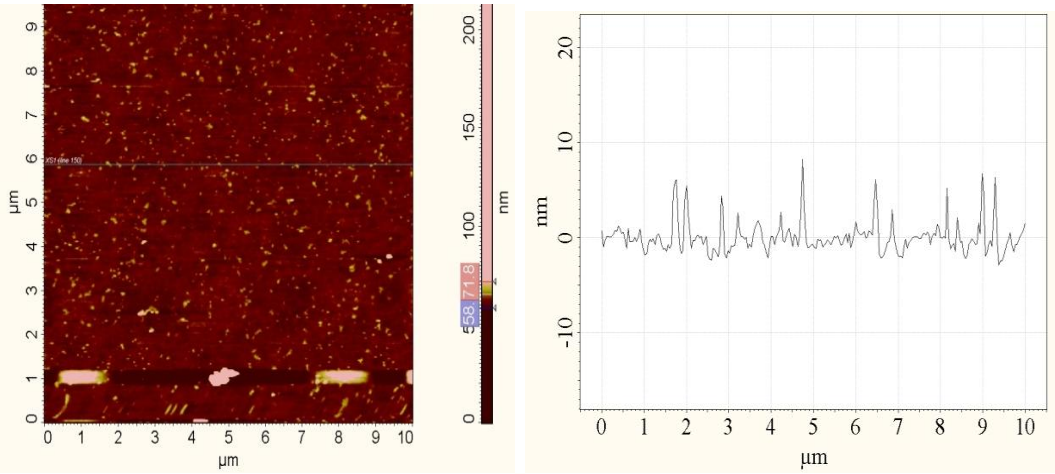


Figure 8.7: Surface morphology of diluted magnetic nanoparticles (10^{-6}) with corresponding size distribution graph of particles along the reference line



8.3.2. Preparation of silica coated magnetic nanoparticles

Magnetic nanoparticles are very sensitive to oxidation and agglomeration due to large surface area, high chemical reactivity and magnetic dipole interactions. Under ambient conditions, rapid oxidation occurs resulting in the deposition of oxide layers than can change the particle properties. Agglomeration in to larger clusters also affects the use of magnetic nanoparticles. To protect magnetic nanoparticles from oxidation and agglomeration, encapsulation using carbon, silica, metal oxides, organic polymers or surfactants can be carried out (Faraji et al., 2010). Silica coating of magnetic nanoparticles have several advantages like protection of magnetic core, stability under aqueous conditions, easy surface modifications, easy control of inter-particle interactions by variation of shell thickness and creation of abundant silanol groups which can be easily activated by functional groups (Deng et al., 2005; Lu et al., 2007). Silica coated nanoparticles prepared by the procedure as outlined in section 8.2.2 showed oxidation as evident from the brown supernatant. Exposure to high temperature without a reducing atmosphere might have caused oxidation of magnetite during the encapsulation. Hence silica coating of magnetic nanoparticles were done by the modified protocol as mentioned in 8.2.3. The magnetic properties of nanoparticles were maintained even after

silica coating as evident from by their spiking effect under an external magnetic field (Fig 8.8). SEM analysis indicated silica coating to decrease serrations in the nanoparticles, creating smooth surfaces (Fig 8.9).

Figure 8.8: Spiking effect of silica coated magnetic nanoparticles under the influence of an external magnetic field.

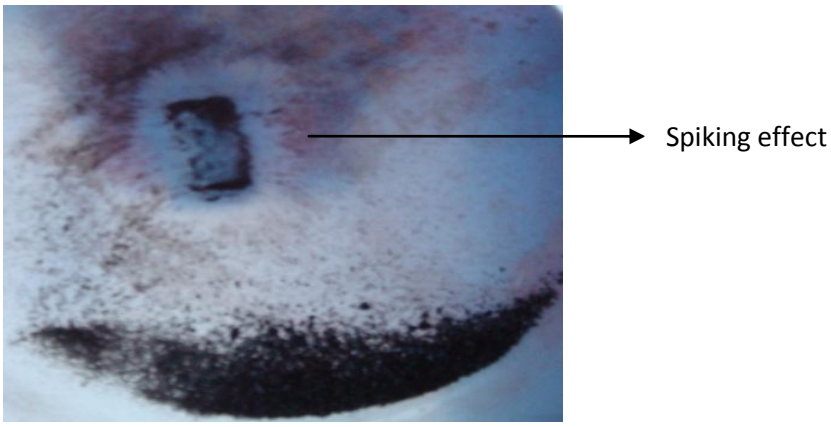
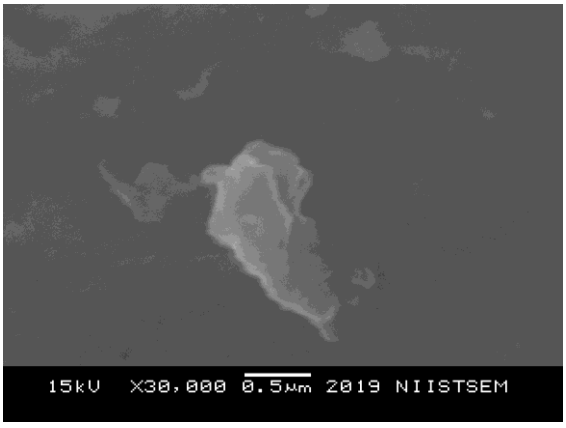
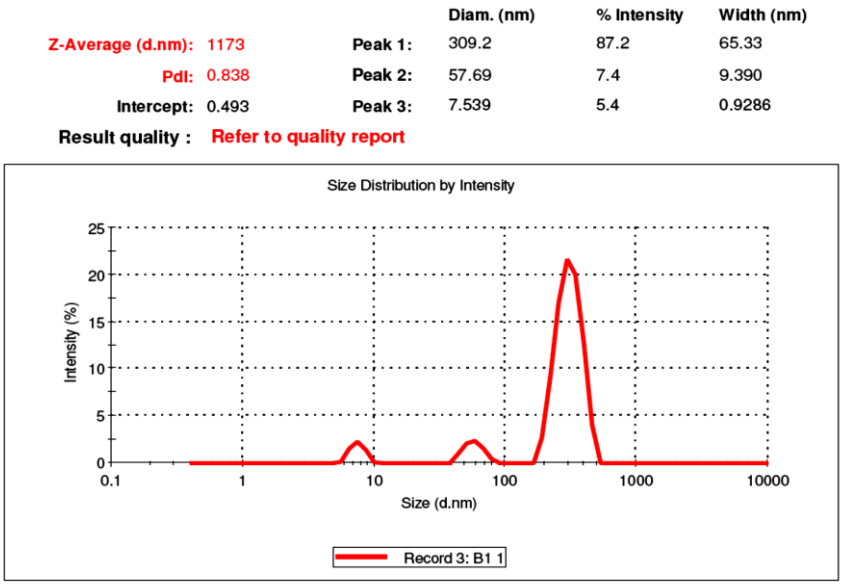


Figure 8.9: SEM image of silica coated magnetic nanoparticles



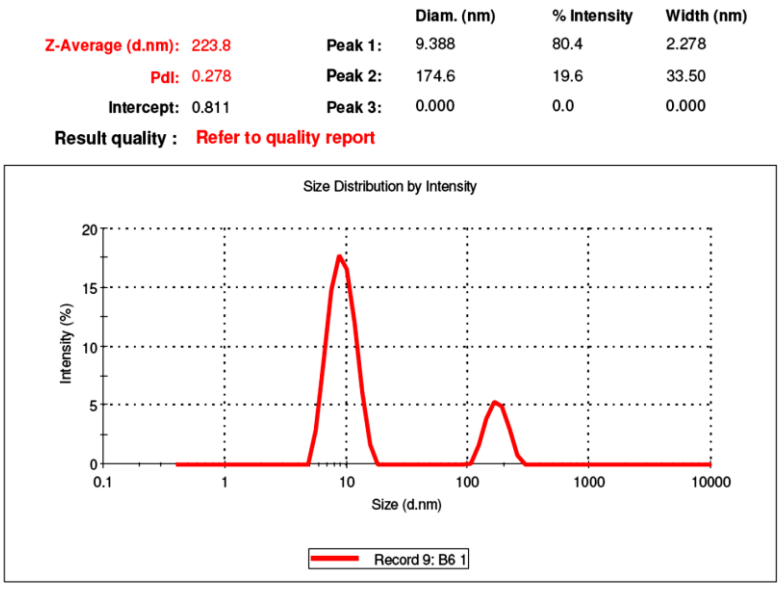
Silica coating of magnetic nanoparticles caused increase in particle size. DLS analysis reveals three peaks of size intensity, the major one corresponding to a size of 309 nm (Fig 8.10). However, compared to uncoated nanoparticles, the highest average size got reduced from 8000 nm to 309 nm indicating the decreased agglomeration due to silica coating.

Figure 8.10: DLS analysis of silica coated magnetic nanoparticles



With addition of tween 80, agglomeration got reduced and major peak of size got shifted to 9.4 nm. However, an additional peak of size intensity corresponding to 175 nm was present (Fig 8.11).

Figure 8.11: DLS analysis of silica coated magnetic nanoparticles in presence of 0.01 % v/v tween 80



8.3.3. Immobilization of β-glucosidase on to silica coated magnetic nanoparticles

The silica coated magnetic nanoparticles were functionalized using APTES and β-glucosidase enzyme was immobilized by covalent binding with glutaraldehyde reagent. The enzyme immobilized nanoparticles were separated by a magnet and washed thrice with citrate buffer and stored at 4 °C in citrate buffer (pH 4.8, 0.05 M). The immobilization efficiency varied with the isoforms. Among the three isoforms, BGL 5 showed high immobilization rate with a total of 1149 U activity against the initial 2000 U provided for immobilization (Table 8.1).The immobilized enzymes showed 100 % storage stability at 4 °C for 1 month. Lipases immobilized on magnetic nanoparticles had a constant activity over one month of storage. The enzyme-nanoparticle composites showed only 15 % activity loss over one month, probably by desorption or denaturation (Dyal et al., 2003). Beta-glucosidase from *Trichoderma reesei* immobilized on to magnetic nanoparticles showed 98 % activity retention with a stability of at least 45 days (Valenzuela et al., 2011). Beta-glucosidase from *Agaricus arvensis* immobilized on silicon oxide nanoparticles showed improved thermal stability with a 288 fold enhancement of $t_{1/2}$ at 65 °C (Singh et al., 2011). On immobilization to magnetic aluminum nitride nanoparticles, β-glucosidase showed an activity retention of 78.4 % (Pan et al., 2008).

