EXTRACELLULAR β-GLUCOSIDASE PRODUCTION BY MARINE *Aspergillus sydowii* BTMFS 55

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By

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Extracellular β-Glucosidase production by marine *Aspergillus sydowii* BTMFS 55**" is based on the original research work carried out by Mr. Madhu K. M. under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.

M. CHANDRASEKARAN

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Extracellular β-Glucosidase production by marine *Aspergillus sydowii* BTMFS 55**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin, under the guidance of Prof. (Dr.) M. Chandrasekaran, Professor in Biotechnology, and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

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Dedicated to My teacher
Prof. Santhamma Peter

ABBREVIATIONS

%	-	Percentage
(M_r)	-	Relative molecular weight
°C	-	Degree Celsius
A	-	Absorbance
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
BSA	-	Bovine serum albumin
CD	-	Czapek-Dox
cfu	-	Colony forming unit
cm	-	Centimeter
CMC	-	Carboxy methylcellulose
CMCase	-	Carboxy methylcellulase
Da	-	Dalton
DEAE Cellulose	-	Diethyl amino ethyl cellulose
DEPC	-	Diethyl pyrocarbonate
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DNS	-	3,5 - Dinitro salicylic acid
dNTP	-	Deoxyribonucleotide triphosphate
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
Fig	-	Figure
FPA	-	Filter paper activity
g	-	grams

g/L	-	Grams per litre
h	-	Hours
HCl	-	Hydrochloric acid
kDa	-	Kilo dalton
K_i	-	Dissociation constant
K_m	-	Michaelis constant
M	-	Molar
mg	-	milligram
mg/gIDS	-	Milligram per gram initial dry substrate
ml	-	milliliter
mm	-	millimeter
mM	-	Millimolar
nM	-	nanomole
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
*p*NPG	-	*para*-Nitrophenyl-β-D-glucopyranoside
rDNA	-	Ribosomal DNA
rpm	-	Revelutions per minute
rRNA	-	Ribosomal RNA
RS	-	Rice straw
RT	-	Room temperature
SDS	-	Sodium dodecyl sulphate
SmF	-	Submerged fermentation
sp.	-	Species
SSF	-	Solid state fermentation
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine

Tris	-	Tris (hydroxy methyl) aminomethane
U	-	Unit
U/gIDS	-	Units per gram initial dry substrate
UV	-	Ultraviolet
V	-	Volt
V/V	-	Volume/Volume
V_{max}	-	Maximal velocity
W/V	-	Weight/Volume
W/W	-	Weight/ Weight
WB	-	Wheat bran
β-GL	-	β-glucosidase
μ	-	Micron
μg	-	microgram
μl	-	microlitre
μM	-	micromole

CONTENTS

Chapter 1

INTRODUCTION

The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, cellulases, amylases, amidases, esterases and lipases. A recent rough estimate of the world market for industrial enzymes put the sales at around 95,000 tons of commercial product. Around 80% of all industrial enzymes are hydrolytic in action and they are used for depolymerization (i.e. breaking down of complex molecules in to simpler molecules), and 90% of them are produced from microorganisms by fermentation.

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the availability of great variety of catalytic activities, the possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995).

Microbial enzymes are considered as useful tools for carrying out a variety of industrially important biotransformations. Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded the enzyme demand.

Chapter 1

Life on Earth depends on photosynthesis, which results in production of plant biomass having cellulose as the major component. Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (Lynd, 1999). Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply. The central technological impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass. A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products in a single process step via a cellulolytic microorganism or consortium. Such "consolidated bioprocessing" offers very large cost reductions, if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties (Lynd, 1996).

Cellulases are a complex of enzymes found in secretions of microorganisms such as fungi and bacteria. They are also present in the digestive system of some gastropod species. This enzymatic complex can hydrolyze cellulose until it reaches small oligosaccharides and glucose molecules. The cellulase market includes mainly enzymes to discolor and soften fabric. Cellulases are also used in formulation of domestic and industrial detergents; extraction of oils, essences, alkaloids, and starch; hydrolyzing lignocellulosic material to produce glucose syrup, production of juices, baby foods, skin products, digestive stimulation products, animal feed, silo-stimulating products, and adjuvant for beer malt; and treatment of organic waste (Kubicek et al., 1993).

The saccharification of cellulose is an interesting alternative for the disposal of residual plant biomasses such as sugarcane bagasse and wastes from forestry activities. Cellulase complex secreted by filamentous fungi is formed by three major enzyme components, the endoglucanases, the cellobiohydrolases (exoglucanases) and the β-glucosidases, which are not regarded as legitimate cellulases but play an important role in the hydrolysis of cellulose (Goyal et al., 1991). β-Glucosidases complete the degradation process through hydrolysis of cellobiose and other short oligosaccharides to glucose (Medve, 1997). β-glucosidases constitutes a major group among glucoside hydrolase and they occur ubiquitously in all three (archea, eubacteria and eukarya) domains of living organism.

The β-glucosidase has been the focus of recent research because of their important roles in a variety of fundamental biological processes. *Trichoderma reesei* (Sandgrena et al., 2005), *Aspergillus* sp.(Riou et al., 1998), *Penicillium* sp., (Castellanos et al., 1995; Jørgensen et al., 2005; van-Wyk, 1999) are the major β-glucosidase producers among the microbial kingdom.

The glucose inhibition is a common characteristic of β-glucosidases and it is an important constraint for industrial use of this enzyme. Most microbial β-glucosidases that catalyze the hydrolysis of cellobiose are very sensitive to glucose inhibition, which limits their activity (Gueguen et al., 1995; Saha et al., 1995). Furthermore, the enzyme is also inhibited by its own substrate, cellobiose (Yan and Lin, 1997). In this respect, the availability of β-glucosidase insensitive to glucose and cellobiose inhibition will have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol.

β-Glucosidase is capable of hydrolyzing anthocyanins that are the main coloring agents found in foods of vegetable origin (Hang, 1995; Martino et al., 1994). The main interest in this enzyme is related to its possible applications in food processing industry (such as the production of wine and fruit juices) for improving organoleptic product properties (Shoseyov et al., 1990). Also, β-glucosidase is used in the synthesis of glycoconjugates by reversing the normal hydrolytic reaction (Hirofumi et al., 1991; Toshio and Susumi, 1985). The production of ethanol from lignocellulosic residues is considered to be one of the major applications of β-glucosidase, with baker's yeast, *Saccharomyces cerevisiae*. Ethanol produced from biomass is today the most widely used biofuel when blended with gasoline. As the carbon dioxide released by combustion is recycled into biomass, the use of biofuels can significantly reduce the accumulation of greenhouse gas. Biomass conversion to ethanol has been advocated for a long time due to its potential to foster sustainable energy supply, reduce green house gas emissions, boost rural economies and reduce the country's dependence on foreign oil.

Several industrial enzymes are derived from terrestrial sources. Whereas, the marine environment, which encompasses about 71 percent of the Earth's surface, and potentially a vast resource for useful enzymes, remains largely unexplored. Marine microorganisms take an active part in the mineralization of complex organic matter through degradative pathways in their metabolism in marine environments, and contribute to the secondary production in the sea. Complex polysaccharides, such as cellulose, lignin, pectin, xylan, starch, proteins, fats, sugar, urea, aromatic and aliphatic hydrocarbons, and several other organic compounds which reach the marine environment, besides the dead plants and animal residues, are all degraded by marine microorganisms. Their participation in the degradation of organic compounds, and retting of ropes and fibers, testify to their potential as a rich source of hydrolytic

enzymes of industrial importance (Chandrasekaran, 1996). There are very few reports on β-glucosidase from marine environment on fungi (Fischer et al., 1996; Park et al., 2005; Swiatek et al., 1996). It is only advantageous to have more potential strains that produce enzymes with varied potentials. In this context, in the present study, an attempt was made to isolate a potent β-glucosidase producing fungus from the marine environment and to develop a bioprocess towards industrial production and explore their possible application for bioethanol production.

OBJECTIVES OF THE STUDY

From the literature available it was inferred that marine fungi is an under explored domain of microroganisms which has enormous potentials for industrial enzymes, particularly polysaccharases that could be utilized for bioconversion of polysaccharide reserves in the environment. Hence, the present study was planned mainly to explore the potentials of marine fungi for β-glucosidase production in the context of requirement for newer and better strains for prospective industrial applications.

Thus, the main objectives of the present study included,

1. The screening and isolation of a potent β-glucosidase producing fungus from the marine environment.
2. The process optimization for maximal enzyme production under submerged and solid state fermentations.
3. Enzyme purification and characterization.
4. Application studies.

Chapter 2

REVIEW OF LITERATURE

2.1 Cellulose

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates) and a few bacteria. Despite great differences in composition and in the anatomical structure of cell walls across plant taxa (Table 2.1) a high cellulose content- typically in the range of approximately 35 to 50% of plant dry weight is a unifying feature (Lynd et al., 1999). In a few cases (notably cotton bolls), cellulose is present in a nearly pure state. In most cases, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30% of plant dry weight (Lynd et al., 1999).

Biotechnology of cellulases and hemicellulases began in early 1980s in animal feed and food applications (Voragen, 1992). During the last two decades, the use of cellulases and hemicellulases has increased considerably, especially in textile, brewery and wine as well as in pulp and paper industries (Godfrey and West, 1996b; Harman and Kubicek, 1998; Uhlig, 1998). These enzymes account for approximately 20% of the world enzyme market (Mantyla et al., 1998), mostly from *Trichoderma* and *Aspergillus* (Godfrey and West, 1996b; Uhlig, 1998). Details of some commercial cellulases are presented in Table 2.2.

Table 2.1 Chemical composition of some typical cellulose containing materials (Hon, 1996)

Source	Composition (%)			
	Cellulose	Hemi cellulose	Lignin	Extract
Hardwood	43 – 47	25 35	16 24	2 – 8
Softwood	40 – 44	25 – 29	25 31	1 – 5
Bagasse	40	30	20	10
Coir	32 – 43	10 – 20	43 49	4
Corn cobs	45	35	15	5
Corn stalks	35	25	35	5
Cotton	95	2	1	0.4
Flax (retted)	71	21	2	6
Flax (unretted)	63	12	3	13
Hemp	70	22	6	2
Henquen	78	4 – 8	13	4
Istle	73	4 – 8	17	2
Jute	71	14	13	2
Kenaf	36	21	18	2
Ramie	76	17	1	6
Sisal	73	14	11	2
Sunn	80	10	6	3
Wheat straw	30	50	15	5

2.2 Cellulase – Classification

The saccharification of cellulose is an interesting alternative for the disposal of residual plant biomasses such as sugarcane bagasse and wastes from forestry activities. Cellulose gives sufficient substrate for human nutrition and bio-industries. A prerequisite to do such is to convert this polymer into glucose, the monomeric end-product of enzymatic degradation carried out by cellulases (Wang et al., 2005). It is an unbranched glucose polymer composed of an anhydro-β-1,4-glucose units linked by a β-1,4-D-glycosidic bond. Cellulolytic enzymes degrade cellulose by cleaving

this glycosidic bond. Cellulase itself is an enzyme complex consists of three enzymes: (i) endo- β-1,4-glucanase (E. C 3.2.1.4), (ii) exo-cellobiohydrolase (E. C 3.2.1.91), and (iii) β-glucosidase (β-D-glucosidic glucohydrolase; E. C 3.2.1.21). Endoglucanase and exocellobiohydrolase act synergistically upon cellulose to produce cellobiose, which is then cleaved by β-glucosidase to glucose. Both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose (Breznak and Brune, 1994). Thus the crystalline cellulose is efficiently hydrolyzed by the synergistic action of all three types of cellulases.

Table 2.2 Activity titres of some commercial cellulase (Nieves et al., 1998)

Product name	Company	Source	FPU/ml	β-glucosidase U/ml
Biocellulase TRI	Quest Intl. (Sarasota, FL)	*T. reesei*	68	200
Biocellulase A	Quest Intl.	*A. niger*	0.29	32
Celluclast 1.5L	Novo Nordisk (Danbury, CT)	*T. reesei*	61	26
Cellulase TAP106	Amano Enzyme (Troy, VA)	*T. viride*	0.42	17
Cellulase AP30K	Amano Enzyme	*A. niger*	0.17	60
Cellulase TRL	Solvay Enzymes (Elkhart, IN)	*T. reesei*	95	170
Econase CE	Alko-EDC (New York)	*T. reesei*	40	46
Multifect CL	Genencore Intl. (San Fran.)	*T. reesei*	64	30
Multifect GC	Genencore Intl.	*T. reesei*	65	59
Spezyme #1	Genencore Intl.	*T. reesei*	74	48
Spezyme #2	Genencore Intl.	*T. reesei*	72	53
Spezyme #3	Genencore Intl.	*T. reesei*	65	53
Ultra Low Microbial (ULM)	Iogen (Ottawa, Canada)	*T. reesei*	88	176

Cellulases play a critical role in a wide range of important processes ranging from biosphere maintenance (carbon recycling) (Berner, 2003; Melillo, 2002) to the generation of potentially sustainable energy sources such as glucose, ethanol, hydrogen and methane (Kamm and Kamm, 2004; Wyman, 2003; Zhang and Lynd, 2005).

2.3 Cellulolytic organisms

In nature there are many microorganisms, fungal and bacterial, that produce enzymes that are capable of catalyzing the hydrolysis of cellulose. These microorganisms can be found in plant debris and soil, i.e. where degradation of plant material takes place (Tomme et al., 1995). The cellulolytic organisms can be sorted into two different subcategories depending on how the cellulolytic microorganism organizes its enzymes.

One class of cellulolytic microorganisms has cellulolytic enzymes that are organized into multienzyme complexes called cellulosomes. In these complexes the individual enzyme molecules are anchored onto a common scaffold. Several different types of enzymes, with different types of catalytic specificities, e.g. endoglucanases and cellobiohydrolases, can be complexed together. (Bhat 2000).

The second class of cellulolytic organisms produces enzymes that are not attached to one another, but act independently. Examples of fungi from this class are *H. jecorina* and *Humicola grisea*, and of bacteria, *Streptomyces lividans* and *Cellulomonas fimi*. Although most of the cellulose degradation is conducted by bacteria and fungi, cellulases have also been isolated from blue mussel (Xu et al., 2000), termites (Watanabe et al., 1992), and plants, e.g. *Arabidopsis* (Williamson et al., 2002).

Fungi are well known agents of decomposition of organic matter in general and of cellulosic substrates in particular (Montegut et al., 1991). Cellulolytic filamentous fungi (and actinomycete bacteria) have the ability to penetrate cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles (Eriksson et al., 1990). Members of genera that have received considerable study with respect to their cellulolytic enzymes include *Bulgaria, Chaetomium*, and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); and *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium*, and *Trichoderma* (Deuteromycetes). Many fungal strains secrete higher amounts of cellulases than bacterial ones, with *Trichoderma* (Godfrey and West, 1996b) as the leading one. Other fungal species were shown to be interesting cellulase producers, such as *Humicola* (Takashima et al., 1997) or *Aspergillus* (Uhlig, 1998).

The filamentous fungus *Trichoderma reesei* is an ideal cellulolytic model organism for studying cellulase degradation since it is one of the most efficient cellulose degrading organisms known, and secretes large amounts of all cellulases needed for degradation of crystalline cellulose. This system contains numerous endoglucanases and two exoglucanases that correspond to approximately 80% of the total secreted protein, but its β-glucosidase activity is very low. It has long been the major workhorse for commercial production of cellulases and industrial strains can produce in the excess of 40 g/l of protein (Sandgrena et al., 2005; Teeri et al., 1998).

2.4 Microbial β-Glucosidase

The β-glucosidase has been the focus of much research recently because of their important roles in a variety of fundamental biological processes (Hansson and Ablercreutz, 2002). Widely distributed in bacteria, fungi, plants, and animals, β-glucosidase is vital for many biological processes, such as biomass conversion (cellulolysis) by microbes/insects, biogenesis of various functional molecules (e.g., terpenols, flavonoids, phytohormones) from glycoside precursors, cyanide-based biodefense (CN releasing from cyano-glucoside), and degradation of various potentially harmful metabolites (e.g., glycosylceramides) (Bhatia et al., 2002). It is also used for the synthesis of oligomers and other complex molecules (such as alkyl-glucosides) by transglycosylation (Hansson and Ablercreutz, 2002). The different microbial groups, which produce the β-glucosidase enzyme as extracellular fraction, are summarised in Table 2.3.

Filamentous fungi such as *Trichoderma* and *Aspergillus* species are well known and efficient β-glucosidase producers. *T. reesei* produces β-glucosidases at low levels compared to other fungi such as *Aspergillus* species (Reczey et al., 1998). Furthermore, the β-glucosidases of *T. reesei* are subject to product (glucose) inhibition (Chen et al., 1992), whereas those of *Aspergillus* species are more glucose tolerant (Decker et al., 2001; Gunata and Vallier, 1999; Yan and Lin, 1997). The levels of *T. reesei* β-glucosidase are presumably sufficient for growth on cellulose, but not sufficient for extensive in vitro saccharification of cellulose. *T. reesei* cellulase preparations, supplemented with *Aspergillus* β-glucosidase, are considered most often for cellulose saccharification on an industrial scale (Reczey et al., 1998).