Table 8.1: Efficiency of immobilization

Enzyme used	Total BGL activity in magnetic nanoparticles (U)	Efficiency of immobilization (% activity retention after immobilization)
BGL 2	211.6	10.58
BGL 3	469.02	23.45
BGL 6	1149.58	72.48

DLS analysis of enzyme immobilized nanoparticles revealed a major peak of size corresponding to 139 nm and a minor peak of 2 nm (Fig 8.12). Addition of tween 80

resulted in the shift of peak towards 9.6 nm, still having a second peak corresponding to a size of 133 nm (Fig 8.13).

Figure 8.12: DLS analysis of enzyme immobilized magnetic nanoparticles

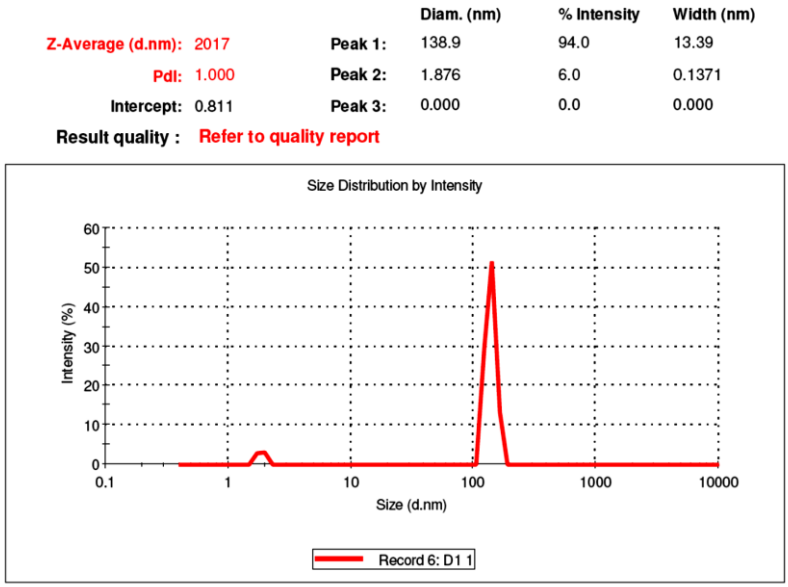
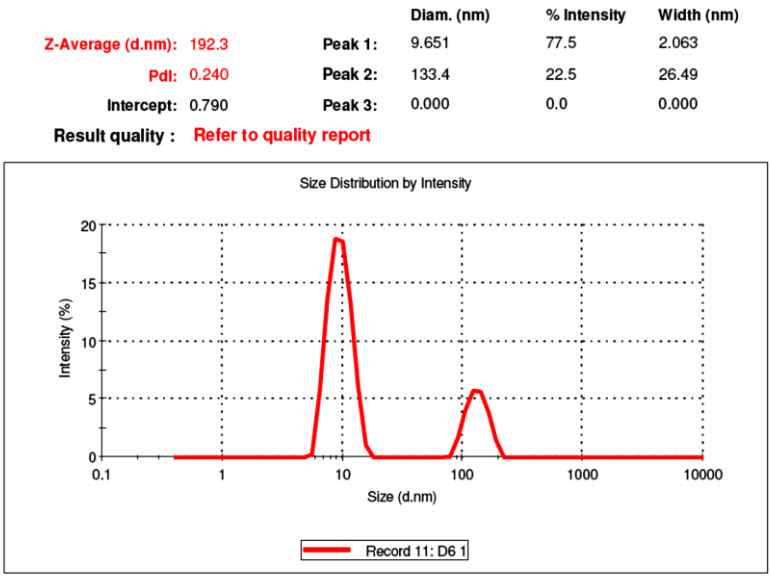


Figure 8.13: DLS analysis of enzyme immobilized magnetic nanoparticles in presence of 0.01 % v/v tween 80



8.3.4. Polyelectrolyte coating of immobilized enzyme

Surfactants or polymers are often employed to passivate the surface of nanoparticles to avoid agglomeration. It creates a electrostatic or stearic repulsion that disperses the nanoparticles and keeps them in a colloidal state (Lu et al., 2007). Polyelectrolyte coating of immobilized enzymes can enhance enzyme stability and also reduces the attack of proteolytic enzymes. Polymer coated catalase was found to be stable against protease degradation, retaining 100 % activity after 100 minutes incubation, while uncoated catalase losing more than 90 % of activity under similar conditions (Caruso et al., 2000).The immobilized isoforms were coated thrice with polyelectrolyte with alternating +ve and –ve electrolyte. The total activity of immobilized enzymes after polyelectrolyte coating is given in table 8.2. Isoforms varied to their activity retention after polyelectrolyte coating. Maximum retention of activity was shown by BGL 3.

Table 8.2: Enzyme activity after polyelectrolyte coating

Enzyme used	Total BGL activity in magnetic nanoparticles (U)	% activity retention after polyelectrolyte coating compared with initial activity in immobilized nanoparticles
BGL 2	141.34	66.80
BGL 3	442.64	94.38
BGL 6	927.36	80.67

Dynamic light scattering analysis showed two peaks of size intensity corresponding to 92 nm and 681 nm (Fig 8.14).Polymer coating has thus increased the average size of nanoparticles. Addition of tween 80 reduced agglomeration as evident from the reduced average size of peaks to 9.3 and 82 nm (Fig 8.15).

Figure 8.14: DLS analysis of polymer coated enzyme immobilized magnetic nanoparticles

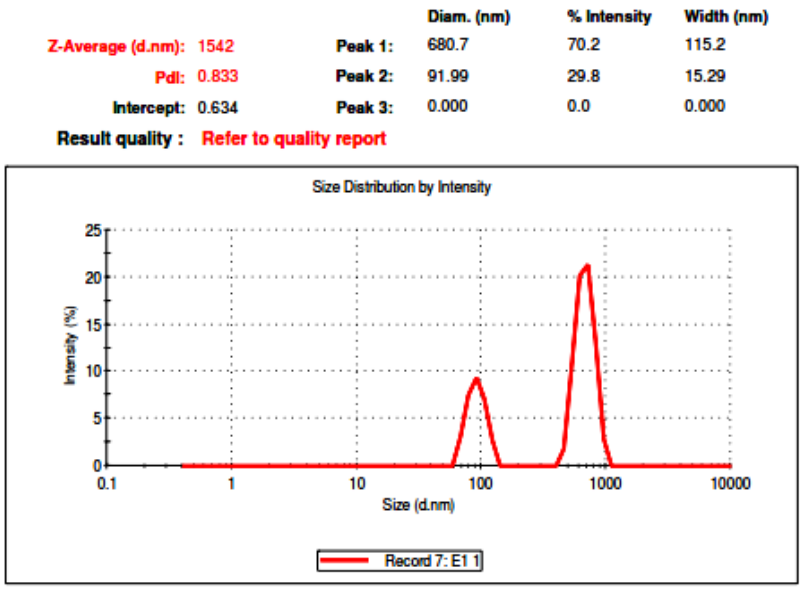
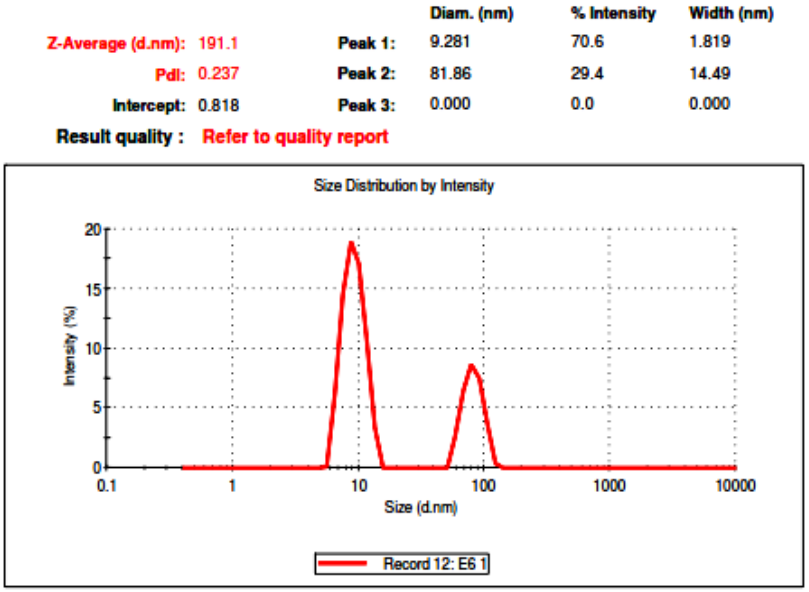


Figure 8.15: DLS analysis of polymer coated enzyme immobilized magnetic nanoparticles in presence of 0.01 % v/v tween 80



8.4. Conclusions

The recovery and reuse of enzymes by immobilization has been a matter of interest in catalysis research. Gel entrapment is the most widely used technique but has the demerit of diffusional resistance that can result in accumulation of products causing enzyme inhibition. Nanoparticles offer greater surface area for enzyme immobilization and remain in colloidal or near colloidal state. Magnetic nanoparticles have the greatest advantage of easy recovery using an applied magnetic field. The present study was successful in synthesizing magnetic nanoparticles. The stability of the produced nanoparticles was enhanced by silica coating and β-glucosidase was effectively immobilized on to the nanoparticles. Additional protection in the form of polyelectrolyte covering was provided which might significantly reduce proteolytic cleavage by proteases present in crude cellulase preparations used in hydrolysis.