The β-glucosidase gene *bgl*1 from *T. reseei* was cloned and sequenced by (Barnett et al., 1991). Later, Fowler (1993) studied β-glucosidase from the null strain of *T. reesei* to explore its role in the cellulase enzyme system. The hydrolysis of cellulose and induction of other cellulolytic enzyme components led Fowler to the conclusion that extracellular β-glucosidase is required for the induction of the other cellulase enzymes.

β-Glucosidases are exo-type glycoside hydrolases that cleave β-glucosidic bonds from the non-reducing ends of their substrates. It reduces cellobiose inhibition by hydrolysing cellobiose to glucose, which allows the cellulolytic enzymes to function more efficiently (Saha et al., 1994; Xin et al., 1993). However, most microbial β-glucosidases that catalyze the hydrolysis of cellobiose are very sensitive to glucose inhibition, which limits their activity (Gueguen et al., 1995; Saha et al., 1995). Furthermore, the enzyme is also inhibited by its own substrate, cellobiose (Schmid and Wandrey, 1987). In this respect, the availability of β-glucosidase insensitive to glucose and cellobiose inhibition will have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol.

In addition to its physiological functions, β-glucosidase is of great interest as a versatile industrial biocatalyst. Being indispensable for an efficient cellulase system, β-glucosidases hydrolytic activity on cellodextrins (particularly cellobiose) not only enables the final step of the cellulolysis of lignocellulosics (to yield glucose), but also relieves the product inhibition on cellobiohydrolase and endoglucanase. Both of the catalytic functions of β-glucosidase are critical to various bio-treatment/biorefinery processes, such as bio-ethanol production (Bhatia et al., 2002; Xu, 2004).

Table 2.3 Major groups of β-glucosidase producers

Major Group	Microorganism	Reference
Fungi	*Aspergillus tubingensis*	Decker et al., 2001
	A. niger	Yan and Lin, 1997
	A. oryzae	Riou et al. 1998
	A. aculeatus	Kawaguchi et al., 1996
	Penicillium echinulatum	(Dillon et al., 2005)
	Phanerochaete chrysosporium	Lymar et al., 1995
	Pyrococcus furiosus	Kempton and Withers 1992
	Thermoascus aurantiacus	(Parry et al., 2001)
	Trichoderma reesei	Saloheimo et al.,2002
	T. koningii	Wood and McCrae, 1982
	Volvariella volvacea	Li et al., 2005
	Neurospora crassa	Yazdi et al., 2003
	Botrytis cinerea	(Gueguen et al., 1995)
	Fusarium oxysporum	(Christakopoulos et al., 1994)
	Thermomyces lanuginosus	Lin et al., 1999
Bacteria	*Vibrio cholerae*	Park et al , 2002
	Azospirillum irakense.	Faure et al., 1999
	Clostridium thermocellum	Ait et al., 1982
	Alicyclobacillus sp.	Lauro et al., 2006
	Bacillus polymyxa	Gonzalez et al., 1990
	Agrobacterium sp.	Pouwels et al., 2000
	B. circulans	(Paavilainen et al., 1993)
	Pseudomonas sp	Her et al., 1999
	Paenibacillus sp.	Shipkowski & Brenhley, 2005
Yeast	*Candida peltata*	(Saha and Bothast, 1996)
	Saccharomycopsis fibuligera	(Machida et al., 1988)
	Pichia anomala	Kohchi and Toh-e, 1985
	Debaryomyces sp	Villena et al., 2006
Actinomycetes	*Streptomyces* sp.	(Ozaki and Yamada, 1992)

2.5 Classification and Structure of β–Glucosidase

β-Glycosidases have been classified into one of the 57 families of glycosidases (Henrissat 1996). In Henrissat's classification, β-glucosidases are classified as Family 1 glycosidases based on amino acid sequence similarity and substrate specificity. A remarkable feature of the enzymes belonging to this family is their wide range of substrate preference despite high sequence homology. These enzymes display broad substrate specificity with respect to the aglycone portion of the substrate. In this classification, enzymes with different substrate specificities are found in the same family, indicating an evolutionary divergence to acquire new specificities as is found in Family 1.

The three dimensional structure of enzymes from several glycosyl hydrolase families have been determined, together with the mechanism of glycosidic bond hydrolysis. The active-site topologies were classified into three classes regardless of whether the enzyme is inverting or retaining (Davies and Henrissat 1995). These three topologies were classified as (1) pocket or crater, (2) cleft or groove and (3) tunnel. Other enzymes in the same group showed similar folding with the same catalytic residues. Family 1 of glycosyl hyrolases is classified under pocket-crater topology. This topology is optimal for recognition of the nonreducing saccharide extremity found in monosaccharides such as β-glucosides, β-galactoside, sialic acid and neuramic acid. On the other hand, these enzymes are not very efficient on fibrous substrates such as native cellulose, which does not have free chain ends (Bhatia et al., 2002).

The three dimensional structures of four family 1 enzymes have been determined. These include a phospho-β-galactosidase from *Lactoccus lactis* (Wiseman, 1995),and a glucosidase from *Bacillus polymyxa* (Sanz-Aparicio et al.,

1998). These enzymes certainly show a common structural folding pattern providing insight into the molecular and mechanistic basis of substrate binding sites. The 3D structure data show that all four enzymes share the same 8 (β/α) barrel topology, and a crater-like active site. These enzymes also have the same catalytic mechanism belonging to the same glycosyl hydrolase family (Family 1). The general mechanism of all Family 1 β-glucosidases are similar due to their sequence and folding similarity and shows the retention of the anomeric configuration via the double-displacement mechanism proposed first by Koshland (1953).

2.6 Mode of action of β-Glucosidase

The enzyme β-glucosidase catalyzes the hydrolysis of glycosidic linkages formed between the hemiacetal -OH group of a cyclic aldose or glucose and the –OH group of another compound viz., sugar, amino-alcohol, aryl-alcohol or primary, secondary, or tertiary alcohols (Bhatia et al., 2002). This reaction proceeds via the following steps:

1. During glycosylation, an enzymic nucleophile attacks the anomeric (C1) center of the substrate glycoside (1), resulting in formation of a covalently linked α-glycosyl enzyme intermediate (2) through an oxocarbenium ion-like transition state (Withers and Street, 1988). Thus, the anomeric configuration at C1 is reversed as shown in Figure 2.1.

2. Another active residue of the enzyme serves as the acid base catalyst and donates a H^+ to the glycosidic oxygen, thereby assisting in departure of the agylcone (or other glycone, as in disaccharides) group.

3. The glycosyl-enzyme intermediate (2) is hydrolyzed via general base-catalyzed attack by water at the anomeric center to release β-glucose as the product (3). The trans-addition of an –OH group results in the net retention of the β-anomeric

configuration. The nucleophilic residue also acts as the leaving group in the deglycosylation step. Both formation and hydrolysis of the enzyme-glycosyl intermediate occurs via an oxocarbenium ion-like transition state.

The reactions for biosynthesis of glycoconjugates occur either by reverse hydrolysis or by transglycosylation. The two-step mechanism employed by retaining enzymes such as β-glucosidases allows these enzymes to transglycosylate. In reverse hydrolysis, the substrate (1) has an H in place of R (Figure 2.1). The enzyme-glycosyl intermediate is intercepted by R'OH where R' is another sugar, yielding a disaccharide product (4). The reaction is under thermodynamic control. In the transglycosylation method, the substrate (1) has R in place of H and is an "activated" donor. The enzyme-glycosyl intermediate may be trapped by a nucleophile other than water, viz., aryl or alkyl alcohol (as R'OH) to yield a new glycoside. Here, the efficiency of the formation of the product is determined by competition between water and the acceptor R'OH for the enzyme-glycosyl intermediate. The reaction is under kinetic control (Bhatia et al., 2002).

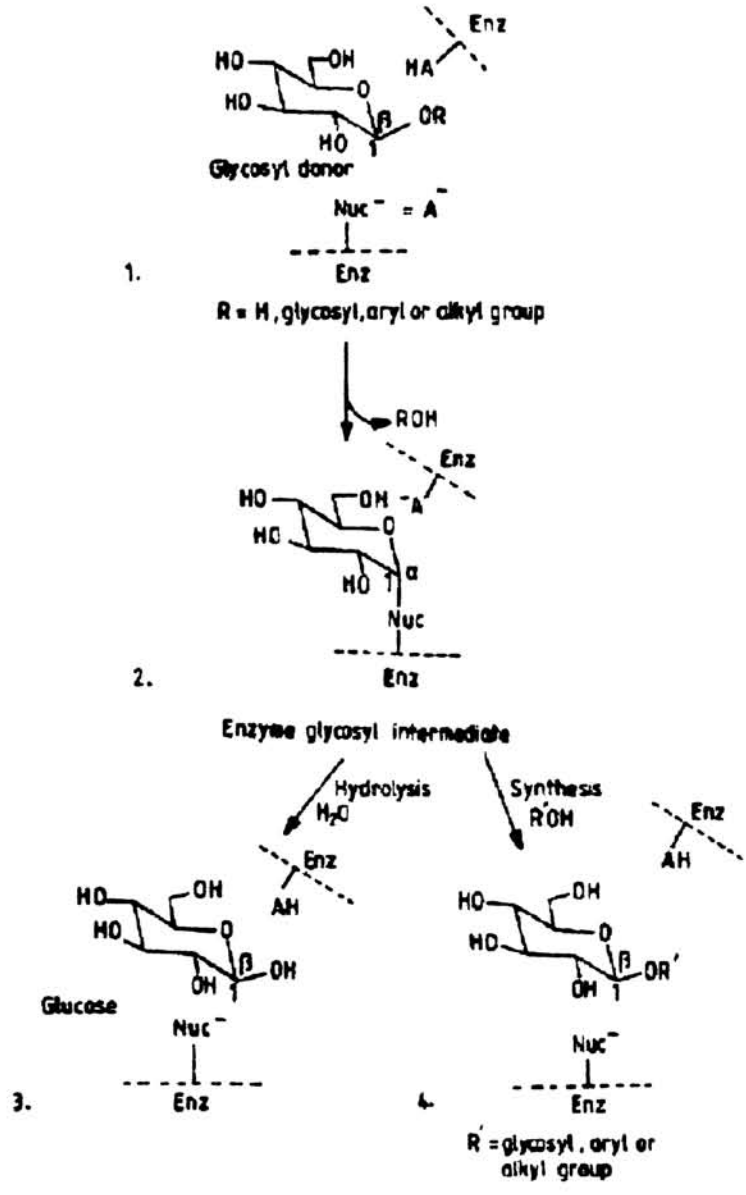

Figure 2.1 Reaction mechanism of β-glucosidase (Bhatia et al., 2002)

2.7 Fermentation and media development for β-Glucosidase

Most enzyme manufacturers produce enzymes using submerged fermentation (SmF) techniques with enzyme titres in the range of grams per liter (Harvey and McNeil, 1993). Submerged fermentations are normally used in the fermentation industry because they are easier to handle and control the fermentation process (Iwashita, 2002). The growth and production of β-glucosidase on different carbon sources under submerged fermentation by various fungal strains are enlisted in Table 2.4. The major drawbacks of solid state fermentation (SSF) compared to submerged include the difficulties in controlling important culture parameters (heat removal and mass transfers) that have not been overcome completely (Bandelier et al., 1997; Ramesh and Lonsane, 1990). But now a days researchers are interested on SSF because of the higher productivity and the use of low cost media. About 30 40% of the production cost of many industrial enzymes is accounted by the cost of growth substrate (Hinnman, 1994). The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials as substrates (Wizani et al., 1990). In SSF process the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells (Lonsane et al., 1985). Filamentous fungi play a key role in SSF for their hyphal development allows them to effectively colonise and penetrate the solid substrate (Machida, 2002; Pandey et al., 2000). The growth and production of cellulase enzymes by some cellulolytic strains on lignocellulosic materials are summarized in Table 2.5.

Table 2.4 Comparative data for growth and β-Glucosidase productivity in submerged fermentations by various fungal strains

Fungal strain	Carbon source	Reference
Aspergillus tubingensis	Rutin / Glucose	(Decker et al., 2001)
Monascus purpureus	CMC	(Daroit et al., 2007)
Stachybotrys sp.	Avicel	(Amouri and Gargouri, 2006)
Debaromyces pseudopolymorphs	Cellobiose	(Villena et al., 2006)
Penicillium echinulatum	Cellulose	(Camasola et al., 2004)
P. etchellsii	Cellobiose	(Wallecha and Mishra, 2003)
Volvariella volvacea	CMC	(Li et al., 2005)
Acremonium persicinum	Laminarin/Glucose	(Pitson et al., 1997)
A. niger	Quercetin/Rutin/ Cellobiose/Glucose	(Gunata and Vallier, 1999)
Neurospora crassa	Cellulose	(Yazdi et al., 2003)
A. aculeatus	Cellobiose/Glucose	(Murai et al., 1998)
Orpinomyces sp.	Avicel	(Chen et al., 1994)
A. fumigatus	Glucose/Cellobiose	(Ximenes et al., 1996)
	Wheat straw/ Filter paper	
Phanerochaete chrysosporium	Cellulose	(Igarashi et al., 2003)
Thermoascus auranticus	Glucose / Wheat bran	(Parry et al., 2001)
A. oryzae	Quercetin/Rutin Sucrose/Cellobiose /Glucose	(Riou et al., 1998)

Table 2.5 Cellulase enzyme yields by some fungal strains grown on different lignocellulosic biomass

Organism	Substrate	Enzyme activity (IU g^{-1})			Reference
		FPA	CMCase	β-GL	
Aspergillus ustus	Rice straw	5.8	12.6	15.8	(Shamala and Sreekantiah, 1986)
	Wheat bran	3.8	11.8	60	
Penicillium capsulatum	Beet pulp	2.7	632	14.8	(Considine et al., 1988)
T. reesei	Beet pulp	7.7	704	4.6	" "
T. reesei ZU-02	Corn cobs	--	--	59	(Xia and Cen, 1999)
T. reesei + A. niger	Sugar cane Bagasse	--	129	21.7	(Gutierrez-Correa et al., 1999)
Talaromyces emersonii	Wheat bran/ Beet pulp	18	265	33	(Tuohy et al., 1990)
Thermoascus auranticus	Wheat straw	4.3	956	46.1	(Kalogeris et al., 1999)
Sporotrichum cellulophilum	Wheat bran	--	45	--	(Kim et al., 1985)
Humicola lanuginose	Beet pulp, Cellulose	--	1.7	46.8	(Grajek, 1986)
A. niger	Rice straw	19	130	94	(Kang et al., 2004)

2.7.1 Effect of carbon and nitrogen sources

Most microbial cellulases are inducible enzymes, and they are secreted when the microorganisms grow on cellulose substrates (Kubicek et al., 1993; Mach and Zeilinger, 2003). The β-glucosidase enzyme production was also affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate is of importance (Kang et al., 2004).

There are a number of reports regarding the role of glucose in the synthesis of cellulase enzyme as an inducer (Hulme and Stranks, 1971; Reese and Levinson, 1952) or repressor (Canerascini et al., 1979). In possessing β-glucosidase activity inducible by different cellulosic inducers, *Myothecium thermophila* D-14 resembles *T. reesei* (Mandels and Reese, 1960) and *Streptomyces venezuelae* (Chatterjee and Vining, 1982), in which the enzyme is inducible and repressed by easily metabolizable carbohydrates.

The effect of different carbon sources on enzymatic biosynthesis in the supernatant fluid of *Debaryomyces pseudopolymorphus* demonstrated that the β-glucosidase synthesis was depending strain (Villena et al., 2005). The yeast showed an increased biomass as well as enzyme production with cellobiose as the sole carbon source. The yeast grew well on both cellobiose and glucose containing medium, although the cell mass was about 1.2 times lower in glucose containing media than in cellobiose containing media (Villena et al., 2006).

The effect of a variety of carbon sources on high glucose tolerant β-glucosidase production by *A. oryzae* as investigated by Riou et al. (1998). The organism grew well on every substrate tested, although the cell mass yield was about five times lower on quercetin or lactose containing media than on glucose containing media. A high yield was also obtained on non-β-glucosidic substrates, such as glucose or maltose. The best substrate for the production of high glucose tolerant β-glucosidase form was found to be quercetin. rutin, lactose, and sucrose were also found to be good inducers of high glucose tolerant β-glucosidase.

The β-glucosidase from *Candida peltata* was seemed to be a constitutive enzyme regardless of the presence or absence of cellobiose. The highest level of β-glucosidase activity (117 mU/ml) was obtained in Xylose grown whole culture broth.

Glucose was a good growth substrate for production of β-glucosidase (93 mU/ml). About 55 to 89% of β-glucosidase activity was found in the culture supernatant, depending on the carbon source used (Saha and Bothast, 1996). The use of chitin as the carbon source for the production endo-β-1,6-glucanases from *Trichoderma harzianum*, was also reported (Cruz et al., 1995).

In *A. niger,* under solid state fermentation, β-glucosidase synthesis was induced by substrate, except with the addition of the cellulose powder. The highest level of β-glucosidase was produced when 20% corn bran or wheat bran was added along with radicle as the solid substrate. CMC also resulted in 16.7% enhancement in β-glucosidase production when added with 20% (w/w) of the radicle (Fadel, 2000).

Penicillium echinulatum could produce the β-glucosidase enzyme in high titer, after 96 h of incubation in the presence of 1% lactose. The same strain also showed a higher β-glucosidase activity at 72 h with 1% cellulose as the carbon source (Sehnem et al., 2006). Lactose is a low commercial cost disaccharide, and it is generated in large amounts during the production of cheese and other dairy products. It is a potential source of carbon in fermentation processes for cellulase production, and the advantage is that it is soluble (Seiboth et al., 2002).

Production of cellulase is influenced by the nitrogen source and nitrogen level in the medium (Desai et al., 1982). It was reported that an addition of 2% ammonium sulphate could enhance the β-glucosidase enzyme production 20% more (Fadel, 2000). Ammonium sulphate was shown to facilitate the cellulase and β-glucosidase production in *Penicillium funiculosum*, *Myrothecium* sp, *Chaetomium cellulolyticum*, *T. reesei*, A. *niger* and *A. terreus* (Chahal et al., 1996; Rao et al., 1995).

In *Monascus purpureus* the presence of peptone in the culture media played a critical role in the production of high levels of β-glucosidase (Daniel et al., 2007). Corn steep liquor and soy meal are cheap substrates that were successfully employed as complex nitrogen sources in β-glucosidase production, contributing to reduced costs of enzyme production (Gomes et al., 2000; Kang et al., 2004).

2.8 Purification of β-Glucosidase

Most of the commercial applications of enzymes do not always need homogeneous preparation of the enzyme. However, a certain degree of purity is required, depending upon the final application, in industries such as fine chemicals, pharmaceuticals and cosmetics. Besides, purification of the enzyme is a must for understanding the 3D structure and the structure-function relationships of proteins (Aires-Barros et al., 1994; Saxena et al., 2003; Taipa et al., 1992). For industrial purposes, the purification strategies employed should be inexpensive, rapid, high yielding and amenable to large scale operations. They should have the potential for continuous product recovery, with a relatively high capacity and selectivity for the desired product. Various purification strategies used for β-glucosidase have been reviewed and some are listed in Table 2.6.

The culture filtrates were extracted by filtration over cheesecloth or centrifugation and the crude enzyme supernatant is subjected to various concentration methods such as ammonium sulphate precipitation (Zhang et al., 2007), ultra filtration (Endo et al., 2001; Rajoka et al., 2006), precipitation using solvents such as chilled ethanol (Saha, 2004), acetone (Umezurike, 1991) etc. After the concentration step, the enzyme is applied for various chromatographic columns depending on the nature of the protein.

Table 2.6 Various strategies employed for the β-glucosidase enzyme purification

Microorganism	Concentration method	Column matrices	MW of BGL (kDa)	Reference
Aspergillus japonicus		Source 15 Q	28	(Grishutin et al., 2006)
Pichia etchelsii	$(NH_4)_2SO_4$	DEAE Sepharose Sephadex G-25	52	(Roy et al., 2005)
P. etchellsii	$(NH_4)_2SO_4$	DEAE Sepharose Sephacryl S-400 Hydroxyapatite	340	(Wallecha and Mishra, 2003)
Talaromyces thermophilus	Ultrafiltration	DEAE Sepharose	50	(Nakkharat and Haltrich, 2006)
A. oryzae		Q- Sepharose FF	120	(Langston et al., 2006)
A. niger	Ethanol fractionation	CM- Sepharose Q- Sepharose Sephacryl S-300	360	(Yan et al., 1998)
Thermomyces lanuginosus	Amicon P-30 $(NH_4)_2SO_4$	Sephadex G-150 Q-Sepharose QAE-Sephadex A-25	200	(Lin et al., 1999)
Phanerochaete chrysosporium	Ultrafiltration	DEAE Sephadex Sephacryl S-200 FPLC-Mono Q	114	(Lymar et al., 1995)
A. niger	$(NH_4)_2SO_4$	Octayl-Sepharose Q-Sepharose Sephadex S-200 D-Zephyr	118 190	(Masson and Pellerin, 1998)
A. tubingensis		DEAE Sephadex DEAE Sepharose Sephacryl S-200 Mono P HR	290	(Decker et al., 2001)

A. oryzae	$(NH_4)_2SO_4$	Ultrogel AcA- 44 DEAE 5PW	130	(Riou et al., 1998)
A. terreus		DEAE-CMC Cellulose ConA-agarose DEAE Cellulose	--	(Workman and Day, 1982)
Orpinomyces sp.	Ultrafiltration $(NH_4)_2SO_4$	Q Sepharose Mono P HR Superose 12	87	(Chen et al., 1994)
Aureobasidium pullulans	$(NH_4)_2SO_4$	CM Bio-Gel A Bio-Gel A Sephacryl S-200	340	(Saha et al., 1994)
Neurospora crassa		Sephacryl S-200 SP-Sepharose	178, 106 & 43	(Yazdi et al., 2003)
Mucor racemosus	$(NH_4)_2SO_4$	DEAE Sephadex SP Sephadex Sephadex G-150	91	(Borgia and Mehnert, 1982)
A. fumigatus	Ultrafiltration	Sephacryl S-200 S-Sepharose	130	(Ximenes et al., 1996)
Schizophyllum commune	Ethanol precipitation	Bio-Gel A Bio-Gel P-200	97	(Desrochers et al., 1981)
Aureobasidium sp.		DEAE Toyopearl 650S S-Sepharose TSK-Gel Toyopearl HW	331	(Hayashi et al., 1999)
T. reesei	$(NH_4)_2SO_4$	Sephadex G-20		(Krishna et al., 2000)
Gaeumannomyces graminis	$(NH_4)_2SO_4$	Sepharose 4B	250	(Dori et al., 1995)
Candida wickerhamii		Concanavalin A HPLC-AX300 Sephadex G-75 Hydroxyapatite	180	(Skory et al., 1996)
Stachybotrys sp.	$(NH_4)_2SO_4$	Sephadex G-25 FPLC Mono Q	85	(Amouri and Gargouri, 2006)

2.9 Properties of fungal β-glucosidases

β-Glucosidases from several microorganisms have been studied extensively and the various properties of fungal β-glucosidase (viz. molecular weight, pH and temperature optima, enzyme kinetics) reported in literature are summarized in Table 2.7. However, a brief account of individual properties is presented in the following sections.

2.9.1 Optimum pH and Temperature

The optimal pH of most of the β-glucosidases is 5.0 and the enzyme showed stability in the range of pH 3.0 to 12.0. Whereas, in the case of optimal incubation temperature for the maximal enzyme production 50°C was mostly reported and the temperature stability ranged from 30-80°C. The characteristic features of pH and temperature activity and stability of some β-glucosidase producing strains are displayed in Table 2.7.

2.9.2 Substrate Specificity and Enzyme kinetics

β-Glucosidases display broad specificity with respect to both the aglycone and the glycone moieties of their substrates. In fact, β-glucosidases from all sources have a similar specificity for the glycone (glucose) portion of the glucoside. However they vary with respect to aglycone specificity. The natural substrates of β-glucosidases include steroid β-glucosides and β-glucosyl ceramides of mammals, cyanogenic β-glucosides and glucosinolates of plant secondary metabolism, and oligosaccharide products released from digestion of the cellulose of plant cell wall (Clarke 1987). Artificial substrates such as benzyl, nitrophenyl and methylumbelliferyl-glycosides are frequently used during the purification and characterization of glycosidases. These

substrates allow the easily assay of enzyme velocities of hydrolysis and substrate affinities.

β-Glucosidases with very broad substrate specificity have been isolated from many fungi. The purified β-glucosidase of *A. oryzae* efficiently hydrolyzed natural (gentiobiose, laminaribiose, cellobiose, lactose and sophorose) as well as synthetic (*p*NPG) substrates (Riou et al., 1998). The purified enzyme of *Stachybotrys* sp. showed high velocity and affinity towards *p*NPG, compared to salicin, cellobiose and CMC (Amouri and Gargouri, 2006). The substrate specificity of the purified *Talaromyces thermophilus* β-glucosidase BGL1 was determined towards various arylglycosides (*o*-NPGal, *o*-NPGlu) and disaccharides. BGL1 has higher binding affinities for *o*-NPGlu, cellobiose as compared to the corresponding galactose-containing molecules (*o*-NPGal, lactose). In addition to the disaccharide substrates cellobiose and lactose, BGL1 showed significant activity with gentiobiose , while it showed no activity with xylobiose and maltose (Nakkharat and Haltrich, 2006). β-glucosidase from *Thermomyces lanuginosus*-SSBP was active on both aryl-β-glucoside and cellobiose with similar specific activities under the assay conditions. The purified enzyme, however, exhibited less but significant α-D-glucosidase activity when maltose and *p*-nitrophenyl-α-D-glucopyranoside were used as the substrates. Salicin and gentiobiose were not favorable substrates for the purified enzyme (Lin et al., 1999).

The reaction kinetics of the purified enzyme is determined from the Lineweaver – Burk plot. A wide range of K_m (Michaelis constant) and V_{max} (maximum velocity) values of microbial β-glucosidase toward *p*NPG and cellobiose have been reported by many investigators and the K_m value of some fungal β-glucosidases are enlisted in Table 2.7.

Table 2.7 Properties of some fungal β-glucosidases

Source	pH		Temperature (°C)		K_m (mM)		K_i (mM)	MW (kDa)	Reference
	Optimum	Stability	Optimum	Stability	*p*NPG	CB			
Aspergillus oryzae	5.0	3.0 – 7.0	50	20 – 45	0.55	7	136	43	Riou et al., 1998
Aureobasidium pullulans	4.5	4.0 – 6.5	75	60 – 85	1.17	n.d	5.65	165	Saha et al., 1994
Aspergillus niger	5.0	5.0 – 7.0	55	10 – 60	21.7	n.d	543	105	Yan & Lin, 1997
Thermoascus auranticus	4.5	n.d	80	40 – 70	0.11	0.64	0.29	120	Parry et al., 2001
Neurospora crassa	5.0	5.0 – 9.0	55	4 & 37	n.d	1.5	n.d	106	Yazdi et al., 2003
Candida peltata	5.0	4.0 6.0	50	30 45	2.3	66	140	43	Saha & Bothast, 1996
Aureobasidium sp.	4.0	2.2 – 9.8	80	30 - 80	n.d	8.85	n.d	331	Hayashi et al., 1999
Aspergillus fumigatus	5.0	n.d	65	n.d	0.075	1.36	3.5	130	Ximenes et al., 1996
Aspergillus phoenicis	5.0	4.0 – 8.0	60	30 – 50	0.58	n.d	n.d	77	Zhang et al., 2007
Stachybotrys sp.	5.0	n.d	50	4 – 50	0.3	2.2	n.d	85	Amouri& Gargouri, 2006
Thermomyces lanuginosus	6.0	5.0 – 12.0	65	40 – 55	0.075	n.d	0.55	200	Lin et al., 1999
Phanerochaete chrysosporium	5.2	4.0 - 8.0	45	n.d	0.096	2.3	0.27	114	Lymar et al., 1995
Volvariella volvacea	6.4	5.6 – 8.0	50	n.d	0.2	n.d	n.d	380	Lil et al., 2005
Trichoderma reesei	4.8	n.d	50	n.d	n.d	1.1	26	n.d	Cascalheira & Queiroz, 1999

(n.d not determined; *p*NPG *p*-nitrophenyl β-D-glucopyranoside; CB - cellobiose; K_i glucose inhibition constant; MW molecular weight)

2.9.3 Effect of metal ions, inhibitors and compounds

Two intracellular β-glucosidases (A and B) from the *Neurospora crassa* mutant cell-1 has been purified and the effects of metal ions on the activity were determined (Yazdi et al., 2003). Cu^{2+} strongly increased the activity of both enzymes, while, Sn^{2+} and Mn^{+2} severely inhibited them. Ag^{+} and Hg^{2+}, greatly reduced the activity of β-glucosidases B. Pb^{2+}, Zn^{2+} and Co^{2+} had nearly no effect on the activity of β-glucosidases B, but slightly inhibited β-glucosidases A. Mg^{2+} slightly increased the activity of β-glucosidases A and decreased that of β-glucosidases B.

N-bromosuccinimide, $KMnO_4$, and Zn^{2+} strongly inhibited the purified β-glucosidase enzyme of *Acremonium persicinum* (Pitson et al., 1997). The β-glucosidase from *Debaryomyces pseudopolymorphus* was moderately inhibited by 10mM Ca^{2+}, Mg^{2+} and Zn^{2+} and at the same concentration of the Co^{2+}, K^{+} and Na^{+} cations, the enzyme showed no inhibition. It was found that the enzyme was moderately activated by 1mM acetic acid and ethanol, but was inhibited by the same compounds at 10 mM. The β-glucosidase showed 70% of maximum residual activity with 1mM glucose, and was moderately activated by this hexose at 10 mM. It showed slight activation in the presence of 1mM glycerol (Villena et al., 2006).

2.9.4 Effect of Ethanol on β-glucosidase activity

Some β-glucosidase could preferentially utilize alcohols rather than water as acceptors for the glycosyl moiety during catalysis of *p*NPG, resulting in elevated reaction rates, which suggests that ethanol increases the hydrolysis rate of *p*NPG by acting as an acceptor molecule for glucose (Pemberton et al., 1980; Saha et al., 1994).

This property is advantageous for the use of the enzyme in the simultaneous saccharification and fermentation of cellulosic materials to ethanol.

Activation of enzyme by ethanol was observed for β-glucosidase from *Dekkera intermedia* (Blondin et al., 1983), *Fusarium oxysporum* (Christakopoulos et al., 1994) *A. tubingensis* (Decker et al., 2001), *A. oryzae* (Riou et al., 1998), *Aureobasidium pullulan* (Saha and Bothast, 1996), *Candida peltata* (Saha et al., 1994) and *A. niger* (Yan and Lin, 1997).

2.10 Glucose tolerant β-Glucosidase

β-Glucosidase reduces cellobiose inhibition by hydrolyzing cellobiose to glucose, which allows the cellulolytic enzymes to function more efficiently (Saha et al., 1994). Most of the microbial β-glucosidases reported are highly sensitive to glucose inhibition, which limits their activity (Gueguen et al., 1995;Saha et al., 1995). Moreover the enzyme is inhibited by its own substrate, cellobiose and hence the glucose tolerant β-glucosidase have a significant industrial importance such as in the production of fuel ethanol by the enzymatic conversion of the cellulosic biomass to glucose (Yan and Lin, 1997).

Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases that limits their use in enzymatic hydrolysis of plant products (Gueguen et al., 1995; Painbeni et al., 1992; Saha et al., 1995; Woodward and Wiseman, 1982). Most microbial enzymes show inhibition constants (K_i) of 0.6 to 8 mM for glucose (Ozaki and Yamada, 1992).

β-glucosidases from *A. tubingensis* (470 & 600 mM), *A. oryzae* (953 mM), *Pyrococcus furiosus* (300 mM), *Candida peltata* (1.4 M) and *A. niger* (543 mM) are some of the reported high glucose tolerant fungal β-glucosidase producers (Decker et al., 2001; Gunata and Vallier, 1999; Kengen et al., 1993; Saha and Bothast, 1996; Yan and Lin, 1997). Yan and Lin (1997) also reported a β-glucosidase enzyme with a lower K_i of 0.543 from *A. niger* (Table 2.4).

A cloned β-glucosidase (BglB) from *Microbispora bispora* was also activated two to threefold in the presence of 2 to 5% (0.1 to 0.3 M) glucose and did not become inhibited until the glucose concentration reached about 40% (Wright et al., 1992). Perez-Pons et al. (1995) reported that glucose in the range of 0.45 to 3.6% (25 to 200 mM) enhanced the rate of *p*NPG hydrolysis, isolated from a *Streptomyces* sp.

2.11 Recombinant β-Glucosidases

The β-glucosidase genes from a large number of bacterial, mold, yeast, plant, and animal systems have been cloned and expressed in both *E. coli* and eukaryotic hosts such as *S. cerevisiae* and filamentous fungi.

Fungal β-glucosidases have been cloned in eukaryotic expression systems such as *Trichoderma reesei* (Barnett et al., 1991), *Aspergillus* sp. (Takashima et al., 1999), *S. cerevisiae* and *Pichia pastoris* (Dan et al., 2000), with the exception of β-glucosidase from *Talaromyces emersonii* (Morrison et al., 1990) and *Phanerochaete chrysosporium* (Li and Ranganathan, 1998) that were cloned in *E. coli*. Mostly, cloned fungal β-glucosidases were extracellular but a few, such as recombinant Bgl A encoded by *Aspergillus kawachii*, were localized in the periplasmic space of the host *S. cerevisiae* (Iwashita et al., 1999). Yeast β-glucosidase genes have been expressed in eukaryotic hosts such as *S. cerevisiae* or *Candida* sp., with the exception of β-

glucosidases from *Candida wickerhamii* and *Pichia etchellsii*, whose genes were expressed in a prokaryotic expression system of *E. coli* (Sethi et al., 2002; Skory and Freer, 1995). In general, the enzymes expressed in *E. coli* were localized intracellularly as Bgl I from *P. etchellsii* (Pandey and Mishra, 1995) or to periplasmic space as BglII from the same yeast (Sethi et al., 2002). However, BGL1 and BGL2 of *Saccharomycopsis fibuligera* cloned in *S. cerevisiae* were secreted into the extracellular medium (Machida et al., 1988).