Chapter 9

Anti β-glucosidase antibody production and its applications

Chapter 9

Anti β-glucosidase antibody production and its applications

9.1. Introduction

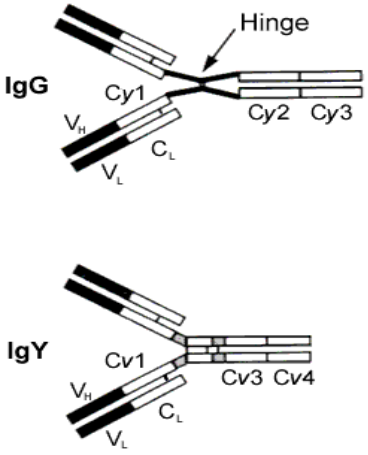
Antibodies against β-glucosidases are important in studies on β-glucosidase expression by microbes and in cells of higher order organisms. Anti BGL antibodies have been used to study Gaucher's disease (Grace et al., 1997), for determination of the catalytic mechanism of BGL action (Fabbro and Grabowski, 1991; Fischer et al., 1996), for studies on screening, isolation and detection of novel β-glucosidases (Jinfeng et al., 2007; Moranelli et al., 1985; Skory and Freer, 1995) and in localization of BGL in tissues, mycelia or plant cells (Nikus et al., 2001; Saloheimo et al., 2002; Yildiz et al., 2006). BGL antibodies also could be used for single step purification of β-glucosidases as in fermentation broths which will aid in obtaining relatively pure enzyme. This is important, since pure BGL is required in some applications like transglycosylations for production of industrially and pharmaceutically important compounds/metabolites. While there are several antibodies against human BGLs, very few are reported for fungi.

Several reports indicate successful production of antibodies in rabbit against microbial enzymes. (Pfyffer et al., 1989; Liu et al., 1993; Zia et al., 2012; Ziglari et al., 2008;) Rabbit anti β-glucosidase antibody has been raised against β-glucosidases from *Volvariella volvacea* (Cai et al., 1999), *Trichoderma reesei* (Jackson and Talburt, 1988; Saloheimo et al., 2002; Sprey, 1986), *Debaryomyces pseudopolymorphus* (Villena et al., 2006) and *Uromyces fabae* (Haerter and Voegele, 2004). The major disadvantage of using rabbit as source of antisera include difficulty in purification of antibodies, low yield and distress to the animal that occurs from immunization, collecting of blood samples and bleeding (Michael et al., 2010).

An alternative to producing antibody in mammals is to raise them in avian (e.g. Chicken) species. Avian sera contain three principal classes of immunoglobulins – IgA,

IgM and IgY. IgY (molecular weight of 180 kDa) is identical to IgG of mammals but has some variations from the typical IgG structure (Figure 9.1). The birds lay eggs and thus transfer maternal immunoglobulins onto their offspring within the egg contents. Thus the egg yolk becomes rich in IgY, while the albumin contains IgA (Tizard, 2002) .

Figure 9.1: Structures of Mammalian IgG and Avian IgY



Antibodies from chicken have several advantages compared to mammalian IgG. The antibody amount generated from mammals per bleed (40 ml blood) is around 200 mg (per month) while a single egg can carry an IgY load of 50-100 mg. As chicken lays 5-7 eggs per week, the total IgY will be around 1500 mg (Kovacs-Nolan and Mine, 2004). Moreover, the antibodies can be easily purified by simple precipitation techniques (Gassmann et al., 1990). Chicken IgY antibodies are widely used in the treatment of infectious diseases of the gastrointestinal tract. Also IgY antibodies can be used to compact H5N1 infections especially as antibody impregnated air filters (Kamiyama et al., 2011). In addition to their therapeutic importance, IgY is widely used in biological and medical research as diagnostic molecules. Since they significantly reduce cross reactivity, their use in immunological assays can reduce interference that would be caused by IgG (Larsson et al., 1992).

IgY can be used for immuno-affinity chromatography to isolate and purify target molecules. Due to the high affinity of antibodies, the target molecules can easily be

isolated from crude preparations. They are already reported for purification of lactoferrin (Li-Chan et al., 1998) and in the isolation and separation of IgG antibodies from colostrum, cheese whey and milk (Akita and Li-Chan, 1998). However the IgY molecules need to be bound on to the column by some strong covalent or non-covalent forces. The use of IgY immobilized magnetic particles offer a greater advantage as they can be applied directly into solution containing target molecules and can be recovered by applying a strong magnetic field.

In the present study, anti β-glucosidase antibody was produced from rabbit and chicken. The antibody from chicken was used in the localization studies of β-glucosidases and also was immobilized on to magnetic nanoparticles. The antibody immobilized magnetic particles were successfully utilized for capturing β-glucosidases from crude enzyme extracts. Beta-glucosidase immobilized magnetic nanoparticles could capture anti β-glucosidase IgY antibodies and hence offer a novel method of immuno-affinity purification of anti β-glucosidase IgY antibodies.

9.2. Materials and Methods

9.2.1. Immunization of Rabbit

Two New Zealand white rabbits procured from Amrita Institute of Medical Sciences, Ernakulum, India, were used in the present study. For immunization, isoform BGL 6 was used. 1.0 ml of enzyme was mixed with 1.0 ml of Freund's complete adjuvant (Merck, India). The antigen-adjuvant was used for immunizing rabbits by intramuscular and subcutaneous injection. Booster doses were provided in every 2 weeks interval for 2 months consisting of enzyme and Freund's incomplete adjuvant (Merck, India).

9.2.2. Antisera collection

Before immunization of the rabbit, blood was taken from the marginal ear vein. The blood was kept undisturbed under 15 °C overnight and centrifuged. The clear

supernatant was taken as normal rabbit serum. After 4 weeks of first immunization, blood was collected from the marginal ear vein and antiserum that contain anti β-glucosidase antibody was isolated as stated above.

9.2.3. Immunization of chicken

Two chickens were immunized with purified BGL 6 isoform as antigen. 1.0 ml of enzyme was mixed well with 1.0 ml of Freund's complete adjuvant. The antigen-adjuvant was used for immunization by subcutaneous injection. Booster doses were provided every 3 weeks interval for 2 months consisting of enzyme and Freund's incomplete adjuvant. The eggs before immunization and after 2 months of immunization were collected and stored at 15 °C.

9.2.4. Isolation of IgY from eggs

The egg shell was removed and the yolk was carefully taken discarding the egg white. IgY from yolk was isolated using chicken IgY purification kit (Merck, India) as per the protocol given by the supplier. The IgY so obtained was dissolved in 0.01 M phosphate buffered saline (pH 7.4) containing 15 mM sodium azide and stored at 4 °C.

9.2.5. Double immuno-diffusion assay

For immuno-diffusion, 1 % low melting agarose was used. The agarose was dissolved in 0.01 M phosphate buffered saline (pH 7.4) by heat and poured onto clean glass slides. After solidification, wells were created. To one well 20 µl antisera from rabbit or anti β-glucosidase IgY was added and to the other the antigen (purified BGL isoforms) were added. The gel was incubated under moisture overnight and observed for precipitin lines. For detection of BGL activity in the precipitin lines, the gel was incubated in 0.005 M Methyl-umbelliferyl β-D glucopyranoside (MUG) for 5 minutes at 30 °C. The gel was then visualized under a UV-trans-illuminator.

9.2.6. Beta-glucosidase localization studies

9.2.6.1. Preparation of fungal mycelium

Fungal spores of *Byssochlamys fulva* NII 0930 were inoculated in PDA plates and a sterile cover slip was inserted at an angle of 20° to agar surface, 1 cm away from the spot of inoculation. The plates were incubated at 30 °C for 3 days and the cover slip overlaid with fungal mycelium was removed for further studies.

9.2.6.2. Fluorescent microscopic studies

Localization studies were conducted using a modified protocol of Cai et al. (Cai et al., 1999). The cover slips coated with fungal mycelia were briefly heat fixed and then chemically fixed with 2 % glutaraldehyde solution for 30 minutes. The cover slips were washed for 5 minutes each in six changes of 10 mM sodium phosphate buffer (pH 7.4) and then quenched for 2 h in a blocking solution consisting of the same buffer containing 1 % w/v BSA and 0.02 % sodium azide (Albumin Azide Buffer- AZB) and normal rabbit serum (1:30 dilution). Hyphae were then incubated for 2 h in a solution containing anti β-glucosidase IgY in AZB (1:100 dilution). Control experiments were incubated with pre immune IgY in AZB (1:30 dilution). After four 5 minutes wash with ABZ buffer, the test and control samples were incubated for 15 minutes with rabbit anti-chicken immunoglobulin G-Fluorescein isothiocyanate (FITC)-labeled antibody (Merck, India) diluted to 1:500 with ABZ . The samples were washed thoroughly with 10 mM sodium phosphate buffer, air dried and mounted in glycerol. The samples were visualized under a fluorescence microscope (Leica, Germany).

9.2.7. Immobilization of anti β-glucosidase antibody to magnetic nanoparticles

Silica coated, APTES functionalized magnetic nanoparticles were prepared as outlined under appropriate section of chapter 8. Immobilization protocol as outlined by Can et al (Can et al., 2009) was used with minor modifications. 1.53 g of silica coated

nanoparticles was dispersed in 60 ml 5 % glutaraldehyde. The suspension was kept overnight with stirring at 4 °C. The nanoparticles were recovered using a magnet and washed thrice with de-ionized water. To this, anti β-glucosidase antibody (equivalent to 500 mg of protein) in phosphate buffered saline (pH 7.4, 0.01 M) was added and incubated at 4 °C for 5 h with mild agitation. For control, albumin immobilized magnetic nanoparticles were synthesized using a similar procedure as outlined under appropriate sections of chapter 8 with a modification of the use of albumin (500 mg) instead of anti β-glucosidase antibody. After incubation, the nanoparticles were separated by a magnet and washed thrice with phosphate buffered saline (pH 7.4, 0.01 M). The immobilized nanoparticles were stored at 4 °C in phosphate buffered saline (pH 7.4, 0.01 M). The particles were diluted and total bound protein was determined as outlined in section 2.5.3. To confirm the binding of anti β-glucosidase antibody, the immobilized nanoparticles were incubated for 15 minutes with rabbit anti-chicken immunoglobulin G-fluorescein isothiocyanate (FITC)-labelled antibody diluted to 1:500 with ABZ. The nanoparticles were recovered by a magnet and were washed thoroughly with 10 mM sodium phosphate buffer, air dried and mounted in glycerol. The samples were visualized under a fluorescence microscope (Leica, Germany).