2.12 Marine microorganisms

Among the three major habitats of the biosphere, the marine realm which covers 70% of the earth's surface provides the largest inhabitable space for living organisms, particularly microbes (Das et al., 2006). Currently, microbes from terrestrial sources are employed for industrial production of enzymes, although the potential for synthesis of several novel enzymes by marine microorganisms has been recognized. In India, a number of studies have been made on detection of enzymes including phosphates, arylsulfatase, chitinase, L-asparaginase, L-glutaminase, amylase, protease, lipase, cellulase, urease, and lactamase produced by marine bacteria, cyanobacteria, and fungi (Chandrasekaran, 1996). The marine environment is a virtually untapped source of novel micro-biodiversity and therefore of new metabolites.

Marine fungi are the important intermediaries of energy flow from detritus to higher trophic levels in the marine ecosystems. They require sea water for the completion of their life cycle. More than 500 species of marine fungi have been described (Jones, 1983). Marine filamentous fungi have been reported on a variety of detritus decaying wood, leaves, seaweeds, sea grasses, calcareous and chitinous

substrates. As most fungal isolates that have been obtained from marine source so far belong to genera well known from the terrestrial environment e.g. *Aspergillus*, *Cladosporium*, *Penicillium*. It appears that they originate from the land and are washed into the sea where they are trapped by filter-feeding invertebrates (König et al., 2006). Cellulolytic marine fungi appear to utilize cellulose growth substrates throughout the salinity range 0-34% (Pointing et al., 1998).

The gene encoding β-glucosidase of the marine hyperthermophilic eubacterium *Thermotoga neapolitana* (bglA) was subcloned and expressed in *E. coli*. The molecular mass was determined to be 56.2 kDa. The recombinant BglA was active toward *p*NPG, cellobiose and lactose (Park et al., 2005). The molecular cloning and characterization of a β-Glucosidase from *Vibrio cholerae* was reported by Park et al. (2002). BglA was molecularly cloned into *E. coli*, and the protein BglA was over expressed and purified to apparent homogeneity. BglA is 65 kDa (574 amino acids) with an N-terminal amino acid sequence predicted by the gene sequence, suggesting that the enzyme is cytoplasmic. The purified enzyme exhibited optimal activity with *p*NPG, cellobiose, and higher oligosaccharides of cellulose. The extremely thermostable wild type and recombinant β-glucosidases, from *Pyrococcus furiosus*, served as catalysts for the biotransformation of new glucoconjugates at elevated temperatures (Fischer et al., 1996).

The distribution pattern of marine bacteria in Taiwan Strait has been investigated and reported by Zheng et al. (2002). The study was carried out in order to have better understanding of the β-GlcA, as well as its relation to marine bacterial biomass, productivity and environmental factors in Taiwan strait by using fluorogenic model substrate (FMS) technique.

2.13 Applications of β-Glucosidase

The hydrolytic activity of fungal originated β-glucosidase is exploited in several biotechnological processes: (i) for increasing the rate and extent of saccharification of cellulosic material by hydrolyzing the cellobiose which is inhibitor of cellulases (Woodward and Wiseman, 1982), (ii) for flavour enhancement in fruit juices and derived beverages through the liberation of flavour compounds from glucosidic precursors (Shoseyov et al., 1990), (iii) for debittering of citrus juices through the hydrolysis of pruning (Roitner et al., 1984) and (iv) for detoxification of cassava through the hydrolysis of cyanogenic glycosides (Birk et al., 1996). The major applications of β-glucosidase detailed below.

2.13.1 β-glucosidase in flavour industry

Microbial β-glucosidases are widely used in the flavour improvement industry. β-glucosidases are key enzymes in the release of aromatic compounds from glucosidic precursors present in fruits and fermentating products (Gueguen et al., 1995). Indeed, many natural flavour compounds, such as monoterpenols, C-13 norisoprenoids, and skimate-derived compounds, accumulate in fruits as flavourless precursors linked to mono or diglycosides and require liberation by enzymatic or acidic hydrolysis (Vasserot et al., 1995; Winterhalter et al., 1997). Microbial β-glucosidases can also be used to improve the organoleptic properties of citrus fruit juices, in which the bitterness is in part due to a glucosidic compound, naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside), that is hydrolyzed by a α-rhamnosidase and a β-glucosidase (Roitner et al., 1984). Monoterpenols in grapes (e.g., linalol, geraniol, nerol, citronelol, α-terpineol and linalol oxide) are linked to diglucosides, which contribute to the flavor of wine (Gunata et al., 1994). The

enzymatic hydrolysis of these compounds requires a sequential reaction, which produce monoglucosides. Subsequently, monoglucosides are hydrolyzed by the action of β-glucosidases. Endogeneous β-glucosidases from grape are not sufficient to process the hydrolysis of monoterpenyl-glucosides. The grape enzymes display limited activity towards these glucosides and a large fraction of the aromatic compounds remains unprocessed in mature fruit. The addition of glucose-tolerant exogenous β-glucosidase isolated from fungi (e.g. *A. oryzae*) was shown to improve the hydrolysis of glucoconjugated aromatic compounds and enhance wine quality (Riou et al., 1998).

2.13.2 β-glucosidases in Ethanol production

Biomass is the earth's most attractive alternative among fuel sources and most sustainable energy resource and is reproduced by the bioconversion of carbon dioxide. Ethanol produced from biomass is today the most widely used biofuel when blended with gasoline (e.g., E10 [gasoline containing 10% ethanol]). As the carbon dioxide released by combustion is recycled into biomass, the use of biofuels can significantly reduce the accumulation of greenhouse gas. Of the biomass materials, cellulose, a major component of the cell wall of plants, is the most abundant and renewable carbohydrate. In recent years, it has been proposed that waste cellulosic biomass could be used as a cheap and readily available sugar to replace starchy materials in fermentation. A process of this kind is needed to solve environmental problems such as global warming and to construct a society independent of fossil fuels.

Ethanol is commonly derived from corn grain (starch) or sugar cane (sucrose) (Gray et al., 2006). Sucrose can be fermented directly to ethanol, but starch is hydrolyzed to glucose before it can be fermented, generally by *Saccharomyces*

cerevisiae (Sorensen et al., 2005). Ethanol fermentation from starch can be improved by utilizing better enzymes and strains and preferably hydrolyze the starch from whole grains without a chemical pre-treatment and with simultaneous liquefaction, saccharification and fermentation (Bhargava et al., 2005). Simultaneous saccharification and fermentation (SSF) is an economically favourable technology for bio-ethanol production from lignocellulosics. Compared to separate hydrolysis and fermentation (SHF) the final ethanol yield is higher, less energy is required and production costs are minimized (Alfani et al., 2000; Kadar and Reczey, 2004).

However, the starch biomass material, as well as sugar cane, is limited and for renewable biofuel to be able to compete with fossil fuel, a cost-efficient process of an even more abundant renewable resource is needed. Agricultural and forest biomass are available in large enough quantities to be considered for large-scale production of alcohol based fuels. Urban wastes are an additional source of biomass; it is estimated that cellulose accounts for 40% of municipal solid waste (Tomme et al., 1995). Cellulose-based products can be competitive with products derived from fossil resources provided processing costs are reduced (Wyman, 2003).

The fermentation step is usually performed by *S. cerevisiae* or *Zymomonas mobilis*, but this can be a disadvantage, since the temperature has to be reduced from the hydrolysis step, which is better performed at higher temperature, at least 50°C (Stenberg et al., 2000). Thermoactive yeast, *Kluyveromyces marxianus*, active up to 50°C, performed equally well as *S. cerevisiae* (Singh et al., 1998), but even higher temperatures are desired. The fermentation can also be done by thermo-active anaerobic bacteria. Some thermophiles isolated from Icelandic hot springs performed quite well in ethanol production from lignocellulolytic hydrolysates (Sommer et al., 2004).

Enzymatic cellulose hydrolysis to glucose is today predominantly carried out by fungi, e.g. *Trichoderma* sp, *Penicillium* sp and *Aspergillus* sp (Galbe and Zacchi, 2002). Researchers have also developed ethanologenic bacteria (Guedon et al., 2002; Wood and Ingram, 1992; Zhou and Ingram, 2001) and yeast (Cho and Yoo, 1999; Fujita et al., 2002) that can produce ethanol from cellulosic materials. The recombinant *Klebsiella oxytoca* SZ21 developed by Zhou et al. was able to directly produce ethanol from amorphous cellulose (Zhou and Ingram, 2001). To reduce the cost of ethanol production from cellulosic biomass, recombinant microorganisms with the ability to ferment cellulose have been developed by many researchers (Cho and Yoo, 1999; Guedon et al., 2002; Zhou and Ingram, 2001). These whole-cell biocatalysts with the ability to degrade cellulose have several advantages: conversion of cellobiose and glucose, which inhibit cellulase and β-glucosidase activities; lower sterilization requirements, as glucose is immediately taken up by cells and ethanol is produced; and a single reactor (Medve, 1997; Teeri et al., 1998). There are reports regarding the simultaneous and synergistic saccharification and fermentation of amorphous cellulose to ethanol with the use of only a recombinant yeast strain co-displaying three types of cellulolytic enzyme, namely, *T. reesei* EGII and CBHII and *A. aculeatus* BGL1 (Fujita et al., 2004). The production of ethanol from wheat bran by using a recombinant *E. coli* (strain FBR5) that can ferment mixed multiple sugars to ethanol was also reported (Dien et al., 2000). *S. cerevisiae* has recently been modified with genes from a fungal xylose pathway and from a bacterial arabinose pathway, which resulted in a strain able to grow on both pentose and hexose sugars with improved ethanol yields (Karhumaa et al., 2006).

It has been shown that cellulases from different origins, with different temperature optima ranging from mesophilic to thermophilic, can be matched together and still exhibit substantial synergism in the degradation of cellulosic material

(Sheehan and Himmel, 1999). An endoglucanase from *Acidothermus cellulolyticus*, which was fused to *T. reesei* cellobiohydrolase and expressed in *T. reesei* was used for enhancing saccharification yields (Bower et al., 2005). Endoglucanase and cellobiohydrolase activity is however not sufficient, as the degradation product (cellobiose) inhibited the former enzymes and blocked further depolymerization of the cellulose. It is suggested that to solve this product inhibition, β-glucosidases have to be added, or engineered into production strains that are able to ferment cellobiose and cellotriose to ethanol (Wood and Ingram, 1992).

2.13.2.1 Ethanol production in India

India imports nearly 70% of its annual crude petroleum requirement, which is approximately 110 million tons. The prices are in the range of US$ 50-70 per barrel, and the expenditure on crude purchase is in the range of Rs.1600 billion per year, impacting in a big way, the country's foreign exchange reserves (Oil Prices touched a record high of $76 per barrel). The petroleum industry now looks very committed to the use of ethanol as fuel, as it is expected to benefit sugarcane farmers as well as the oil industry in the long run. Ethanol (Fuel Ethanol) can also be produced from wheat, corn, beet, sweet sorghum etc. Ethanol is one of the best tools to fight vehicular pollution, contains 35% oxygen that helps complete combustion of fuel and thus reduces harmful tailpipe emissions. It also reduces particulate emissions that pose a health hazard (http://www.ethanolindia.net/).

Demand for ethanol has increased substantially due to its use in gasoline as a fuel oxygenate in high-octane fuels (Lyons, 2004). Ethanol demand is anticipated to continue to increase in response to the declining use of methyl tetrabutyl ether (MTBE); a petroleum-based fuel oxygenate that has been shown to be a groundwater

pollutant and a possible carcinogen (Lyons, 2004).

2.13.3 Other applications

Levels of serum glycosylhydrolases, β-glucosidase and β-galactosidase, have been used as sensitive markers in post-diagnosis of hepatic ischemia-reperfusion injury and recovery, because there is a marked increase in the concentration of these enzymes following liver injury. The alterations in the levels of lysosomal β-glucosidases, β-galactosidases, and β-glucuronidases have been used as a diagnostic tool to detect premalignant and malignant lesions of oral mucosa in hamsters, as activities of these enzymes were elevated markedly only in the carcinoma stage (Balasubramanian et al., 1996). The H-antigen of *Histoplasma capsulatum*, secreted a 120-kDa glycoprotein, was found to exhibit β-glucosidase activity. It could elicit cell mediated immunity and humoral immunity and thus was used for serodiagnosis of histoplasmosis (Fisher and Woods, 2000).

Chapter 3

MATERIALS AND METHODS

3.1 Microorganism

Fungal strains isolated from the sediment and seawater of the coastal areas of Kerala is available in the culture collection of the Microbial Technology Laboratory of Department of Biotechnology, Cochin University of Science and Technology, India, were used.

3.2 Medium

The cultures were grown at room temperature (28±2°C; RT) for 5 days in GMY (Glucose, Maltose and Yeast extract) fungal medium with the following composition (w/v): glucose-1%, malt extract-1%, NH_2NO_3-0.1%, $MgSO_4$-0.05%, yeast extract-0.1%, NaCl-1%, and pH-5.5. Cultures were maintained on the slants of the same media at 4°C, and sub cultured periodically.

The medium used for the preliminary screening and submerged fermentation was Czapek-Dox (CD) minimal medium with the composition of $NaNO_3$- 0.2, K_2HPO_4 - 0.1, $MgSO_4.7H_2O$- 0.05, KCl- 0.05, $FeSO_4$ -0.001, $ZnSO_4$ (1%)- 0.1, $CuSO_4$ (0.5%)- 0.1, NaCl- 1, pH- 7.8 and 1% CMC as sole carbon source.

3.3 Screening and selection of potential fungal strain for the Cellulase production

Potential strain that produce cellulase was screened and selected from among a large collection of isolates by subjecting them to preliminary and secondary screening.

3.3.1 Inoculum Preparation

For the selected fungal strains, conidial inoculum was prepared from a freshly raised 8 days old GMY agar slant by dispersing the spores in 0.1% Tween 80 prepared in sterile physiological saline. One millilitre of this spore suspension (22 x 10^8 cfu/ml) was used as inoculum.

3.3.2 Preliminary Screening

Preliminary screening of the fungal isolates for cellulase production was carried out using plate assay as detailed below.

The cultures were grown on the Czapek-Dox (CD) mineral agar medium containing 1% Carboxymethyl cellulose (CMC) as the sole carbon source. The prepared agar plates were spot inoculated with 8 day old fungal spores and incubated for 5 days at room temperature. After the incubation, the agar medium was flooded with an aqueous solution of Congo red (0.1%) for 15 min. The stain solution was then poured off, and the plates were further treated with 1M NaCl for 15 min. The visualized zones of hydrolysis was stabilized by flooding the agar with 1M HCl, which change the dye colour to blue and inhibits further enzyme activity (Teather & Wood 1982).

The cultures were selected on the basis of the presence of a clearing zone around the colony.

3.3.3 Secondary Screening

Secondary screening was carried out in shake flasks containing 100ml CD minimal medium. Selected cultures were inoculated with prepared inoculum (sec 3.3.1) and the flasks were incubated at room temperature (28±2°C) on a rotary shaker, at 150 rpm. After 120 h of incubation the fungal biomass was removed by centrifugation and the clarified supernatant was used as the source of enzyme. The samples were taken and their β-glucosidase activity was assayed as detailed under section 3.5.1.1. The cellulase enzyme profile of the selected culture was also checked as detailed under section 3.5.1.1 and 3.5.1.2.

3.4 Identification of the fungal strain

The identification of the selected strain was done at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and a molecular approach for identification was also made based on 28S ribosomal DNA sequence homology (O' Donnell, 1993).

3.4.1 Molecular approach for strain identification

3.4.1.1 Genomic DNA isolation from *A. sydowii* BTMFS 55

The mycelia mat of *A. sydowii* was harvested from GMY medium, washed with sterile distilled water and blot dried. Approximately 1g of mycelia was frozen in liquid nitrogen and disrupted into fine powder using a sterile mortar and pestle. This powder was transferred into a 50 ml capacity centrifuge tube containing 16 ml of pre

heated (65°C) CTAB buffer and incubated at 65°C for 30 min. in a water bath followed by incubation at room temperature. Equal volumes of chloroform: isoamyl alcohol (24:1) was added to the tubes and mixed thoroughly to form an emulsion. Subsequently it was centrifuged at 10,000 rpm for 10 min. and the aqueous layer was saved into a new centrifuge tube using cut tip. The chloroform: isoamyl alcohol extraction was repeated twice and the aqueous phase was collected carefully. Sodium acetate (pH 5.2) was added to the aqueous phase at a final concentration of 300 mM and DNA was precipitated by the addition of 2/3 volumes of ice-cold isopropanol. The DNA strands were spooled out gently with the aid of a glass rod, washed with 70% ethanol, air dried briefly, and then dissolved in Milli Q water (Millipore, USA).

3.4.1.2 Solutions and Buffers

All buffers and solutions were prepared in deionised water unless otherwise mentioned.

Chloroform: Isoamyl alcohol	24:1
Sodium acetate (pH 5.2)	3 M
Ethidium bromide	10 mg/ml

TAE (50X)

EDTA – 0.5 M (pH 8.0)	100 ml
Glacial Acetic acid	57.1 ml
Tris	242 g

TE buffer

EDTA (pH 8.0)	1 mM
Tris-HCl (pH 8.0)	10 mM

CTAB buffer (DNA extraction buffer)

Tris-HCl (pH 8.0)	100 mM
NaCl	1.4 M
EDTA (pH 8.0)	20 mM
CTAB	2% (w/v)

DNA Loading buffer (Type III)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol	30%

3.4.1.3 PCR amplification of 28S rRNA gene

Molecular identification of the selected fungal strain was done using a primer pair for 28S rDNA, NL1F (Table 3.1). A portion of the 28S rRNA gene was amplified from the genomic DNA (O'Donnell, 1993).

Table 3.1 Primer sequence used for the molecular identification of the fungal strain

Primer	Sequence (5'-->3')	Amplicon	Reference
NL1F	GCATATCAATAAGCGGAGGAAAAG	~700 bp	O'Donnell, 1993
NL4R	GGTCCGTGTTTCAAGACGG	"	"

The fungal DNA was prepared, and the resulting DNA was used as a template to amplify the 28S ribosomal DNA with the primers, NL1F and NL4R (Table 3.1) PCR was carried out using 1X PCR buffer, 1µl Taq polymerase (Genei, Bangalore), and 1µl each of the NL1F and NL4R primers. The PCR reaction was carried out for 35 cycles of denaturation at 94°C for 30 sec., annealing at 62°C for 30 sec., and extension at 72°C for 1 min using an Eppendorf Master Cycler personal.

Products after PCR amplification were subjected to sequencing, followed by homology analysis. Nucleotide sequences were determined by the ABI Prism 310 genetic analyzer using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequence was established by comparing the sequences obtained with the 28S rRNA gene sequences in the database using BLAST software (Altschul et al., 1990) and the phylogenetic tree was constructed using the dendrogram implemented in CLUSTAL W (http://align.genome.jp).

3.4.1.4 Agarose gel electrophoresis (Sambrook et al., 2001)

(i) Agarose gel with a concentration of 0.8% was prepared for electrophoresis of the PCR products.

(ii) 5µl of the PCR products was loaded on to the gel and electrophoresed at 80 volts until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. The 500 bp DNA ladder (Genei, Bangalore) was used as the marker.

(iii) The gel was stained in freshly prepared 0.5mg/ml Ethidium bromide solution for 10 min.

(iv) The gel was viewed on a UV- Transilluminator, and image captured with the help of Digi Doc system (Bio-Rad).

3.5 Enzyme production by *A. sydowii* under Submerged Fermentation (SmF)

β-Glucosidase production by the selected fungal strain under submerged fermentation (SmF) was optimized towards maximal production. SmF studies were conducted in Czapek-Dox broth and the various bioprocess parameters that influence enzyme production were optimized.

3.5.1 Analytical Methods

3.5.1.1 Enzyme Assay

β-glucosidase activity was assayed by a reaction mixture containing 1ml of 0.1% *p*-Nitrophenyl β-D-glucopyranoside (*p*NPG) in 0.1 M Glycine-NaOH buffer (pH 8.0), and 0.5ml of diluted enzyme solution (Endo et al, 2001). The reaction was carried out at 50^0C for 30min.and stopped by addition of 2ml of cold 1M Na_2CO_3. *p*-Nitrophenol (*p*NP) released into the solution was quantified at 410nm. One unit of enzyme activity was defined as the amount of enzyme producing 1μmol of a *p*-NP per ml per minute.

CMCase activity was determined by measuring reducing sugar by the DNS method with glucose as the standard (Miller, 1959). The reaction mixture consisting of 1ml of 1% CMC in 50mM Glycine-NaOH buffer (pH 10.0) and 0.5ml of crude enzyme solution was incubated for 30 min. at 50°C. One unit of CMCase activity was defined as the quantity of enzyme required to catalyse the formation of 1μm of glucose per minute.

Filter paper activity was determined by measuring reducing sugar by the DNS method with glucose as the standard (Miller, 1959). The reaction mixture consisting of 50mg of Whatman filter paper (1x 6 cm), 1ml 50mM Glycine-NaOH buffer (pH

10.0) and 1ml of diluted enzyme solution was incubated for 1hour at 50°C. 3ml of DNS reagent was added to terminate the reaction and OD at 540nm was read. One unit of FPA was defined as the quantity of enzyme releasing 1μm of glucose from filter paper per ml per hour.

3.5.1.2 Protein Estimation

Protein content was determined according to the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as the standard and was expressed as milligram protein per millilitre (mg/ml).

Reagent

(a) 2% solution of sodium carbonate in 0.1 N NaOH

(b) 0.5% solution of cupric sulphate in distilled water

(c) 1% solution of sodium potassium tartrate in distilled water

(d) *Working reagent: To 100 ml of solution (a), 1ml each of solution (b) and solution (c) were added.

(e) *1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water

*Prepared fresh before use.

Estimation

An aliquot of 0.2ml of sample was made up to 2 ml with distilled water and 5 ml of freshly prepared working reagent (d) was added. The contents were mixed thoroughly and incubated for 10 minutes at RT. 0.5ml of solution (e) was added and incubated for 30 minutes followed by measuring the absorbance at 750 nm in a UV-Visible spectrophotometer (Shimatzu, Japan).

3.5.1.3 Dry weight estimation

Mycelial dry weight was determined after centrifugation of fermentation broth at 10,000 rpm for 10 min. The pellet was washed twice with physiological saline, centrifuged and dried at 80°C to constant weight. The dry biomass is expressed in dry cell weight (Domingues et al. 2001).

3.5.2 Optimization of bioprocess variables for β-glucosidase production by *A. sydowii* under Submerged Fermentation (SmF)

Various process parameters that influence β-glucosidase production by *A. sydowii* under SmF were evaluated to effect maximal enzyme production using CD medium. Enzyme production was carried out in 250ml Erlenmeyer flasks containing 100ml CD medium. 1% spore suspension prepared as mentioned above was used as the inoculum and the cultures were incubated at RT for 120 h at 150 rpm on a rotary shaker, unless otherwise specified. The biomass was removed by filtration followed by centrifugation and the supernatant was used as the crude enzyme for assay.

Strategy adopted for the optimization was to evaluate the effect of each parameter for maximal β-glucosidase production, and incorporate the same variable at its optimized level in the subsequent experiment while evaluating the next parameter for its optimal level (Sreeja et al., 2006)

The parameters studied included the following in the sequential order: (i) various carbon sources as substrate at 0.5% level (w/v) (CMC, cellulose powder, cellobiose, glucose, xylose, lactose, maltose, mannose, arabinose and sucrose), (ii) initial pH content of the medium (3.0- 11.0), (iii) incubation temperature (25, 30°C (RT) & 35°C), (iv) additional carbon source at 0.5% (w/v) level (starch, xylan,

sorbitol, glycerol, cellobiose, rhamnose, salicin, fructose, galactose, mannitol, and xylose), (v) additional nitrogen sources at 0.5% (w/v) (organic nitrogen sources viz., soybean meal, yeast extract, malt extract, beef extract, peptone, casein, and urea; inorganic nitrogen sources viz., ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium dihydrogen phosphate and di-ammonium hydrogen phosphate), (vi) additional NaCl (0.5-5% (w/v) level), (vii) effect of Tween 80 (0-0.5%), and (viii) inoculum concentration (0.5-5%). The culture was harvested after 5 days of incubation on a shaker at 150 rpm. A time course experiment was conducted using the various optimized levels of the variables.

3.5.3 Time course study under optimised conditions

Time course experiment was conducted with the optimized condition determined after optimization of various variables. The conditions selected included the following:

- Czapek-Dox minimal media
- 2 % glucose
- pH 9.0
- 0.5% ammonium sulphate
- 0.5% NaCl
- 4 ml inoculum with spore count of 22 x 10^8cfu/ml
- Incubation at room temperature (30°C)

The inoculated flasks were incubated for 14 days and samples were drawn on each day and assayed to record the enzyme activity, protein content and specific activity.

3.6 Enzyme production by *A. sydowii* under Solid State Fermentation (SSF)

Bioprocess for production of β-glucosidase by *Aspergillus sydowii* under Solid State Fermentation was optimised using wheat bran as solid substrate as described below.

3.6.1 Solid substrate medium

Commercially available wheat bran (WB) was used as the solid substrate medium for β-glucosidase production. Wheat bran, sorted into varying particle sizes by sieving through mechanical sieve of different mesh size, was dried in sunlight for one day and stored in airtight containers.

Ten grams of WB, with particle size <425 μ, taken in 250ml Erlenmeyer flasks was moistened with distilled water, so that the final moisture content was 60% (w/v) after inoculation. The contents were mixed thoroughly, autoclaved at 121°C for 30 minutes and cooled to room temperature. This was the general procedure followed for the solid substrate preparation for SSF studies unless otherwise specified.

3.6.2 Inoculum Preparation

The spores were scraped from a freshly raised 8-day-old ME agar slant using an inoculation needle under strict aseptic conditions and the spore suspension obtained was adjusted to a concentration of 22 x 10^8 cfu/ml using physiological saline containing 0.1% Tween 80. One millilitre of this prepared inoculum was used for inoculating each flask unless otherwise mentioned.

3.6.3 Inoculation and Incubation

The WB medium (10 g) prepared in 250 ml conical flasks was inoculated with 1ml of the prepared inoculum, mixed thoroughly and incubated in a slanting position in the BOD incubator at 30°C. The humidity inside the incubator was maintained using distilled water. At intervals of 24hrs, the contents inside the flask were mixed thoroughly by mechanical shaking. After the desired period of incubation (up to 9 days), the enzyme was extracted from the moldy wheat bran (MWB).

3.6.4 Extraction and recovery of enzyme

β-Glucosidase from the fermented WB was extracted by the simple contact method of extraction using distilled water as extractant (Suresh and Chandrasekaran, 1999). Ten volumes of distilled water per gram MWB (based on initial dry weight of the substrate) was added to the flask and the extraction were performed by agitation at room temperature in a rotary shaker for 30 min at 150 rpm. The slurry was then squeezed through cheese cloth (Prabhu and Chandrasekaran, 1996) and clarified by centrifugation at 10,000 rpm at 4°C for 15 min. The clear supernatant was used as crude enzyme for assay of β-glucosidase.

3.6.5 Analytical Methods

3.6.5.1 Estimation of dry weight of the substrate (Wheat bran)

One gram of moistened wheat bran was dried in oven at 105°C for 2hrs and weighed. The drying was continued until a constant weight was obtained for WB. Dry weight of the substrate was calculated by subtracting the moisture content from the wet weight.

3.6.5.2 Enzyme Assay

β-glucosidase activity was performed as detailed under sections 3.5.1.1 Enzyme activity was expressed as Units per gram Initial Dry Substrate (U/gIDS).

3.6.5.3 Protein Estimation

Protein content was estimated as described under the section 3.5.1.2 and was expressed as milligram per gram of Initial Dry Substrate (mg/gIDS).

3.6.5.4 Specific Activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/ mg protein.

$$\text{Specific activity} = \frac{\text{Enzyme activity (U/ gIDS)}}{\text{Protein (mg/gIDS)}}$$

3.6.6 Optimisation of Bioprocess variables for β-glucosidase production by *A. sydowii* under Solid State Fermentation (SSF)

The impact of various process parameters influencing β-glucosidase production by *A. sydowii* under SSF were optimised towards maximal enzyme production. The parameters optimized included initial moisture content of the solid substrate medium, incubation time, particle size, initial pH of the medium, incubation temperature, additional carbon and nitrogen sources, sodium chloride concentration and inoculum concentration. Strategy adopted for the optimization was to evaluate

the effect of each parameter on β-glucosidase production at its optimum level for maximal β-glucosidase production, and incorporate the same variable at its optimized level in the subsequent experiment while evaluating the next parameter for its optimal level (Sreeja et al., 2006)

Solid substrate medium preparation, inoculum preparation, inoculation and incubation and extraction of enzyme were done as described earlier. In each case, samples were used for assaying the enzyme activity, protein content and specific activity.

3.6.6.1 Initial moisture content

Impact of initial moisture content on β-glucosidase production was evaluated by adjusting the moisture content of the WB to various levels ranging from 30% to 90% and assaying the enzyme yield. Moisture content of the WB medium was adjusted with varying volume of distilled water such that after autoclaving, the initial moisture content before inoculation was 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% (v/w).

3.6.6.2 Incubation time

Optimal incubation time for maximal enzyme production was determined by incubating the inoculated media for a total period of 9 days and analyzing the samples at a regular interval of 24 h for enzyme activity, protein content and specific activity.

3.6.6.3 Particle size of the substrate

Optimal particle size of WB that support maximal enzyme activity was evaluated using the culture grown in WB medium of different particle size i.e., <425µ, 425µ, 600µ and WB without sieving.

3.6.6.4 Incubation temperature

Optimal incubation temperature for maximal enzyme production was determined by incubating the inoculated WB media at 25°C, 30°C (RT) and 35°C and determining the enzyme activity, protein content and specific activity.

3.6.6.5 Initial pH of the medium for enzyme production

The effect of initial pH of the WB medium that support maximal enzyme production was determined by subjecting the fungal strains to various pH levels 5 - 10 adjusted in the moistening medium with various buffers including Acetate buffer (pH 5), Phosphate buffer (pH 6-8) and Glycine-NaOH buffer (pH 9-10).

3.6.6.6 Additional Carbon source

The effect of additional carbon sources on enzyme synthesis by the fungus was evaluated by incorporating various sugars at a concentration of 1% (w/w) level in the WB medium. The carbon sources tested included glucose, xylose, fructose, sucrose, maltose, cellobiose, CMC, and cellulose. Further, the optimal concentration of the additional carbon source, which supported maximal enzyme production, was also studied by providing the carbon source at various concentrations in the medium.

3.6.6.7 Additional Nitrogen source

Effect of additional nitrogen sources on β-glucosidase production was evaluated using both organic and inorganic nitrogen sources at a concentration of 1% (w/w) level in the WB medium. The nitrogen sources analyzed included organic substrates such as soybean meal, malt extract, yeast extract, peptone and inorganic compounds such as ammonium sulphate, ammonium nitrate, ammonium chloride and di-ammonium hydrogen phosphate. The optimal concentration of the additional nitrogen source which supported maximal enzyme production was also studied by providing the nitrogen source at various concentrations in the medium.

3.6.6.8 Inoculum concentration

Optimal inoculum concentration that supports maximal enzyme production by fungus during SSF was assessed by using different concentrations of conidial inoculum of increasing concentration of 10-50% (v/w) (section 3.5.2).

3.6.6.9 Sodium chloride concentration

The effect of NaCl on enzyme production during SSF by the strain was studied by the addition of sodium chloride to the WB media at various concentrations in the range of 0.5-5% (w/w).

3.6.6.10 Time course study

Time course experiment was conducted with the optimized conditions determined after optimization of various variables. The conditions selected included the following:

- Wheat Bran of particle size <425 μ
- 60% moisture content
- pH 8.0
- 0.5% CMC
- 0.5% peptone
- 0.5% NaCl
- 10% inoculum with spore count of 22 x 10^8cfu/ml
- Incubation temperature of 30°C

Inoculated flasks were incubated for 14 days and samples were drawn on each day and their enzyme activity, protein content and specific activity were evaluated.

3.7 Optimisation of bioprocess variables for β-glucosidase production by *A. sydowii* -Statistical approach

3.7.1 Plackett – Burman experimental design

Plackett–Burman design, an efficient technique for medium component optimization (Naveena et al., 2005), was used to pick factors that significantly influence β-glucosidase production, and to obtain a smaller, more manageable set of factors. It is proved to be a valuable tool for the rapid evaluation of the effects of the various medium components. Eleven variables representing ten nutritional components and initial pH of the medium were used. For each nutrient variable, a high (+) and a low (-) concentration was tested. The main effect of each variable upon β-glucosidase activity was estimated as the difference between both averages of measurements made at the high level (+) and at the low level (-). A 12-run Plackett Burman design (Plackett & Burman, 1946) was used to screen eleven factors, and the

experimental responses were analyzed by the method of least squares to fit the following first-order model:

$Y=\beta_0-\beta_1X_1+\beta_2X_2-\beta_3X_3+\beta_4X_4-\beta_5X_5+\ldots\ldots\ldots\ldots\ldots\beta_{11}X_{11}$, where Y was the predicted response (β-glucosidase yield), β_0, β_1, β_2 $\ldots\ldots\ldots\beta_{11}$ were the regression coefficients, and X_1, X_2,..... X_{11} were the coded levels of the independent variables (Table 3.1 & 3.2).

Table 3.1 Levels of factors chosen the Plackett and Burman design for β-glucosidase production by *A. sydowii*

Variable Code	Variable	Levels	
		+1	-1
A	Moisture (%)	70	50
B	Temperature (°C)	30	25
C	pH	9	7
D	Inoculum (%)	20	10
E	Particle size (μ)	600	425
F	CMC (g/L)	10	2
G	Peptone (g/L)	10	1
H	$(NH_4)_2SO_4$ (g/L)	10	1
J	NaCl (g/L)	7.5	2.5
K	Tween 80 (%)	1	0.5
L	Incubation time (h)	120	72

Table 3.2 Plackett - Burman design matrix for β-glucosidase production by *A. sydowii* under SSF on Wheat bran

	Coded variable level										
Run	***A***	***B***	***C***	***D***	***E***	***F***	***G***	***H***	***J***	***K***	***L***
1	-1	-1	-1	-1	+1	+1	-1	-1	+1	+1	-1
2	-1	+1	-1	+1	-1	-1	+1	-1	+1	-1	-1
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
5	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
6	-1	-1	-1	-1	-1	+1	+1	-1	+1	+1	+1
7	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
8	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
9	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
11	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
12	+1	-1	+1	-1	-1	-1	+1	-1	-1	+1	-1

Plackett- Burman design was applied for the statistical optimization of the production of β-glucosidase under solid state fermentation by *A. sydowii*. Wheat bran (10g) taken in each flasks was added with each of the nutrients as per the matrix design at the corresponding pH and sterilized at 121°C for 30 min. Inoculated flasks were incubated at the appropriate temperatures and the enzyme was extracted after 72 and 120 h, and the enzyme activity was determined.