9.2.8. Use of immobilized anti β-glucosidase antibody for capturing BGL

The immobilized anti β-glucosidase antibody (8 mg total protein) was added to 20 ml of crude 2V acetone precipitated BGL preparation (pH of 7.0) with an activity of 40 U/ml and was kept under shaking at 150 rpm for 30 minutes. The nanoparticles were removed from the BGL preparation using a magnet, washed thrice with phosphate buffered saline (pH 7.4, 0.01 M) and assayed for β-glucosidase activity as outlined in section 2.5.1. The nanoparticles were stirred in citrate buffer (0.05 M, pH 3.5) for 30 minutes to separate the enzyme from the immobilized antibody. The free enzyme was assayed for β-glucosidase activity as outlined in section 2.5.1.

9.2.9. Use of immobilized BGL for capturing anti β-glucosidase antibody

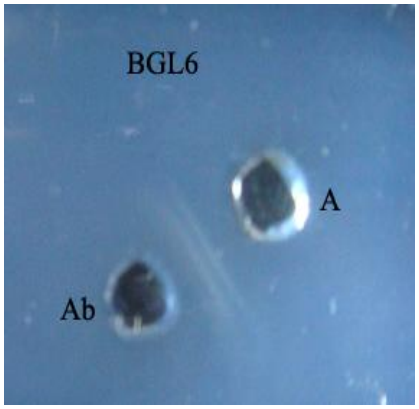
The β-glucosidase (BGL 6) immobilized on magnetic nanoparticles prepared as explained under appropriate sections of chapter 8 were used in the present study. The egg yolk (5 ml) containing anti β-glucosidase antibody was diluted to 100 ml using phosphate buffered saline (pH 7.4, 0.01 M) and magnetic nanoparticles with a total BGL 6 activity of 200 U were added on to the egg yolk solution. As control, instead of anti β-glucosidase antibody containing egg yolk, pre-immune egg yolk was used. This was kept under shaking at 150 rpm for 30 minutes. The nanoparticles were recovered using a magnet, washed thrice with phosphate buffered saline (pH 7.4, 0.01 M). The nanoparticles were stirred in citrate buffer (0.05 M, pH 3.5) for 30 minutes to separate the antibody from the immobilized enzyme. Total free protein was determined as outlined in section 2.5.3. To confirm the binding of anti β-glucosidase antibody, anti β-glucosidase antibody bound magnetic nanoparticles and control were treated with rabbit anti-chicken immunoglobulin G-fluorescein isothiocyanate (FITC)-labeled antibody diluted to 1:500 with ABZ buffer and incubated for 15 minutes. The test and control nanoparticles were recovered using a magnet, were washed thoroughly with 10 mM sodium phosphate buffer, air dried and mounted in glycerol. The samples were visualized under a fluorescence microscope (Leica).

9.3. Results and Discussion

9.3.1. Rabbit anti β-glucosidase antibody

The antisera of rabbits immunized with β-glucosidase showed precipitin line with purified BGL 6 in double immuo-diffusion assay (Figure 9.2).

Figure 9.2: Double immuno diffusion assay of purified BGL 6 against antisera from rabbits

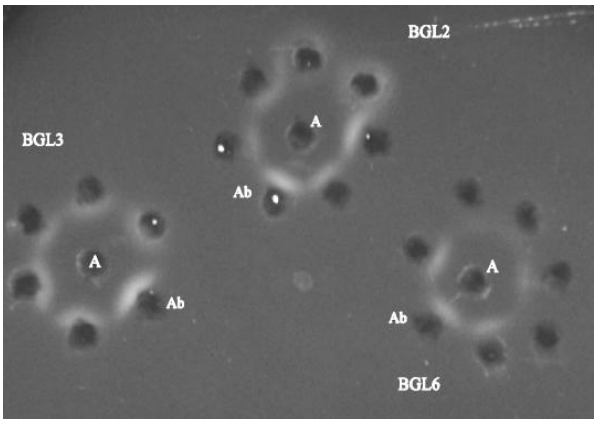


A- Antigen (BGL 6); Ab –antisera from rabbit

9.3.2. Chicken anti β-glucosidase antibody

Chickens immunized with BGL 6 isoform showed the presence of anti β-glucosidase antibody. The IgY antibody was purified and a final concentration of about 60 mg protein was obtained from each egg. The antibody showed broad specificities to all the three isoforms- BGL 2, BGL 3 and BGL 6. Precipitin lines were formed and were visualized as enzyme activity bands with MUG under UV light (Figure 9.3). The presence of enzyme activity in precipitin line indicates the antigen-antibody binding does not affect the catalytic activity of the enzymes. Also, the cross reactivity of antibody against all the three isoforms indicate the presence of similar epitopes in the isoforms. Cross reactivity of polyclonal IgY antibodies, developed against rat liver cytosolic casein kinase, with casein kinase of other tissues and nuclear form of the enzyme has been reported (Goueli et al., 1990). This cross reactivity has been speculated to the homology of immunogenic domains in the enzymes.

Figure 9.3: Double immuno diffusion assay of BGL isoforms against anti β-glucosidase IgY



Precipitin lines as visualized by MUG staining under UV light.
A-antigen (BGL 2 or BGL 3 or BGL 6); Ab- anti β-glucosidase IgY

9.3.3. Enzyme localization studies

Chicken anti β-glucosidase IgY antibodies were used for localizing β-glucosidase enzyme in the fungal mycelium of *B. fulva* NII 0930. Green fluorescence was noted in test mycelium while control mycelium did not show any fluorescence under excitation. The fluorescence was associated with the cell wall as evident from the result of localization studies (Figure 9.4, 9.5).

Figure 9.4. Phase contrast image of fungal mycelium

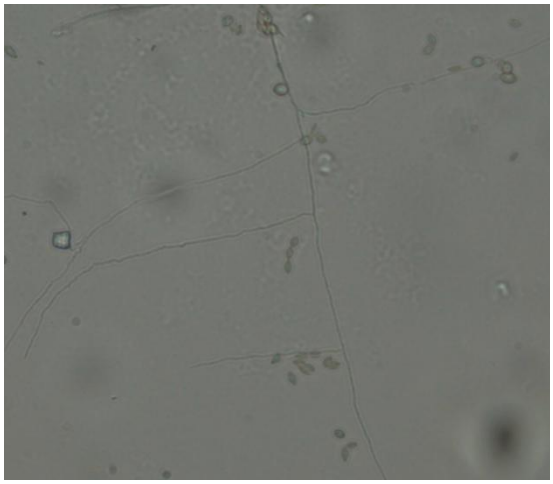
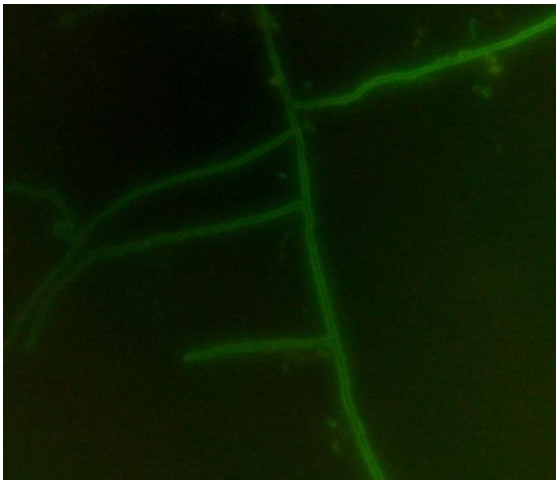


Figure 9.5. Immuno-stain with anti β-glucosidase antibody



Fluorescence in younger mycelium was more compared to mature ones. A similar localization study by Cai et al (1999), using anti β-glucosidase IgG of rabbit showed distribution of β-glucosidase in the apical region of the hyphae of *Volvariella volvacea.* Using a confocal laser scanning microscope, the intracellular localization of the enzyme at hyphal apex and in the apical region, extending 60-70 µm below the hyphal tip of *V. volvacea* was revealed. β-glucosidase was also present in the extracellular region extending approximately 15 µm all around its hyphal tip and trailing back along the length of the hyphae. The region of hyphae away from the apical region was found to be devoid of intracellular BGL but was present in the sheath associated with the hyphal wall. In *Trichoderma reesei*, rabbit anti β-glucosidase antibody was used to localize β-glucosidase in the mycelium by Sprey (1986) and the enzyme was detected in the outer, fibrous exopolysaccharide layer and the outer face of the plasma membrane. Rabbit anti β-glucosidase antibody raised against BGL 11 of *Trichoderma reesei* confirmed the presence of β-glucosidase in cell lysates and its absence in culture supernatant (Saloheimo et al., 2002). In the present study, fluorescence was noted all along the hyphal wall and was absent in the intracellular region. This suggests to the extracellular or cell wall bound localization of β-glucosidase in *B. fulva.* An interesting feature noted was the absence of fluorescence on spore surface (Figure 9.6, 9.7). This indicates the possible lack of spore associated β-glucosidases in *B. fulva* NII 0930.

Figure 9.6. Phase contrast image of conidia bearing hyphae

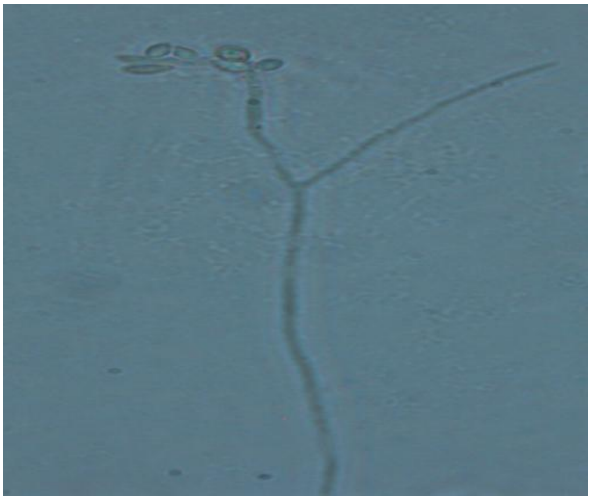
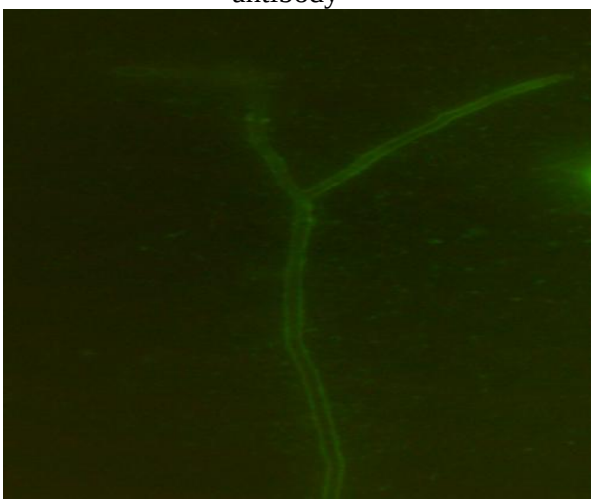