Statistical significance of the model equation was determined by Fisher's test, and the proportion of variance explained by the model was presented by the multiple

coefficient of determination, R^2 value. Design Expert (Version 7.1.0; STATEASE Inc., USA) was used for the matrix design and interpretations.

A validation run was performed to confirm the results after getting the Plackett-Burman results.

3.8 Enzyme Purification

β-glucosidase produced by A. *sydowii* under SSF was purified employing standard protein purification procedures which included ammonium sulphate precipitation, followed by dialysis and ion exchange chromatography as detailed below. Purity of the protein was checked by SDS – PAGE. All the experiments were done at 4°C unless otherwise specified.

3.8.1 Ammonium sulphate fractionation

Ammonium sulphate precipitation was done according to Englard and Seifter (1990). Ammonium sulphate required for precipitating the β-glucosidase enzyme was optimized by its addition at varying levels of concentrations (0-20%, 20-40%, 40-60%, 60-80% and 80-90% saturation) to the crude extract. The resulting precipitate was collected by centrifugation at 10,000 rpm for 10 min. and dissolved in the smallest possible volume of 0.01 M phosphate buffer (pH 8.0), and then dialyzed overnight against the same buffer.

3.8.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against a buffer in order to remove the ammonium sulphate from the precipitate, as detailed below.

(i) The precipitated protein was resuspended in minimum quantity of 0.01M phosphate buffer (pH 8.0).

(ii) Dialysis was conducted in the pretreated dialysis tube (cut off value 12kDa) against 0.01M solution of phosphate buffer of pH 8.0 for 24 h, at 4°C with 6 changes of buffer and assayed for β-glucosidase activity, protein content and specific activity as per the methods described under sections 3.5.1.1 and 3.5.1.2.

3.8.2.1 Pre-treatment of Dialysis Tube

Dialysis tube was treated to remove the humectants and protectants like glycerine and sulphur compounds present in it, and to make the pores of the tube more clear. The treated tube retain most of the proteins of molecular weight 12kDa or greater. The method for the treatment (Sigma Aldrich) is as follows.

(a) The tube was washed in running water for 3-4 h

(b) Dipped in 0.3% (w/v) solution of sodium sulphide at 80°C for 1 min.

(c) Washed with hot water (60°C) for 2 min.

(d) Acidified with 0.2% (v/v) H_2SO_4

(e) Rinsed with hot water (60°C)

3.8.3 Ion Exchange Chromatography

Ion exchange chromatography was done according to Rossomando (1990). Active fraction obtained after ammonium sulphate fractionation followed by dialysis was further purified by ion exchange chromatography using the anion exchanger DEAE Sepharose as the column material.

3.8.3.1 Purification using DEAE Sepharose column

DEAE Sepharose was carefully packed in column without trapping any air bubble. The column was equilibrated with Phosphate buffer of pH 8.0 (0.1M) for overnight.

4 ml of dialyzed sample with protein content of 8.86 mg/ml was applied to the DEAE Sepharose column with height 25 cm; pre-equilibrated with 0.1 M phosphate buffer (pH 8.0). After the complete entry of sample to the column, the unbound proteins were washed with the same buffer until the OD_{280} reached near zero. Stepwise elution was done at a flow rate of 10ml/h using 0-0.5 M NaCl in the same buffer. 5 ml fractions were collected and protein content was estimated by measuring the absorbance at 280nm. The active fractions were pooled and dialyzed overnight against phosphate buffer (0.1 M, pH 8.0); assayed for β-glucosidase activity, protein content and specific activity. Yield and fold of purification was also calculated. This enzyme solution was the purified β-glucosidase preparation used for subsequent studies.

3.8.4 Calculation of yield of protein, yield of enzyme activity and fold of purification

The yield of protein, yield of enzyme activity and fold of purification were calculated as below.

$$\text{Yield of protein} = \frac{\text{Total protein content of the fraction x 100}}{\text{Total protein content of the crude extract}}$$

$$\text{Yield of activity} = \frac{\text{Total activity of the fraction x 100}}{\text{Total activity of the crude extract}}$$

$$\text{Fold of purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}$$

3.9 Characterization of purified enzyme

The purified β-glucosidase was further characterized for their molecular mass determination, zymogram profile and enzyme kinetics as described in the following sections.

3.9.1 Electrophoretic Methods

Active fractions collected after ion exchange chromatography were subjected to electrophoresis by Native-PAGE and SDS-PAGE in a 10% polyacrylamide gel according to the method of Laemmlli (1970) for the purpose of checking purity and molecular mass determination. SDS-PAGE of purified enzyme was carried out under both reductive and non-reductive conditions, i.e., with and without β-mercaptoethanol respectively.

3.9.1.1 Reagents for polyacrylamide gel electrophoresis

1) **Stock acrylamide solution** (30:0.8)

Acrylamide (30%)	-	60 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water (DW)	-	200 ml

Stored at 4°C in amber coloured bottle.

2) **Stacking gel buffer stock** (0.5 M Tris-HCl, pH 6.8)

Tris buffer - 6g in 40 ml DW

Titrated to pH 6.8 with 1M HCl (~48 ml) and made up to 100ml with DW. Filtered with Whatman No.1 filter paper and stored at 4°C

3) **Resolving gel buffer stock** (3M Tris-HCl, pH 8.8)

Tris buffer - 36.3 g

Titrated to pH 8.8 with 1M HCl (~48 ml) and made up to 100ml with DW. Filtered with Whatman No.1 filter paper and stored at 4°C

4) **Reservoir buffer for Native-PAGE** (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g

Dissolved and made up to 1L with DW. Prepared in 10X concentration and stored at 4°C

5) Reservoir buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g
SDS	-	1 g

Dissolved and made up to 1L with DW. Prepared in 10X concentration and stored at 4°C.

6) Sample buffer for Native-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol	-	10% (v/v)
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C.

7) Sample buffer for Reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C.

8) Sample buffer for Non-Reductive SDS PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C.

9) SDS (10%) - 1g in 10ml DW

10) Sucrose (50%) - 5 g in 10ml DW

(Autoclaved at 121°C for 10 minutes and stored at 4°C)

11) Protein Staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

12) Destaining solution

Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

13) Protein Marker for SDS–PAGE

High molecular weight marker mix of Genei, Bangalore, India was used. The composition of the marker mix is as given below.

Components		**MW in Dalton**
Myosin, Rabbit muscle	-	205,000
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	43,000
Carbonic anhydrase	-	29,000
Soybean Trypsin inhibitor	-	20,100
Lysozyme	-	14,300
Aprotinin	-	6,500
Insulin (α and β chains)	-	3,000

3.9.1.2 Native polyacrylamide gel electrophoresis (Native-PAGE)

3.9.1.2.1 Gel preparation

Resolving gel (10%)

Acrylamide: bis-acrylamide (30: 0.8)	-	10 ml
Resolving gel buffer stock	-	3.75 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	16.25 ml
TEMED	-	15µl

Stacking gel (2.5%)

Acrylamide: bis- acrylamide (30: 0.8)	-	2.5 ml
Stacking gel buffer stock	-	5 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.5 ml
TEMED	-	15 µl

Sample buffer (1X)

Native-PAGE sample buffer (2X)	-	1 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.9.1.2.2 Sample preparation

Sample for electrophoresis was prepared by the addition of 100µl of 1X sample buffer to lyophilized sample or 20µl of 2X sample buffer and 10µl of 50% sucrose to 30µl liquid sample, mixed well, and 30µl of the sample and 5µl of marker mix were loaded on to the well of the prepared gel.

3.9.1.2.3 Procedure

(a) The gel plates were cleaned and assembled.

(b) **Resolving gel** - All the components except APS were added in to a beaker, mixed gently, and finally added APS. The mixture was immediately poured into the cast and poured a layer of Butanol over the gel and allowed to polymerize at least for 1h.

(c) **Stacking gel**- All the components of stacking gel except APS were added into a beaker, mixed gently. APS was added finally and poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. This was allowed to solidify at least for 30 min.

(d) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native-PAGE.

(e) The gel was pre run for 1h at 80V.

(f) The protein sample was loaded to the gel.

(g) The gel was run at 80 V until the sample entered the resolving gel.

(h) When the dye front entered the resolving gel, the current was increased to 100 V.

(i) The run was stopped when the dye front reached 1cm above the lower end of the glass plate.

(j) The gel was removed from the cast and stained for at least 1hr in the staining solution.

(k) The gel was destained till the bands became clear and observed under a transilluminator.

3.9.1.3 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

Purified protein was subjected to SDS-PAGE. High molecular weight marker of Genei, Bangalore, India was used as standard.

3.9.1.3.1 Gel preparation

Resolving and Stacking gel was prepared as described in section 3.9.1.2.1

Sample buffer (1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1 ml
50% Sucrose	-	0.4 ml
DW	-	0.6ml

3.9.1.3.2 Sample preparation

Sample for electrophoresis was prepared by the addition of 100µl of 1X sample buffer to lyophilized sample or 20µl of 2X sample buffer and 10µl of 50% sucrose to 30µl liquid sample, mixed well, and 30µl of the sample and 5µl of the high molecular weight markers were loaded on to the well of the prepared gel.

3.9.1.3.3 Procedure

Procedure followed for electrophoresis and staining was essentially the same as described in section 3.8.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.9.1.4 Zymogram

Zymogram or the activity staining was performed by incubating the SDS polyacrylamide gel with a solution of 4-methylumbelliferyl β-D-glucopyranoside at a concentration of 0.01% in Glycine – NaOH buffer (pH 8.0) at 50°C for 10 min. The β-glucosidase activity was visualized under UV light (Parry et al., 2001).

3.9.1.5 Effect of Temperature on enzyme activity and stability

Temperature optimum for enzyme activity was determined by incubating the reaction mixture for 30 min. at different temperatures ranging from 30 to 80°C. The temperature stability of the purified enzyme was determined by incubating the enzyme solution at temperatures in the range of 30-80°C and the enzyme was assayed and the residual activity was estimated at 30 min, 1 h, 12 h and 24 h of incubation. Enzyme activity of the sample kept at 4°C was taken as control.

3.9.1.6 Effect of pH on enzyme activity and stability

The effect of pH on the activity of the purified enzyme was studied by performing the enzyme assay at various levels of pH in the range of 3-10. The buffer systems used included, Citrate buffer (pH 3 to 6), Phosphate buffer (pH 7 to 8), and Glycine-NaOH buffer (pH 9 to 10). Enzyme activity and relative activity were calculated. The pH stability of the enzyme was evaluated by incubating the enzyme

with the buffers described above for 24 h at 4°C and then carrying out the β-glucosidase assay.

3.9.1.7 Substrate specificity

The substrate specificity of the purified enzyme was evaluated by conducting enzyme assay with different substrates such as Cellobiose, Lactose, Maltose, Sucrose, Trehalose, Raffinose and *p*-nitrophenyl β-D-glucopyranoside (*p*NPG). All the substrates were prepared in 0.1M Glycine-NaOH buffer of pH 8.0 at a final concentration of 5mM and 0.5 ml of this solution was used for the assay. Depending on the substrate, the enzyme activity was determined under optimal conditions (50°C, pH 8.0, 30 min.) by measuring the release of reducing sugar (by DNS method, A_{540}) or the release of *p*-nitrophenol (A_{410}).

3.9.1.8 Effect of Substrate concentration and Kinetic studies

The effect of substrate (*p*NPG) concentration on the activity of the enzyme was determined by using different concentrations of *p*NPG (1-4 mM), in the reaction mixture and estimating the enzyme activity. The kinetic parameters such as the substrate concentration (K_m) at which the reaction velocity is half maximum and V_{max} were also calculated from the data using double reciprocal plots (Lineweaver & Burk, 1934).

3.9.1.9 Glucose inhibition

The inhibition of glucose on the enzyme activity was measured by adding various concentrations of glucose (50,100 and 150 mM) into the reaction mixture with *p*NPG (0.5, 1, 2.5 and 3 mM) as substrate. The inhibition constant (K_i) was obtained at the intersection of the line of a Dixon plot (Dixon, 1953).

3.9.1.10 Effect of various metal ions on enzyme activity

The effect of various metal ions on the enzyme activity was evaluated by incubating the enzyme along with different concentrations of various metal ions in the enzyme reaction mixture for 30 min followed by measuring the residual enzyme activity. The metals studied included 1, 5 and 10mM final concentrations of Calcium chloride, Cobalt chloride, Cupric sulphate, Ferric chloride, Magnesium chloride, Manganese chloride, Mercury chloride, Potassium chloride, Silver sulphate and Zinc sulphate which contribute the metal ions, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, K^{+}, Ag^{2+} and Zn^{2+} respectively.

3.9.1.11 Effect of chemical reagents on enzyme activity

Activity and stability in the presence of reducing agents were studied by incubating enzyme solution with 1, 5 and 10mM of β-mercaptoethanol, dimethyl sulfoxide (DMSO), EDTA, diethyl pyrocarbonate (DEPC) and SDS for 30 min and measuring the residual activity.

3.9.1.12 Effect of organic solvents on enzyme activity

Impact of various organic solvents on enzyme activity was evaluated by adding each solvent into the reaction mixture at the final concentration of 10, 20, 30, 40 and 50% (v/v). After incubation of the enzyme with each organic solvent for 30 min, enzyme was assayed for the residual activity.

3.9.1.13 Residual activity

Residual activity is the retained enzyme activity of the sample with respect to the enzyme activity of the control sample expressed in percentage.

$$\text{Residual activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the control (U/ml)}}$$

3.9.1.14 Relative activity

Relative activity is the percentage enzyme activity of the respective sample with respect to the sample for which maximum activity was obtained

$$\text{Relative activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the maximal enzyme activity obtained sample (U/ml)}}$$

3.10 Application studies

As part of application studies, an attempt was made to produce ethanol from rice straw and wheat bran using β-glucosidase and *Saccharomyces cerevisiae* (dry, commercially available). The approach included a pretreatment with H_2SO_4 followed by simultaneous saccharification with enzyme and fermentation by yeast.

3.10.1 Enzymes

Cellulase (Zytek) and β-glucosidase from *Aspergillus sydowii* BTMFS 55 were used; Cellulase contained 78 FPU/mL where β-glucosidase contained 1166 U/ml.

3.10.2 Substrate and pretreatments

Wheat bran and Rice straw were purchased from the local market and the rice straw cut into small pieces before the pretreatment. Five gram each of wheat bran and rice straw was taken in 250 ml Erlenmeyer flask containing 100 ml distilled water with 0.5% H_2SO_4 and subjected to autoclaving (121°C) for 30 min. After cooling the samples, decanted the water and washed the pre-treated substrate with sterile distilled water for 5 more times, to remove the residual glucose and other inhibitors.

3.10.3 Simultaneous saccharification and fermentation

Experiments were carried out in 250ml flasks with 100ml working volume. The substrate was added with 100ml yeast peptone medium (pH 5.0) with 0.5 % Cellulase, 2% β-glucosidase and 1% yeast (dry, w/v) were added, mixed and incubated at 37°C on an orbital shaker at 150 rpm for a total period of 120 h. Samples were taken aseptically and assayed for ethanol content.

3.10.4 Analytical Techniques

The glucose was estimated according to the dinitrosalicylic acid (DNS) method (Miller, 1959). Ethanol was estimated by gas chromatography (Chemito, India) in which a flame ionization detector and stainless steel column packed with Poropak were used. The column oven was operated isothermally at 155°C, and the detector and injection port were kept at 255 and 175°C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 ml/min, and the combustion gas was a mixture of hydrogen and air.

Chapter 4

RESULTS

4.1 Screening of the fungal strains for the Cellulase production

Potential fungal strain capable of producing β-glucosidase was screened from among 100 strains available in the stock culture of Microbial Technology Laboratory, Department of Biotechnology, CUSAT. Initially those strains which produced cellulase enzyme complex were screened through primary screening followed by selection of β-glucosidase producing strain through secondary screening.

4.1.1 Preliminary Screening

From the results presented in Table 4.1 it could be noted that only 10 strains out of 100 were found as positive and were short listed on the basis of the clearing zones produced in the Czapek-Dox agar (CDA) plates by the Congo red staining technique (Fig.4.1). Among the 10 cultures, 5 strains were found to be more potent cellulase producers and were subjected to the secondary screening.

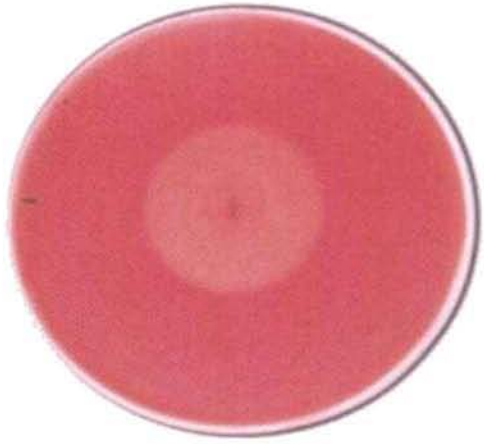

Fig. 4.1 Fungus grown on CMC agar plate showing clearing zone by Congo red staining

Table 4.1 Preliminary screening of cellulytic fungi based on the ratio of the diameter of the clearing zone and that of the colony diameter grown on Czapek-Dox agar

Fungal cultures	Diameter of the clearing zone (mm)	Diameter of the colony (mm)	Clearing zone / Colony diameter (mm)
BTMFS 03	30	15	2.00
BTMFS 08	28	20	1.40
BTMFS 36	36	15	2.40
BTMFS 49	28	17	1.65
BTMFS 55	30	15	2.00
BTMFS 68	32	12	2.67
BTMFS 72	30	18	1.67
BTMFS 73	24	15	1.60
BTMFS 74	32	20	1.60
BTMFS 75	29	14	2.07

4.1.2 Secondary Screening

Secondary screening of cellulolytic fungi included the analytical examination of the crude enzyme produced by the fungi in CD broth at pH 7.8 at room temperature, on a rotary shaker at 150 rpm for 120 h. Based on the β-glucosidase enzyme activity, the strain BTMFS 55, which showed maximal β-glucosidase activity among the groups was selected (Fig.4.2).

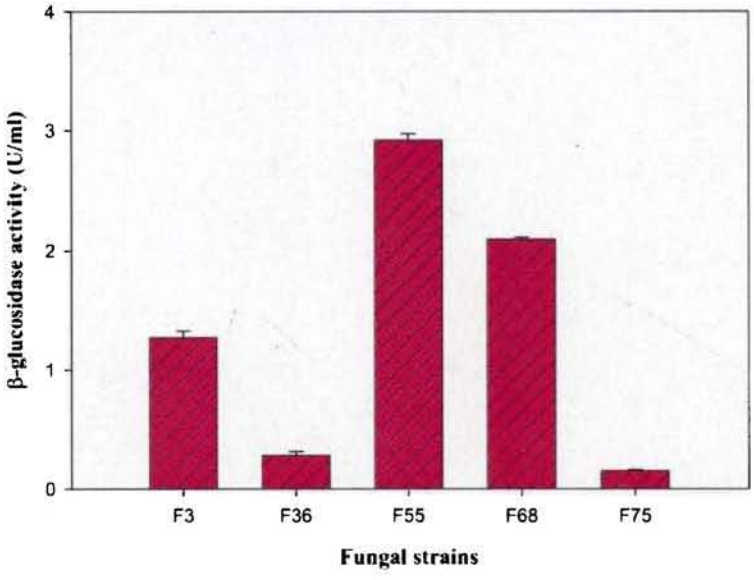

Fig. 4.2 β-glucosidase profile of various fungal strains grown in Czapek-Dox broth

The cellulase enzyme profile of this particular strain was also checked (Fig.4.3). The fungus BTMFS 55 was observed to produce all the three enzymes of the cellulase enzyme system viz. endoglucanase, exoglucanase and β-glucosidase.

However further studies were conducted only for the β-glucosidase enzyme since it was recorded to be at higher level compared to other enzymes of cellulase complex.

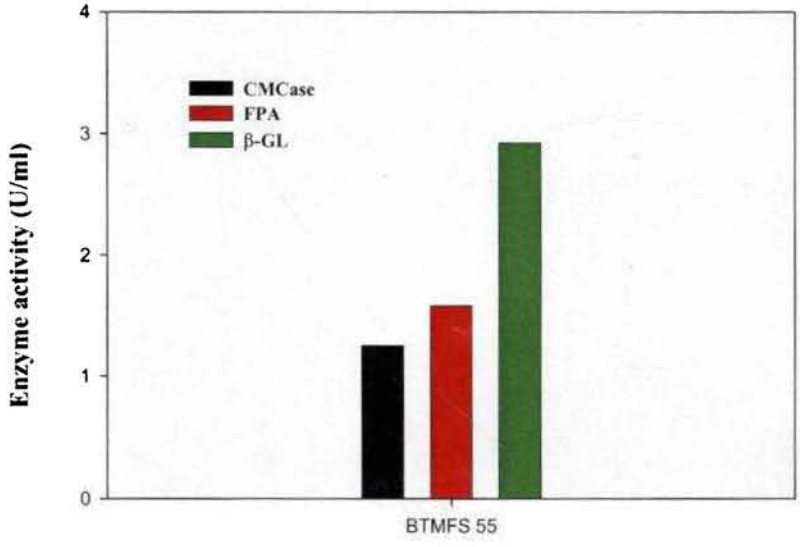

Fig. 4.3 Cellulase enzyme profile of the fungus BTMFS 55

4.1.3 Identification of the selected fungal strain

The fungal strain, BTMFS 55, was later identified as *Aspergillus sydowii* BTMFS 55 by the Microbial Type Culture Collection (MTCC) of Institute of Microbial Technology (IMTECH), CSIR, Govt. of India, Chandigarh. The marine fungus *A. sydowii* BTMFS 55 showed characteristic features of bluish green conidial heads, uncoloured to definitely brown holding biseriate sterigmata and globose to subglobose spores on GMYagar plates (Fig.4.4).

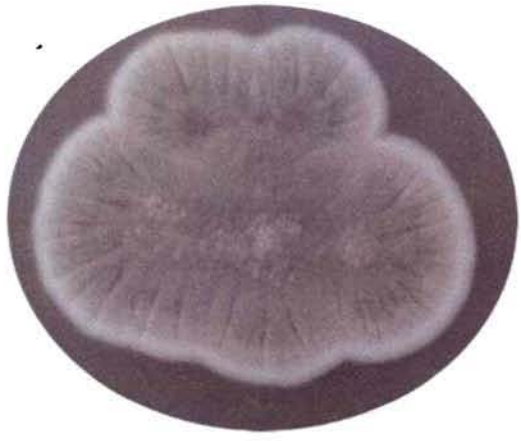

Fig. 4.4 Colony of *Aspergillus sydowii* BTMFS 55 on GMY agar plate

The molecular identification of the fungal strain was done based on 28S rDNA sequence homology. The size of the amplified product was estimated to be approximately 700 bp from agarose gel electrophoresis (Fig.4.5).

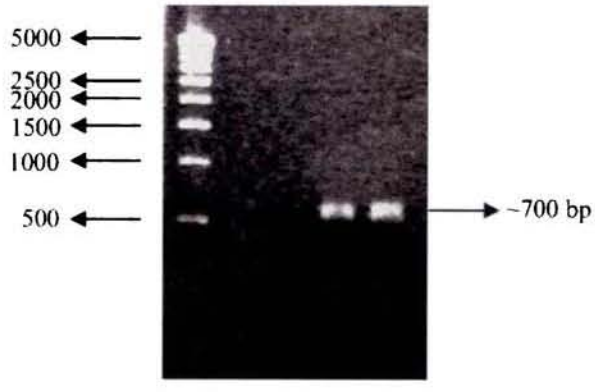

Fig. 4.5 PCR amplicon of 28 rDNA segment amplified with specific primers from the genomic DNA of *A. sydowii* BTMFS 55

4.1.3.1 Ribotyping using partial 28S rRNA gene

Confirmation of identification of *Aspergillus* sp. was done by ribotyping using a primer pair for 28S rDNA. A portion of the 28S rRNA gene (~700 bp) was amplified from the genomic DNA (O'Donnell, 1993) and the amplicon was subjected to sequencing, followed by homology search and analysis. The partial nucleotide sequence obtained after sequencing was of 494 bp and is presented below.

Sequence of AS-55 NL1F

GGCCTCAGTACGGCGAGTGAAGCGGCTAAGAGCTCAAATCTGAAATCTGGCC
CCTCCGGGGTCCGAGTAGTAATGCAGCAGAGGATGCTTCGGGTGCGGCCCCT
GTCTAAGTGCCCTGGAACGGGCCGTCAGAGAGGGTGAGAATCCCGTCTTGGG
CAGGGTGCCCGTGCCCGTGTGAAGCTCCTTCGACGAGTCGAGTTCGTTCTGG
GAATGCAGCTCAAAATGGGTGGTAAATCTCATCTAAAGCTAAATACCGGCCG
GAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAA
CAGAGAGTCAAACAGCACGTGAAGTCGTCGAAACGGGATCGCGCTTGCAACC
AGACTCGGCCTCGGGGTCAGCCAGCATTCGTGCTGGTGTACTTCCCCGGGGC
CGGGCCAGCGTCGGTCTGGGCCGCCGGTCACAGGCCCTTGATTGTTCGTCCT
CGGACTTCTTATCCCTCGGGTCCATG

Partial sequence of the 28S rRNA gene was submitted to GenBank (accession number EF 570064) through BankIt programme, at NCBI site (http://www.ncbi.nlm.nih.gov/BankIt).

4.1.3.2 Phylogenetic tree construction

The identity of the sequence was established using BLAST software (Altschul et al., 1980). The obtained sequence showed 93% identity with already available sequences of *Aspergillus sydowii* in the GenBank.

Phylogenetic tree was constructed using the dendrogram method implemented in CLUSTAL W (Fig.4.6). Tree was constructed using nucleotide evolutionary model based on synonymous and non-synonymous nucleotide substitutions. Tree was visualized using the CLUSTAL W dendrogram, clustalw.dnd (http://align.genome.jp).

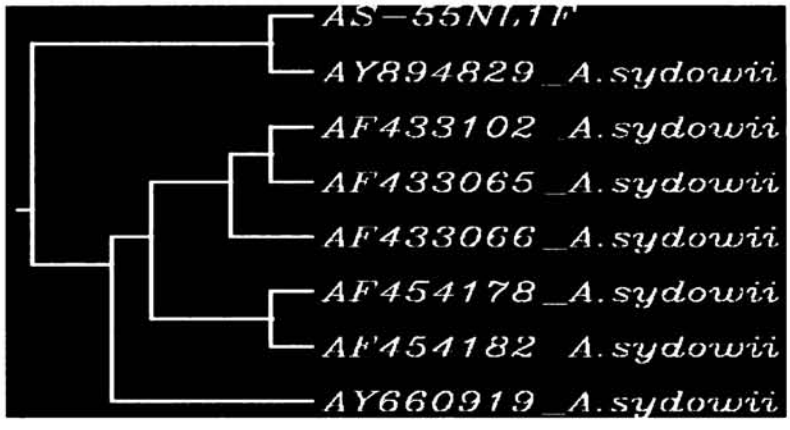

Fig. 4.6 Dendrogram representing the relationship of AS-55 NL1F with other reported *A. sydowii*

4.2 Submerged Fermentation (SmF) of β-Glucosidase by *A. sydowii* BTMFS 55

4.2.1 Optimization of substrates

Aspergillus sydowii BTMFS 55 isolated from seawater produced extracellular β-glucosidase in CD medium utilizing all the substrates evaluated (Fig.4.7) during the course of the study. Highest level of β-glucosidase activity was obtained with glucose (9.9 U/ml) followed by cellobiose (9.1 U/ml), xylose (8.2 U/ml), sucrose (7.9 U/ml), lactose (6.6 U/ml), maltose (6.6 U/ml), mannose (3.9 U/ml), CMC (1.2 U/ml) and cellulose powder (0.06 U/ml). Hence, glucose was selected as a suitable substrate for further studies. However, the specific activity was maximal with xylose (69 U/mg protein) followed by sucrose (65.5 U/mg protein) and glucose (62.7 U/mg protein). Nevertheless, in general, glucose, cellobiose, xylose, lactose, maltose and sucrose supported enhanced levels of β-glucosidase compared to cellulose and arabinose which did not support enzyme production. Whereas, CMC supported very low level of enzyme production.

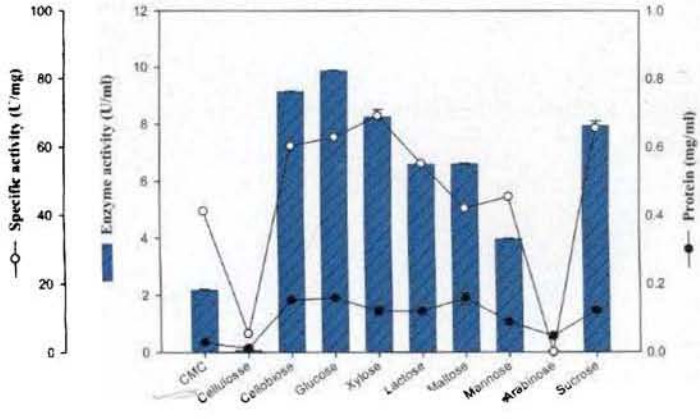

Fig.4.7 Optimisation of substrates for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with different substrates (1%), incubated at RT at 150 rpm and activity was assayed after 120 hrs.

The enzyme activity increased exponentially along with increase in concentrations of glucose up to 2% (12.6 U/ml) and further increase in the concentration of glucose led to slow and steady decrease (Fig.4.8). Hence, 2% glucose was considered as optimum.

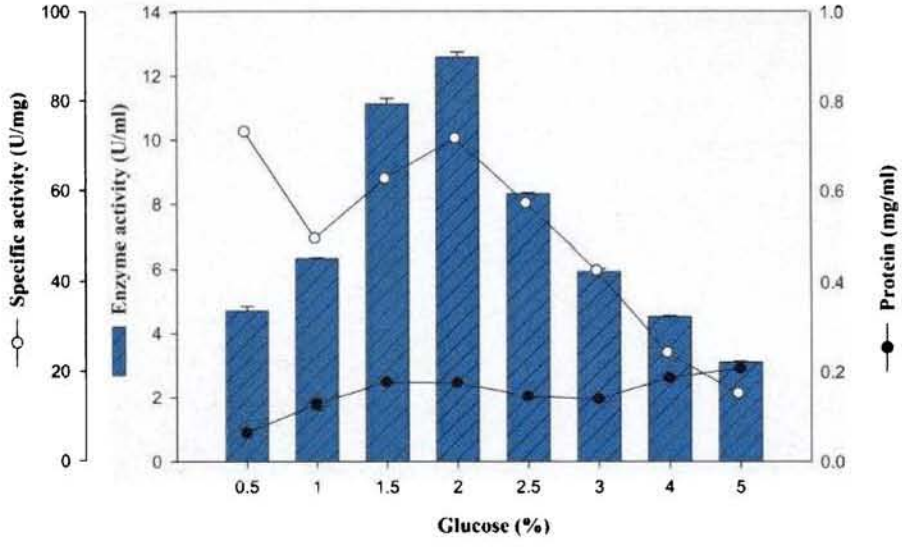

Fig. 4.8 Optimisation of glucose concentration for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with different concentration of glucose, incubated at RT at 150 rpm and activity was assayed after 120 h

4.2.2 Optimisation of pH

A.sydowii showed maximal enzyme activity at pH 9.0 (11.3 U/ml) although enzyme production was recorded at all the pH levels (3-11) tested (Fig.4.9). A considerable level of enzyme activity was recorded over the pH range 3.0 (3.9 U/ml) to pH-11 (8.6 U/ml). There were two peaks, one at p H 6.0 and other at 9.0. It was observed that at pH 7.0 the yield of enzyme was comparatively low (7.9 U/ml). Both the specific activity (85.6 U/mg protein) and protein content (0.13 mg/ml) were also higher at pH 9.0. Hence, pH 9.0 was considered the optimum pH for β-glucosidase production under SmF.

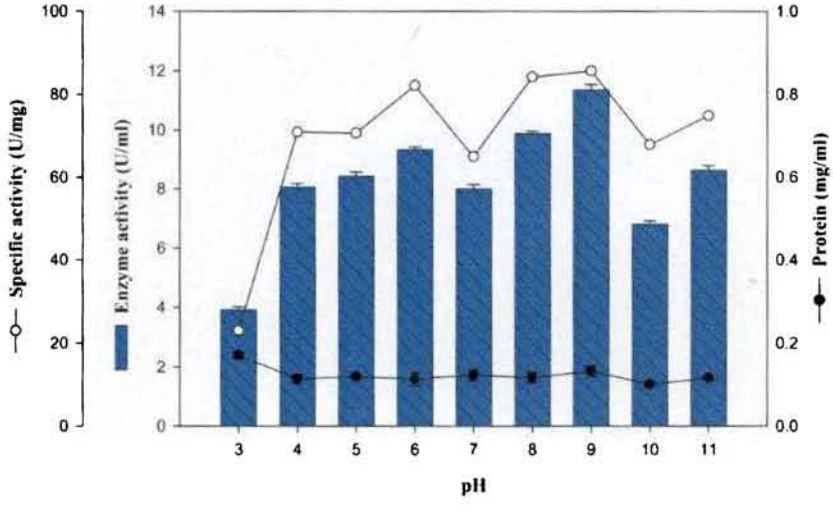

Fig. 4.9 Optimisation of pH for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 0.5% glucose as carbon source, incubated at RT, at 150 rpm at different pH and activity was assayed at 120 hrs.

4.2.3 Optimisation of Temperature

Optimal temperature for maximal production of β-glucosidase was evaluated by incubation at 25°C, 30°C (RT), and 35°C, for 120 h. Maximal enzyme production was observed at RT (12.3 U/ml) with a specific activity of 75.4 U/mg protein (Fig.4.10). There was no enzyme activity in the culture broth incubated at 35°C, while incubation at 25°C supported to some extent enzyme production. The specific activity of the enzyme also exhibited a similar pattern. Hence, 30°C was selected as the optimum incubation temperature.