Figure 9.7. Immunostaining of conidia bearing hyphae with anti β-glucosidase antibody

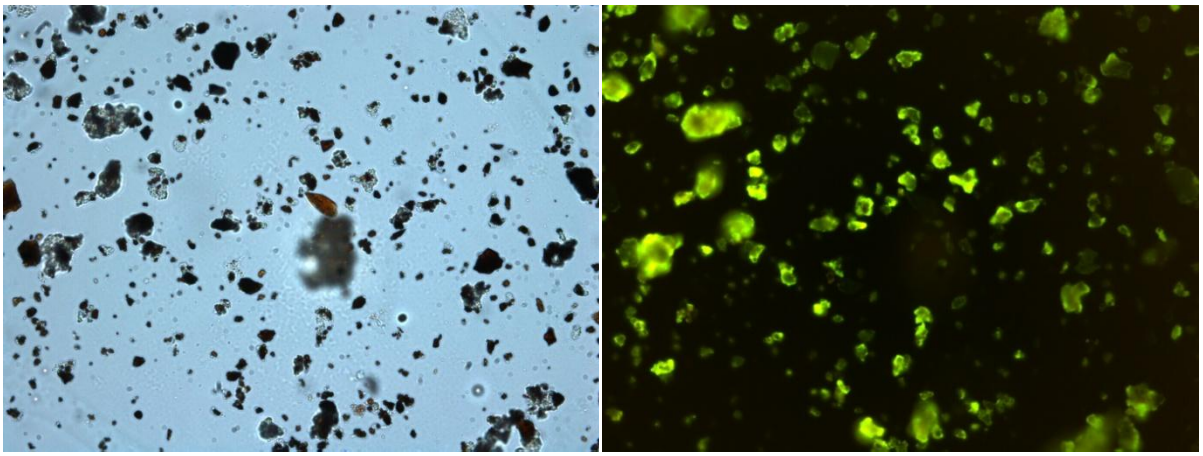


9.3.4. Immobilization of anti β-glucosidase antibody on to magnetic nanoparticles

Chicken IgY was successfully immobilized onto magnetic nanoparticles. After immobilization, 1.5 g of nanoparticles was recovered showing a total protein content of 240 mg. The immobilized antibody treated with rabbit anti-chicken immunoglobulin G-Fluorescein isothiocyanate (FITC)-labeled antibody showed fluorescence on excitement under a fluorescence microscope (Figure 9.8, 9.9). Control experiment showed no fluorescence.

Figure 9.8. Phase contrast image of anti β-glucosidase antibody immobilized nanoparticles

Figure 9.9. Immuno-stain with anti β-glucosidase antibody immobilized nanoparticles



9.2.5. Use of immobilized anti β-glucosidase antibody for capturing BGL

The magnetic nanoparticle immobilized IgY antibody was evaluated for capture of β-glucosidase enzyme from crude concentrated BGL preparation having total of 800 U activity. From the initial 50 mg immobilized antibody (corresponding to 8 mg of total protein) added to the crude enzyme preparation, nanoparticles with total protein content of 7.8 mg were recovered. Enzyme assay revealed that the antibody immobilized nanoparticles could recover only 180 U of activity from the 800 U of total enzyme present in the solution, indicating an efficiency of 22.5 % only. The reason for a low efficiency could be the non-specific binding of the antibody on the magnetic

nanoparticles that resulted in the loss of antigen binding sites in the antibody. However, it is expected that refinement of the methods and a higher loading of antibody on the magnetic nanoparticles would definitely improve the applicability of this method.

9.3.6. Use of immobilized BGL for capturing anti β-glucosidase antibody

Anti β-glucosidase antibody was bound to the immobilized BGL nanoparticles. The total protein concentration of the bound antibody was calculated as 10 µg. No protein was detected in the control. The low binding efficiency of antibody may be attributed to the low protein content of the purified enzyme (high specific activity as explained in section 6.3.1). The anti β-glucosidase antibody bound to BGL immobilized nanoparticles, treated with rabbit anti-chicken immunoglobulin G-Fluorescein isothiocyanate (FITC)-labeled antibody showed fluorescence on excitement under a fluorescence microscope (Figure 9.10, 9.11). Control experiment showed no fluorescence.

Figure 9.10. Phase contrast image of anti β-glucosidase antibody captured by BGL immobilized nanoparticles

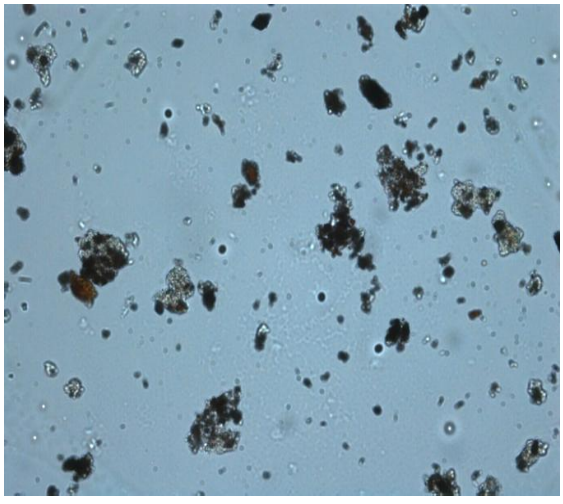
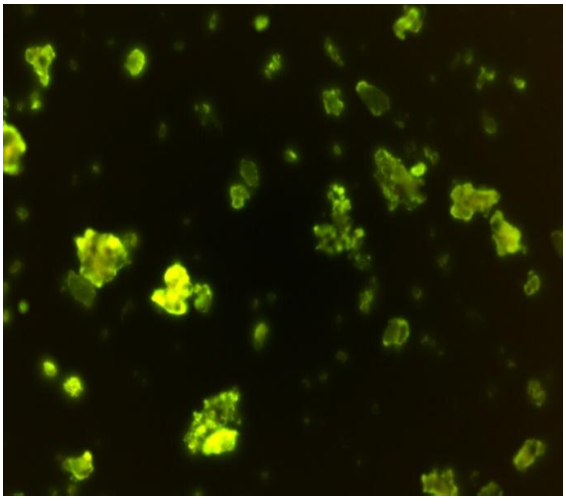


Figure 9.11. Immuno-stain with anti β-glucosidase antibody captured by BGL immobilized nanoparticles



9.4. Conclusions

Anti β-glucosidase antibodies were raised in rabbits and showed precipitin lines with β-glucosidase in immunodiffusion assay. The use of chickens for the production of polyclonal antibodies offers several advantages over the conventional production of antibodies in mammals. This includes high concentration of antibody, easy purification procedure, higher production rates and the absence of distress to the animal as collecting of blood samples and bleeding is not required. BGL 6 was used to immunize chickens but the purified IgY had broad specificities to all the three purified isoforms –BGL 2, BGL 3, BGL 6. This probably indicates the homology of the immunogenic regions in the isoforms. Localization studies using anti β-glucosidase antibodies indicate the cell wall bound and extracellular location of BGL. Also absence of spore bound β-glucosidase was proved using fluorescence labeling. Anti β-glucosidase IgY antibodies were successfully immobilized on to magnetic nanoparticles. Immobilized IgY used for capturing β-glucosidase showed an efficiency of only 22.5 %, which could be due to the nonspecific covalent binding of the antibody on to the magnetic nanoparticles which could lead to reduction in the paratopes for binding the enzyme. Similarly, immobilized BGL nanoparticles when used for capturing the β-glucosidase resulted in a low recovery of the antibody. This was due to the low content of protein used for capturing the antibody. However, the fact that these techniques were successfully used indicates the scope for further refinement and generation of more efficient immobilized catalysts and tools for their recovery.

Chapter 10

Biomass hydrolysis and ethanol production

Chapter 10

Biomass hydrolysis and ethanol production

10.1. Introduction

Efficient hydrolysis of cellulosic biomass would allow its utilization for bioethanol production. Development of technologies for effective conversion of lignocellulosic material to fermentable sugars offers outstanding potential to benefit the society by improved strategic security, decreased trade deficits, healthier rural economies, improved environmental quality, production of useful materials, technology exports and sustainable energy resource supply (Kumar and Pushpa, 2012; Sweeney and Xu, 2012; Zhang et al., 2006). The three sequential steps in biomass to sugar conversion include reduction in the size of biomass, pretreatment and hydrolysis. In the field of biomass-based bioenergy, mechanical size reduction plays a crucial part by increasing bulk density, flowability and digestibility of biomass (Miao et al., 2011). Pretreatment results in the partial degradation of polysaccharidic cell wall, opening fibres and allowing penetration of chemicals or enzymes inside the structure (Tabka et al., 2006). It may be chemical pretreatment using dilute acids, alkali, ammonia, organic solvents; physical, using steam explosion or liquid hot water; biological, using lignin degrading fungi; combinations, like acid catalyzed steam explosion or ammonia fibre explosions etc. Using alkaline pretreatment, most of the lignin and part of hemicellulose are removed and the susceptibility of cellulose to enzymatic hydrolysis is sufficiently increased. Alkali-based methods are more efficient compared to acid hydrolysis for lignin solubilization, leaving behind much of the hemicellulose in an insoluble polymeric form, thus reducing the formation of furan derivatives and phenolics which are potent inhibitors of subsequent fermentation (Boonmee, 2012; Hamelinck et al., 2005; Saha et al., 2005).

Enzymatic hydrolysis of cellulosic substrates is achieved by using hydrolytic enzymes that include endoglucanase, cellobiohydrolase and β-glucosidase. Wood rotting fungi, both ascomycetes and basidiomycetes are good sources of cellulases. However,

many of these fungi produce lower amounts of β-glucosidase. Hence the use of enzyme cocktails with cellulases, xylanases and β-glucosidases sourced from different fungi is now widely practiced. *Trichoderma reesei* is known to be an effective cellulase producer and forms the main source of enzymatic cocktails for cellulose degradation (Schuster and Schmoll, 2010). The cost of cellulase enzymes remains a major concern for the commercialization of lignocellulosic bioethanol (Geddes et al., 2011). One of the possible means of attaining cost reduction of biomass hydrolysis enzymes is the reuse of such enzymes, though this is complicated and challenging due to the adsorption of enzymes to residual lignocellulose (Eriksson et al., 2002). However, immobilization of enzymes onto easily retrievable support matrices like magnetic nanoparticles might aid in the efficient recovery and reuse of enzymes. Since cellulases are enzymes which act on the macromolecular cellulose and the mode of action involves binding of the endoglucanases and cellobiohydrolases on the cellulose polymer through the cellulose binding domains (Lynd et al., 2002), immobilization of endo or exoglucanases will actually result in a decreased efficiency since lesser enzyme would be able to bind to the polymer compared to free enzymes. Immobilization of β-glucosidases however might be feasible since the substrate for the enzyme is a freely diffusible disaccharide – cellobiose. Beta-glucosidases immobilized on to synthetic super-paramagnetic nanoparticles were indeed applied for saccharification of wheat straw and *Eucalyptus globulus* pulps. These nanoparticles could be recovered easily by application of magnetic field and reused twice (Valenzuela et al., 2011).

Sugar cane bagasse is considered as a major feedstock for bioethanol production due to the abundant and centralized availability, especially in the tropics (Cardona et al, 2010). Sugarcane bagasse has a complex structure, composed of 40-50 % cellulose, 25 % hemicellulose and 25 % lignin (Ferreira et al., 2010; Generoso et al., 2012; Ray et al., 2011). Pretreated sugar cane bagasse have been used as a feedstock by several researchers and enzyme manufacturers to test the performance of their enzyme cocktails (Betancur and Pereira Jr., 2010; Buaban et al., 2010; Dupont-Genencor, 2012; Novozymes, 2012; Sukumaran et al., 2009). Ethanol fermentation is usually carried out by unicellular fungi like *Saccharomyces* and *Pichia*. However filamentous fungi are also reported to be ethanol producers. Such ethanol producers can utilize both hexoses and

pentoses for growth and ethanol production. Among the nineteen *Aspergillus* and ten *Rhizopus* screened, three strains of *Rhizopus* were found to produce more than 31 g/L ethanol under anaerobic stress (Skory et al., 1997).

Commercial cellulases from Zytek (Mumbai, India) and Maps Enzyme Limited (Ahmadabad, India) were evaluated and found to be effective in biomass hydrolysis. Enzyme cocktails using these commercial enzymes with additional BGL from *B. fulva* both as free enzymes or in immobilized state were tested for enhancement of hydrolytic efficiency. Reuse of immobilized enzymes was also studied and ethanol production from the hydrolysate was carried out. The filamentous fungi, identified as producers of glucose tolerant β-glucosidases were screened for their ability to ferment glucose and xylose to ethanol.

10.2. Materials and Methods

10.2.1. Hydrolysis of biomass

Saccharification efficiency of enzyme cocktail, created by addition of free or immobilized BGL to commercial cellulase were tested using Avicel® and alkali pretreated sugarcane bagasse as biomass.

10.2.1.1. Pretreatment of Biomass (sugarcane bagasse)

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

overnight at 70 °C in a hot air oven to remove residual moisture. The pretreated feed stock was stored in airtight containers until used.

10.2.1.2. Saccharification of avicel

Enzymatic saccharification was done by incubating 2.5 g of avicel with commercial cellulase (Acid Cellulase, Maps Enzyme Limited) alone or with enzyme cocktails containing free or immobilized BGL 2 of *B. fulva* along with the commercial cellulase preparation (Table 10.1). The saccharification studies were conducted at 50 °C, in 100 ml stoppered Erlenmeyer flasks in a total volume of 25 ml made up with 50 mM citrate buffer (pH 4.8). The flasks were agitated at 100 rpm in a water bath shaker. Hydrolysis efficiencies were measured as the amount of total reducing sugars liberated from biomass according to the reducing sugar assay (section 2.5.4). All the experiments were done in triplicates.

Table 10.1: Saccharification of avicel

Flask	MAP A Enzyme U/g biomass		BGL 2 from *B. fulva* U/g biomass
	Cellulase	BGL	
F1	20 FPU/g	971 U/g	Immobilized BGL 2 (100 U/g)
F2	20 FPU/g	971 U/g	Free BGL 2 (100 U/g)
F3	20 FPU/g	971 U/g	Nil
F4	Nil	Nil	Immobilized BGL 2 (100 U/g)
F5	Nil	Nil	Free BGL 2 (100 U/g)

10.2.1.3. Biomass saccharification (sugarcane bagasse)

Enzymatic saccharification of biomass was studied by incubating 2.5 g of pretreated biomass (sugarcane bagasse) with commercial cellulase (Acid Cellulase, Maps Enzyme Limited) alone or with various enzyme cocktails containing free or

immobilized BGL 2, BGL 3 or BGL 6 isoforms from *B. fulva* along with the commercial cellulase preparation (Table 10.2). The saccharification studies were conducted at 50 °C, in 100 ml stoppered Erlenmeyer flasks in a total volume of 25 ml made up with 50 mM citrate buffer (pH 4.8). The flasks were agitated at 100 rpm in a water bath shaker. Hydrolysis efficiencies were measured as the amount of total reducing sugars liberated from biomass according to the reducing sugar assay (section 2.5.4). All the experiments were done in triplicates.

Table 10.2: Saccharification of pretreated sugarcane bagasse with enzyme cocktail containing immobilized BGL

Flask	MAP A Enzyme U/g biomass		*Immobilized BGL from B. fulva* U/g biomass
	Cellulase	BGL	
A	20 FPU/g	971 U/g	Polymer coated immobilized BGL 2 (100 U/g)
B	20 FPU/g	971 U/g	Immobilized BGL 2 (100 U/g)
C	20 FPU/g	971 U/g	Polymer coated immobilized BGL 2 (50 U/g) + Polymer coated immobilized BGL 3 (50 U/g)
D	20 FPU/g	971 U/g	Immobilized BGL 2 (50 U/g) + Immobilized BGL 3 (50 U/g)
E	20 FPU/g	971 U/g	Polymer coated immobilized BGL 6 (100 U/g)
F	20 FPU/g	971 U/g	Immobilized BGL 6 (100 U/g)
G	20 FPU/g	971 U/g	Immobilized BGL 2 (25 U/g) + Immobilized BGL 3 (25 U/g) + Immobilized BGL 6 (50 U/g)
H	20 FPU/g	971 U/g	Nil

10.2.1.4. Enzyme recovery and reuse

Enzymatic saccharification of biomass was done by incubating 2.5 g of pretreated biomass (sugarcane bagasse) with commercial cellulase (Zytek) alone or with various enzyme cocktails containing free or immobilized BGL 2, BGL 3 or BGL 6 isoforms from *B. fulva* along with the commercial cellulase preparation (Table 10.3).

The saccharification conditions were as outlined in section 10.2.1.3. After hydrolysis, the immobilized BGL were recovered using a strong magnet, washed with 50 mM citrate buffer (pH 4.8) and was reused as BGL supplementation in the second cycle of hydrolysis. The substrate and conditions were similar to the first cycle but instead of fresh immobilized BGL, BGL recovered after the first cycle of hydrolysis were used. After the second cycle of hydrolysis, the immobilized BGL were again recovered and reused in the third cycle.

Table 10.3: Reuse of immobilized BGL for saccharification of pretreated sugarcane bagasse

Flask	Zytek Enzyme U/g biomass		Immobilized BGL from *B. fulva* U/g biomass
	Cellulase	BGL	
F1	30 FPU/g	1455 U/g	Immobilized BGL 2 (100 U/g)
F2	30 FPU/g	1455 U/g	Immobilized BGL 2 (50 U/g) + Immobilized BGL 3 (50 U/g)
F3	30 FPU/g	1455 U/g	Immobilized BGL 6 (100 U/g)
F4	30 FPU/g	1455 U/g	Nil

10.2.2. Ethanol production

10.2.2.1. Filamentous fungi as ethanol producers

Mandels and Weber medium (section 2.2.2), devoid of carbon source was used for the study. Fifty milliliters of media was autoclaved in 250 ml Erlenmeyer flasks. To one set of flasks, filter sterilized glucose and to the other set, filter sterilized xylose were added to a final concentration of 4 % w/v. Spores from fungi were inoculated in the flasks and were incubated at 30 °C, 200 rpm for 2 days. Thereafter, the agitation was reduced to 60 rpm. Samples were taken at regular intervals and ethanol was estimated by gas chromatography (section 2.5.5).

10.2.2.2. Ethanol production using sugarcane bagasse hydrolysate

The suitability of the sugarcane bagasse hydrolysate generated using enzyme cocktail consisting of commercial cellulase and immobilized BGL was tested by using it as substrate for ethanol production. Ethanol production and assay were performed according to sections 2.8 and 2.5.5 respectively.

10.3. Results and Discussion

10.3.1. Saccharification of biomass

10.3.1.1. Saccharification of avicel

Supplementation of commercial cellulase with free or immobilized BGL 2 isoform from *B. fulva* could significantly enhance hydrolysis of avicel (Table 10.4).

Table 10.4: Hydrolysis of avicel by commercial cellulase supplemented with BGL 2

Flask	Enzyme	Reducing sugar mg/g substrate				
		Incubation Time (h)				
		8	16	24	32	48
F1	Cellulase + immobilized BGL 2	416 ± 3.4	588 ± 16.8	623 ± 11.2	661 ± 24.9	757 ± 13.5
F2	Cellulase + free BGL 2	513 ± 20.9	624 ± 6.7	656 ± 6.7	710 ± 1.1	802 ± 6.70
F3	Cellulase (Control)	312 ± 7.3	415 ± 7.9	442 ± 11.2	483 ± 6.7	528 ± 23.9

(* Cellulase used was Acid cellulase from MAPS Enzymes, India)

Among the BGL supplementation, hydrolysis was maximal with free BGL than immobilized BGL. A similar reduction was noted with β-glucosidase immobilized on to super-paramagnetic magnetite nanoparticles that were used in the hydrolysis of wheat

straw and *Eucalyptus globulus* pulp. A 10 % reduction in yield was seen with use of immobilized β-glucosidase compared to free β-glucosidase (Valenzuela et al., 2011). In the present study, with 48 h of incubation, a reducing sugar concentration of 802 mg/g avicel was obtained.

10.3.1.2. Saccharification of alkali pretreated sugarcane bagasse

Several studies indicate the beneficial effect of β-glucosidase supplementation on glucan digestibility. However no universally effective loading of β-glucosidase can been defined, due to the differences in feedstock composition, structure, degree of polymerization, method of pretreatment, apart from the conditions employed for saccharification including solid loading, differences in the enzyme preparations used, duration of hydrolysis and temperature (Kumar and Wyman, 2009). Addition of immobilized BGL from *B. fulva* to commercial cellulase could significantly enhance saccharification of pretreated sugarcane bagasse (Table 10.5).

Table 10.5: Hydrolysis of sugarcane bagasse by commercial cellulase supplemented with immobilized BGL isoforms

Flask	Enzyme	Reducing sugar mg/g substrate	
		16 h incubation	24 h incubation
A	Cellulase + Polymer coated immobilized BGL 2	388 ± 4.5	566 ± 15.3
B	Cellulase + Immobilized BGL 2	533 ± 7.2	613 ± 12.8
C	Cellulase + Polymer coated immobilized BGL 2+ Polymer coated immobilized BGL 3	498 ± 5.4	575 ± 0.7
D	Cellulase + Immobilized BGL 2+ Immobilized BGL 3	505 ± 5.5	610 ± 2.9
E	Cellulase + Polymer coated immobilized BGL 6	490 ± 0.0	543 ± 7.3
F	Cellulase + Immobilized BGL 6	514 ± 13.6	604 ± 4.7
G	Cellulase + Immobilized BGL 2+ Immobilized BGL 3+ Immobilized BGL 6	565 ± 4.5	618 ± 1.5
H	Cellulase (Control)	373 ± 8.5	439 ± 3.7

(* Cellulase used was Acid cellulase from MAPS Enzymes, India)

Combinations of all the three isoforms were more effective than each alone indicating the synergistic action of isoforms in bringing about efficient hydrolysis of biomass. Each isoform vary in their kinetic properties and thus their presence would help the fungus in better survival in its natural habitat. Polymer coated isoforms showed lesser activity compared to the uncoated immobilized isoforms. This may be due to the diffusional limitations preventing easy entry and regress of reactants and products respectively.

10.3.1.3. Enzyme recovery and reuse

The immobilized enzymes could be reused for additional two cycles of hydrolysis (Table 10.6). Immobilized BGL 2 showed a more rapid loss of activity compared to BGL 6 isoform. The supplementation of immobilized BGL in the third cycle was comparable to the commercial enzyme alone. This may be due to loss of enzyme activity but more probably due to the loss of immobilized enzymes by adsorption of nanoparticles on to the un-hydrolyzed substrate. Use of stronger magnetic field may reduce the loss of immobilized enzyme and hence they can be used in subsequent cycles of hydrolysis.

Table 10.6: Reuse of immobilized BGL for hydrolysis of sugarcane bagasse by supplementing with commercial cellulase

Flask	Enzyme	Reducing sugar mg/g substrate		
		1st Cycle	2nd Cycle	3rd Cycle
F1	Cellulase + Immobilized BGL 2	650.3 ± 0.08	590.0 ± 0.10	560.3 ± 0.10
F2	Cellulase + Immobilized BGL 2 + Immobilized BGL 3	690.1 ± 0.01	610.4 ± 0.02	580.2 ± 0.05
F3	Cellulase + Immobilized BGL 6	670.0 ± 0.00	610.0 ± 0.02	590.4 ± 0.02
F4	Cellulase (Control)	570.2 ± 0.01	540.3 ± 0.00	560.1 ± 0.01

(* Cellulase used was Acid cellulase from Zytek, India)

10.3.2. Ethanol production

10.3.2.1. Filamentous fungi as ethanol producers

Fourteen fungi screened as producers of glucose tolerant β-glucosidase were found to produce ethanol from glucose and xylose under oxygen stress.

10.3.2.1.1. Ethanol production with glucose as carbon source

All the fungal strains were found to produce ethanol from the second day of inoculation (Table 10.7). Strain AM 91 was the best among the strains as it could produce 7.89 g/L of ethanol in the second day after fungal spore inoculation. Ethanol production by filamentous fungi is previously reported with some fungal strains having high efficiencies. *Aspergillus awamori*, *A. foetidus*, *A. niger*, *A. sojae*, A. *tamari* were reported to produce ethanol from glucose with a strain of *A. oryzae* producing 24.4 g/L of ethanol from 50 g/L of glucose. However, the best ethanol producers were *Rhizopus javanicus* and *R. oryzae* which could provide an ethanol yield of 100 % of the theoretical value in 72 h (Skory et al., 1997). *Mucor hiemalis* could produce 50 g/L ethanol in the course of fermentation of sugars from wheat and starch hydrolysates (Vinche et al., 2012), while *Mucor hiemalis* and *M. indicus* produce 0.39 g/g ethanol with glucose as carbon source (Millati et al., 2005). In the present study, *Paecilomyces* strains (AM 21, AM 73, AM 75, AM 78, AM 91, AM 98, AM 130 (NII 0930), AM 219) were good producers of ethanol. *Paecilomyces lilacinus* is reported to show anaerobic growth and ethanol production in glucose containing medium (Mountfort and Rhodes, 1991). This indicated the potential of *Paecilomyces* strains as producers of glucose tolerant β-glucosidases and ethanol.

Table 10.7: Ethanol production by filamentous fungi from glucose

Fungal strain	Ethanol concentration (g/L)			
	2nd day	3rd day	4th day	8th day
AM 21	3.95±0.02	5.52±0.13	6.71±0.02	6.71±0.01
AM 27	1.26±0.03	0.79±0.02	1.74±0.02	1.58±0.06
AM 73	1.54±0.06	0.79±0.01	2.49±0.04	5.52±0.06
AM 75	0.83±0.01	1.58±0.01	3.87±0.09	5.52±0.05
AM 78	2.56±0.12	1.58±0.04	3.71±0.08	6.40±0.01
AM 91	**7.89±0.11**	6.71±0.03	6.82±0.11	6.27±0.09
AM 98	7.26±0.08	5.77±0.02	4.93±0.12	4.99±0.09
AM130 (NII 0930)	2.72±0.03	4.73±0.01	6.23±0.01	7.30±0.07
AM 209	1.10±0.04	2.37±0.01	3.71±0.02	4.66±0.01
AM 219	3.40±0.01	7.10±0.02	5.01±0.01	6.59±0.01
AM 317	4.73±0.10	7.10±0.04	3.95±0.04	7.72±0.09
AM 338	5.72±0.09	0.79±0.04	0.79±0.05	7.26±0.09
SA 56	1.26±0.09	1.97±0.10	2.37±0.09	2.37±0.02
SA 58	0.83±0.02	0.79±0.02	3.16±0.01	4.90±0.02

10.3.3.1.2. Xylose as carbon source

The fungal strains could also use xylose as carbon source and produced ethanol (Table 10.8). However the production was comparatively lower than with glucose. But strain AM 91 showed a higher ethanol production of 11.37 g/L in the third day of fungal spore inoculation. In general *Paecilomyces* strains (AM 21, AM 73, AM 75, AM 78, AM 91, AM 98, AM 219) except AM 130 (NII 0930) were good producers of ethanol. *Rhizopus javanicus* and *R. oryzae* were more efficient in converting xylose to ethanol than *Aspergillus* strains. *Rhizopus javanicus* could convert xylose into ethanol in 72 h with a yield of 47 % of theoretical value (Skory et al., 1997), which is comparable to that

produced by AM 91. With xylose as carbon source, *Mucor hiemalis* and *M. indicus* showed an ethanol production of 0.18 g/g and 0.22 g/g respectively (Millati et al., 2005).

Table 10.8: Ethanol production by filamentous fungi in medium with xylose as carbon source

Fungal strain	Ethanol concentration (g/L)			
	2^{nd} day	3^{rd} day	4^{th} day	8^{th} day
AM 21	1.50±0.02	1.19±0.02	1.19±0.09	3.27±0.04
AM 27	1.22±0.02	0.79±0.03	0.87±0.03	1.19±0.04
AM 73	0.83±0.04	1.10±0.03	1.42±0.09	2.76±0.04
AM 75	0.51±0.09	0.79±0.03	1.66±0.08	3.35±0.09
AM 78	1.50±0.01	1.19±0.07	1.19±0.03	3.27±0.02
AM 91	6.08±0.09	**11.37±0.10**	7.89±0.08	6.68±0.09
AM 98	2.72±0.10	0.40±0.09	4.73±0.04	6.75±0.05
AM 130 (NII 0930)	0.51±0.09	0.79±0.05	1.42±0.09	0.51±0.01
AM 209	0.24±0.02	0.08±0.02	0.08±0.01	0.40±0.01
AM 219	5.44±0.02	0.87±0.09	8.78±0.03	7.70±0.05
AM 317	0.28±0.03	0.02±0.09	0.08±0.01	0.71±0.05
AM 338	0.08±0.01	0.08±0.02	0.40±0.01	0.55±0.09
SA 56	0.08±0.03	0.08±0.03	0.08±0.01	0.08±0.05
SA 58	0.08±0.02	0.08±0.01	0.06±0.01	0.16±0.06

Fermentation conditions and the carbon source are critical for ethanol production by filamentous fungi. In a hydrolysate containing 80 % glucose and 20 % xylose, *M. indicus* did not utilize xylose under anaerobic conditions and assimilation started only when glucose got exhausted and mild aerobic conditions (oxygen limited condition) were provided (Millati et al., 2008). Reports indicate the ability of *Paecilomyces* in producing ethanol from xylose at a concentration above 5 % v/v. But ethanol production from xylose becomes commercially feasible only when 50-60 g/L of production is achieved within 36 h with an yield of 0.4 g ethanol/g of sugar (Lee et al., 2000). Even though the yield of ethanol in the present study is low, process optimization may enhance ethanol production.

10.3.3.2. Ethanol from sugarcane bagasse hydrolysate

Enzymatic hydrolysate of sugarcane bagasse, generated by the cocktail containing immobilized BGL was concentrated by evaporation to a reducing sugar concentration of 30, 60 or 120 g/L and was used for the fermentation experiments. The results of the fermentation of alkali pretreated and enzymatically saccharified sugarcane bagasse are given in table 10.9. The maximum ethanol concentration (27.77 g/L) was obtained after 24 h using hydrolysate with 12 % reducing sugar. With the hydrolysate having initial reducing sugar concentration of 6 %, the maximum ethanol concentration obtained was 14.41 g/L and that with 3 % initial sugar, an ethanol concentration of 8.50 g/L was obtained in 24 h. Ray et al (2011) has reported the pretreatment and hydrolysis of sugarcane bagasse using *Cryptococcus albidus* and subsequent fermentation of hydrolysate by *Saccharomyces cerevisiae* where an ethanol concentration of 38.4 g/L was achieved. The process optimization has resulted in an enhancement of nearly 2 fold to 70.0 g/L and may be attributed to the biological pretreatment of sugarcane bagasse using *C. albidus* that have resulted in the absence of inhibitor production.

Table 10.9: Ethanol production from sugarcane bagasse hydrolysate by *Saccharomyces cerevisiae*

Reducing sugar (RS) concentration. (g/L)	Duration of incubation (h)	Ethanol concentration (g/L)	Ethanol yield (%)
30	24	8.50	55.44
60	24	14.41	46.99
120	24	27.77	45.28

With increase in reducing sugar concentration, there is increase in ethanol concentration. However, there is a decrease in the yield of ethanol yield with increased sugar concentrations which may be attributed to the inhibitors present in the hydrolysate, the level of which is enhanced on the concentration of the hydrolysate. This can affect the growth of *Saccharomyces* resulting in lower ethanol yield. The toxic components can stop the growth of microorganism by affecting the rate of sugar uptake with

simultaneous delay in product formation. Increase in toxic compounds can lead to extended incubation time and poor metabolite production. Hence a detoxification step becomes necessary to remove the inhibitors prior to fermentation.

10.4. Conclusion

The study demonstrates the feasibility of BGL isoforms in combination with commercial cellulase in enhancing hydrolysis of Avicel® and alkali pretreated sugarcane bagasse. The use of immobilized BGL and its reuse in subsequent fermentations may help in reduction of the cost of enzyme during biomass to ethanol production. Higher enzyme loading with better recovery of immobilized BGL can enhance the rate of hydrolysis and sugar concentrations. The hydrolysate from sugarcane bagasse could support an ethanol yield of 27.77 g/L (from a reducing sugar concentration of 120 g/L). The lower level of ethanol production may be attributed to the toxic chemicals present in the hydrolysate and points out to the requirement of a detoxification step prior to ethanol fermentation. Levels of ethanol production by filamentous fungal strains, especially of AM 91 seem to be encouraging. With optimization of media components and culture conditions, the production of ethanol could be further enhanced.

Chapter 11

Summary and Conclusion

Chapter 11
Summary and Conclusions

11.1. Summary

Enzymatic conversion of lignocellulosic biomass is projected as the most important route for transportation fuel generation for the future. The major component of biomass is cellulose and conversion of cellulose to its monomeric subunit (glucose) requires the synergistic activity of a group of enzymes referred to as cellulases which include three classes of enzymes namely endoglucanases (EG) which produces nicks in the cellulose polymer exposing reducing and non reducing ends, cellobiohydrolase (CBH) which acts upon the non reducing ends to liberate cellobiose units, and β-glucosidases (BGL) which cleaves the cellobiose to liberate glucose completing the hydrolysis. Glucose can then be fermented to produce ethanol which is a clean and efficient fuel.

Majority of the microbes do not have a complete cellulolytic system capable of generating glucose from natural lignocellulosic material. The best known producer of the complete cellulolytic complex is a filamentous fungus called *Trichoderma reesei.* However, even in hyper-cellulase producing mutants of the same, β-glucosidase production is low. More over most of the β-glucosidase reported are strongly inhibited by glucose. Thus novel microbes secreting glucose tolerant β-glucosidase in high titres needs to be identified, the enzyme so produced can be included in cocktail for effective biomass hydrolysis.

Six hundred and eight fungal strains were isolated from terrestrial and aquatic ecosystems and were screened for glucose tolerant β-glucosidase production. Of the 16 strains screened for production of glucose tolerant β-glucosidase, 8 were identified as *Paecilomyces* by morphological and molecular techniques. Among them, strain AM 130 showed high levels of GBGL production in both SSF and SmF. The fungus identified as

Byssochlamys fulva, the teleomorph of *Paecilomyces*, and was designated as *B. fulva* NII 0930.

Response surface optimizations in solid state fermentation and submerged fermentation using *B. fulva* NII 0930 were carried out and optimum conditions for secreting high levels of glucose tolerant BGL were determined. Optimizations performed using two step statistical design resulted in a significant (4 fold) improvement in production under SmF (750 U/ml to 3200 U/ml) and 2.7 fold improvement (18600 U/gds to 50569 U/gds) in SSF. A solid state bioreactor suitable for cultivation of fungi that are highly aerobic and respond well to cultivation in an agitated reactor was designed. The reactor design provides conditions for containment as well as aseptic operation.

BGL induction studies with different sugars indicated a constitutive nature of BGL secretion. Of the various sugars tested inulin and xylose could induce higher enzyme production while thioglucose was found to repress the same.

Multiple isoforms of enzyme were detected of which three were purified and characterized. One of the isoform (BGL 6) had high glucose tolerance with a *Ki* of 600 mM. BGL 2 had an optimum temperature for activity at 60 °C and pH optimum 4.0. BGL 3 had an optimum temperature for activity at 60 °C and pH optimum of 5.0. The glucose tolerant isoform BGL 6 had an optimum temperature for activity at 50 °C and pH optimum at an acidic pH 6.0 and an alkaline pH 9.0. No transglycosylation activity was noted in BGL from *B. fulva* NII 0930.

Degenerate primers of *bgl* gene were used to amplify genomic DNA and sequencing of amplicons confirmed homology to family 3 glycosyl hydrolases. The translated sequence from 1170 bp nucleotide revealed the presence of a glycosyl hydrolase family 3 C terminal domain. Multiple sequence alignments with similar fungal β-glucosidases indicated that the obtained protein sequence of *B. fulva* shared homology with the C terminal sequences of these fungi. Homology modeling revealed the

presence of the typical α/β sandwich motiff and a fibronectin III like motif further towards the C terminal.

Magnetic nanoparticles were synthesized by co precipiting $FeCl_2$ and $FeCl_3$. Silica coating and functionalization were carried out and the purified BGL were covalently attached to the nanoparticles. The immobilized enzyme was successfully used in enzyme cocktail for saccharification of alkali pretreated sugarcane bagasse and was reused for three cycles of hydrolysis.

Antibody against β-glucosidase was raised in chicken and was purified. Immunolabelling revealed β-glucosidase to be cell wall associated and that spores may not have BGL associated on their wall. Anti β-glucosidase antibody was immobilized on to magnetic nanoparticles. This was used to capture BGL from crude preparations in a single step thus avoiding complex procedures of enzyme purification. Also BGL immobilized magnetic nanoparticles were used to capture IgY developed in chicken egg against the BGL antigen.

The hydrolysate from sugarcane bagasse produced by using enzyme cocktail containing BGL from B. fulva NII 0930 could support an ethanol yield of 27.77 g/L (from a reducing sugar concentration of 120 g/L). Most of glucose tolerant BGL producing fungi identified in the present study were found to produce considerable amount of ethanol from both glucose and xylose.

11.2 Conclusion

The study thus could result in isolation of a new strain of fungus producing glucose tolerant β-glucosidase. Eight strains (including *B. fulva* NII 0930) out of the 16 strains were found to be *Paecilomyces variotii* and their genetic diversity was established through ITS amplification and RAPD analysis. It is concluded that glucose tolerant β-glucosidase production could be a common character exhibited by *P. variotii*. This finding opens up the possibility of selective screening of fungal taxa for glucose tolerant β-glucosidases. The parameters for high BGL production by *B. fulva* NII 0930 and BGL tolerance were optimized using wheat bran (agro residue). Three isoforms

were purified and characterized and their differing properties would help us to understand the evolutionary significance of multiple isoforms. Cloning and sequencing of gene would help to us to understand the mechanism of glucose tolerance. The use of anti β-glucosidase antibody immobilized nanoparticles for enzyme capture allows the recovery of BGL from crude preparations in a single purification step. The BGL from *B. fulva* NII 0930 can be considered as a potent candidate for use in biomass hydrolysis either in free or immobilized state due to its enhanced thermal stability and high activity under hydrolysis conditions.

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APPENDICES

APPENDIX 1

LIST OF ABBREVIATIONS

#	Number
%	Percentage
µ	Micron/ micrometer
µg	microgram
µM	micromolar
µl	microlitre
°C	Degree Celsius
A	Antigen
Ab	Antibody
AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
APTES	Amino propyl tri-ethoxy silane
AZB	Albumin Azide Buffer
BGL	β-glucosidase
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovine Serum Albumin
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
DLS	Dynamic Light Scattering Spectrophotometry
DMSO	Dimethyl Sulphoxide
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
FID	Flame Ionization Detector
Fig	Figure
FITC	Fluorescein iso-thiocyanate
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
GC	Gas Chromatography, Gas Chromatogram
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
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IU	International Units
IUPAC	International Union for Pure and Applied Chemistry.
kDa	kilo Daltons
Ki	Inhibition constant
Km	Michaelis constant

L	Liter
lbs	Pounds Inch $^{-2}$
M	Molar
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
PAH	Poly (allylamine hydrochloride)
PCR	Polymerase chain reaction
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
pI	Isoelectric Point
PMSF	Phenylmethanesulphonyl fluoride
pNP	para-Nitrophenol
pNPG	para –Nitrophenyl β-D glucopyranoside
PS	Polystyrene
RNA	Ribonucleic Acid
rpm	Rotations per minute
rRNA	Ribosomal RNA
RSM	Response Surface Method/Methodology
SB	Sugarcane Bagasse
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope

SmF	Submerged Fermentation
sp	Species
SSF	Solid State Fermentation
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

APPENDIX 2

LIST OF TABLES

APPENDIX 3

LIST OF figures