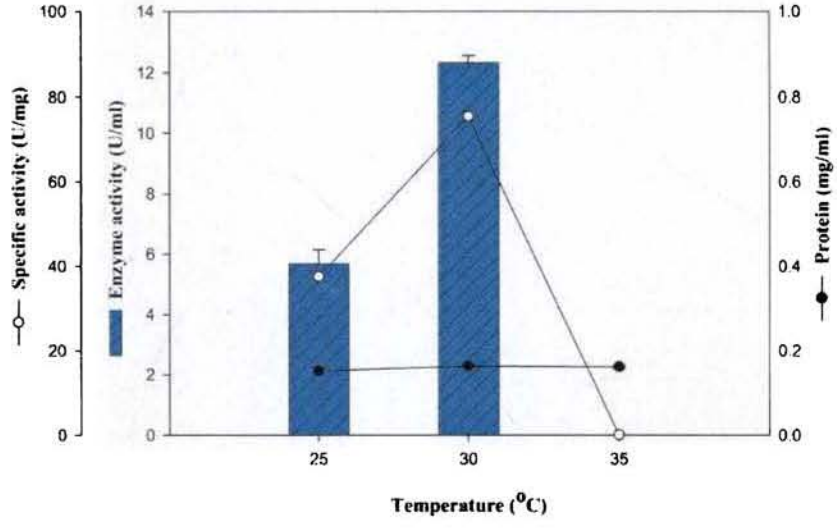

Fig.4.10 Optimisation of incubation temperature for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose, at pH 9.0, at 150 rpm, incubated at different temeperatures and activity was assayed at 120 h

4.2.4 Optimisation of additional Carbon source

Results presented in Fig.4.11 clearly suggest that presence of additional carbon source along with the glucose did not enhance the production of β-glucosidase (starch-8.3 U/ml, xylan-6.6 U/ml, sorbitol-5.6 U/ml, glycerol-3.8 U/ml, Mannitol-5.5 U/ml, cellobiose-6.7 U/ml, rhamnose-1.8 U/ml, salicin-8.1 U/ml, galactose-6.3 U/ml, fructose-2.6 U/ml and xylose-6.5 U/ml). Although there was growth and enzyme production in the presence of additional carbon sources, the enzyme activity was comparatively lesser than the activity recorded with glucose (12.6 U/ml) as the only carbon source. Hence, it is inferred that there is no need for any additional carbon sources for enzyme production.

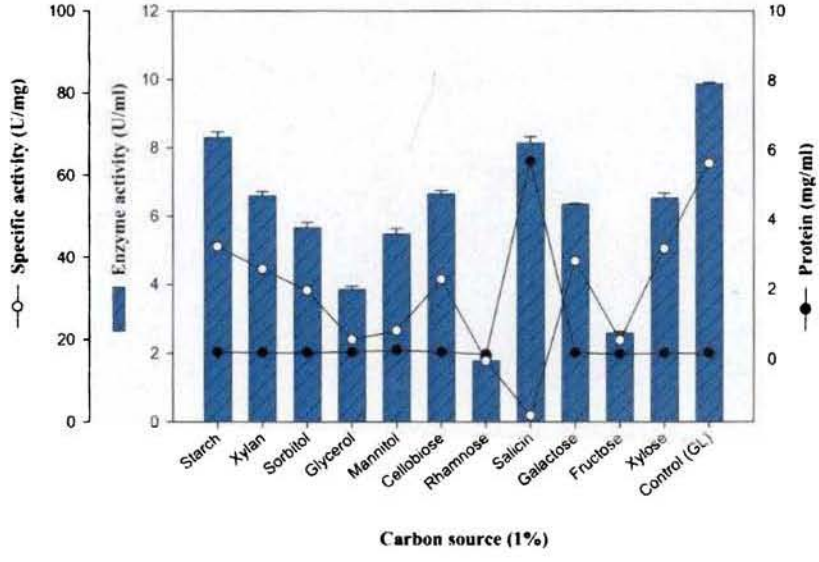

Fig 4.11 Effect of additional carbon sources on β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose with different carbon substrates at pH 9.0, incubated at RT, at 150 rpm and was assayed at 120 h

4.2.5 Optimisation of additional Nitrogen source

Among the nitrogen sources tested as additional nitrogen source (Fig.4.12), ammonium sulphate supported maximal enzyme activity (12.8 U/ml) followed by peptone (11.2 U/ml), malt extract (11 U/ml), yeast extract (9.7 U/ml), beef extract (8.7 U/ml), ammonium nitrate (8 U/ml), ammonium dihydrogen phosphate (6.3 U/ml), casein (6.2 U/ml), ammonium chloride (5.2 U/ml), soybean meal (3.8 U/ml), di-ammonium hydrogen phosphate (1.9 U/ml) and urea (0.35 U/ml). Hence, ammonium sulphate was selected as the additional nitrogen source for the production of β-glucosidase enzyme. It supported a specific activity of 76.6 U/mg protein and a protein content of 0.17 mg/ml. Among the different concentration of ammonium sulphate tested, 0.5% (w/w) was found as optimal for enhanced enzyme production (Fig.4.13).

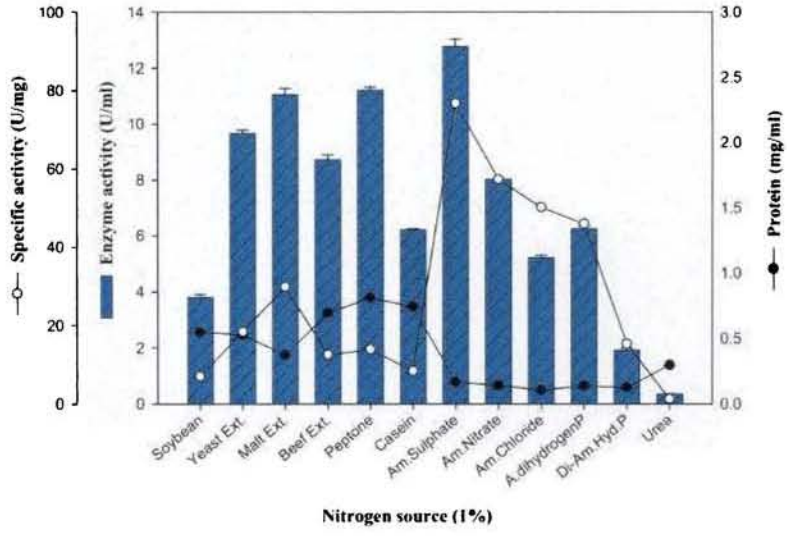

Fig. 4.12 Effect of additional Nitrogen sources on β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose,different nitrogen sources, pH 9.0, RT, 150 rpm and activity was assayed at 120 h

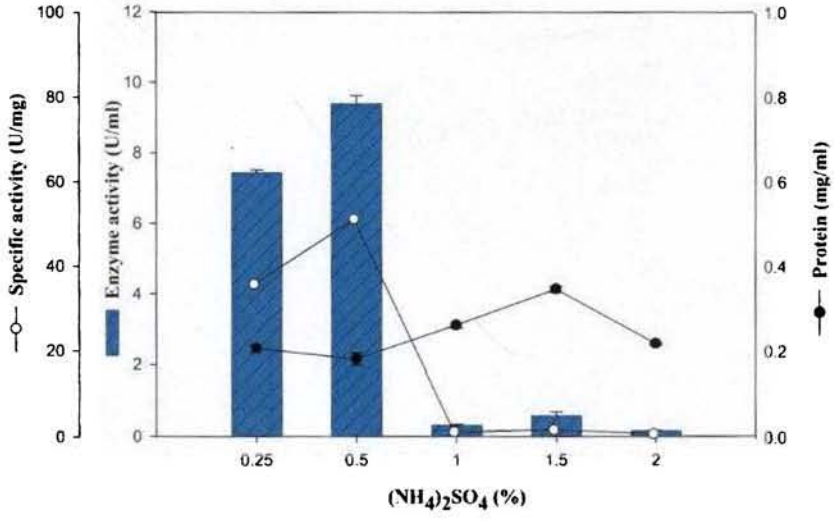

Fig. 4.13 Optimisation of $(NH_4)_2SO_4$ for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media containing 2% glucose, various concentrations of $(NH_4)_2SO_4$, at RT at 150 rpm and activity was assayed after 120 h.

4.2.6 Optimisation of additional NaCl

From the data presented in Fig.4.14, it was observed that β-glucosidase production was maximal only at 0.5% of NaCl (13.9 U/ml), which was higher than that observed with 1% NaCl which was normally used in the CD medium. Increase in concentrations of NaCl led to gradual decrease in enzyme production (6.9 U/ml (0%) to 1.5 (4%)). There was no enzyme activity at 5% NaCl tested. Maximal specific activity was recorded at 1% level (61.4 U/mg protein) followed by 2% (57 U/mg protein) and 0.5% (56 U/mg protein).

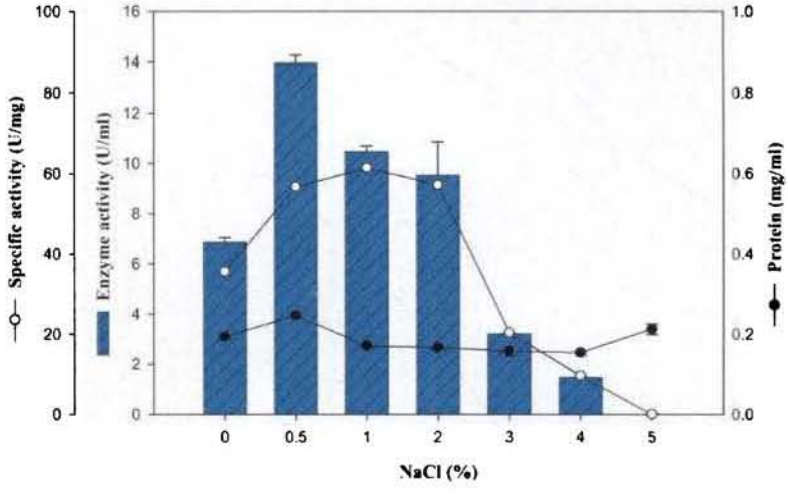

Fig. 4.14 Effect of additional NaCl on β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose, 0.5% ammonium sulphate, pH 9.0, RT, 150 rpm with different concentrations of NaCl and activity was assayed at 120 h

4.2.7 Effect of Tween 80

Low concentration of Tween 80 (0.1%), as surfactant could enhance the production of the enzyme (24.3 U/ml) compared to control (0%-18.8 U/ml) (Fig.4.15). Further, higher concentration of Tween 80 led to gradual decrease (0.65 U/ml at 0.5%). The specific activity of the enzyme at 0.1% was higher (93 U/mg protein) than that of the other concentrations tested. Whereas the protein content was low (0.18 mg/ml) compared to the higher concentrations of Tween 80.

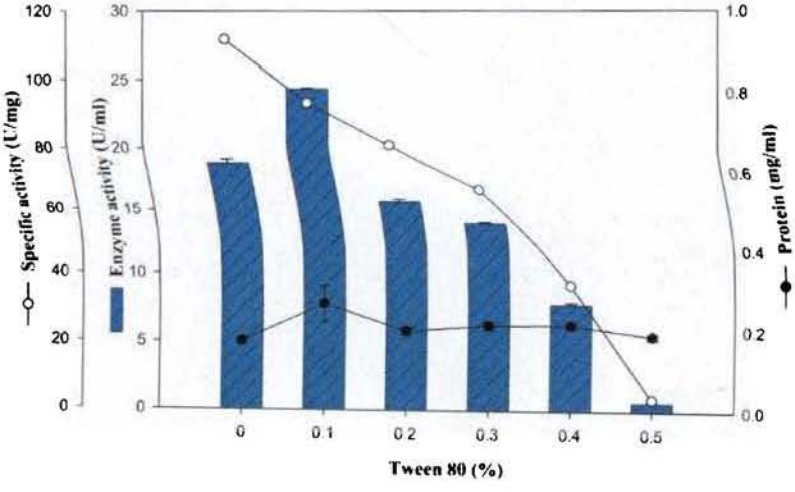

Fig. 4.15 Effect of Tween 80 on β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2 % glucose, 0.5% $(NH_4)_2SO_4$, 0.5% NaCl, pH 9.0, RT, 150 rpm with different concentrations of Tween 80 and activity was assayed at 120 h

4.2.8 Optimisation of inoculum concentration

It was observed that increase in enzyme production increased along with increase in concentration of inoculum up to 4% (12.7 U/ml), and then it gradually declined to at 5% concentration 11 U/ml (Fig.4.16). The specific activity was maximal at 2% inoculum (69.7 U/mg protein). However specific activity of 63.4 U/mg protein could be noted at 4% level with a protein content of 0.2 mg/ml concentration.

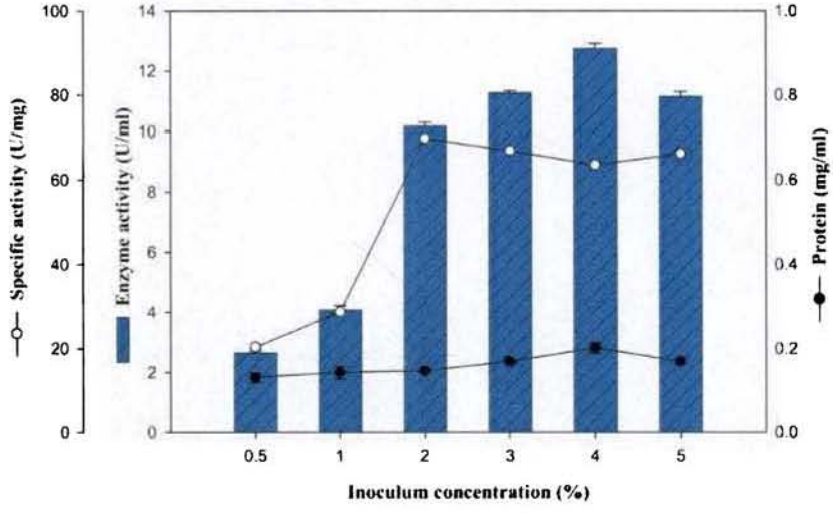

Fig. 4.16 Optimisation of inoculum concentration for β-glucosidase production by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose, 0.5% ammonium sulphate, 0.5% NaCl, 0.1% Tween 80, pH 9.0, RT, 150 rpm with different concentrations of inoculum and activity was assayed at 120 h

4.2.9 Time course studies

Data obtained for the time course experiment presented in Fig.4.17 suggested that considerable levels of β-glucosidase under SmF, could be obtained only after 4 days of incubation (enzyme activity of 9.3 U/ml with a maximal specific activity of 62.2 U/mg protein and a protein of 0.15 mg/ml). The enzyme activity gradually increased to a maximum on the 7 day (14.1 U/ml; specific activity - 51 U/mg protein). The enzyme production remained stable till the 9th day of incubation (13.3 U/ml; specific activity – 44.9 U/mg protein) under the previously optimised condition at pH 9.0. However, the enzyme production declined after 10th day. The maximal biomass (dry weight) was recorded on the 3rd day of incubation (11.21 mg/ml), which decreased later and remained in a steady state from the 6th day on wards (5-9 mg/ml). Whereas, maximal enzyme production at considerable level was recorded from the 6th day onwards, which remained more or less steady with slight variations during the rest of the incubation period.

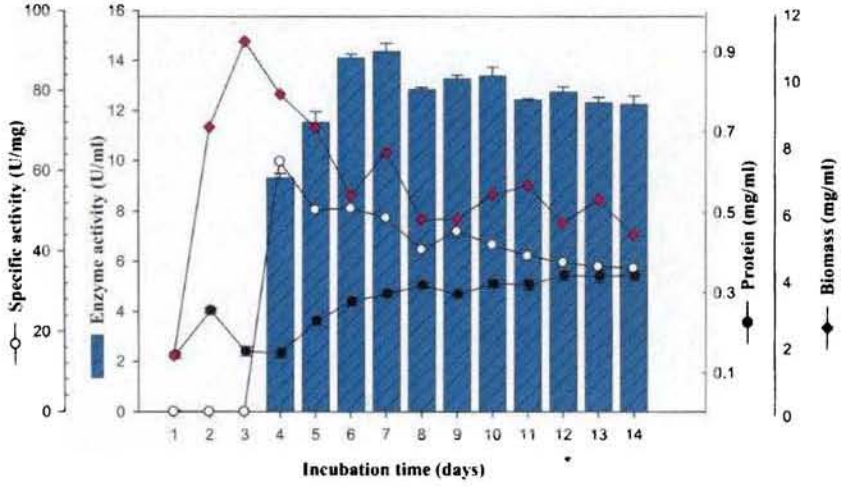

Fig. 4.17 Time course studies of β-glucosidase by *A. sydowii* under SmF: SmF was conducted in CD media with 2% glucose, 0.5% ammonium sulphate, 0.5% NaCl, 0.1% Tween 80, 4% inoculum, pH 9.0, RT.150 rpm.

4.3 Solid State Fermentation (SSF) of β-Glucosidase by *A. sydowii* BTMFS 55

4.3.1 Optimisation of initial moisture content

There was good growth and production of enzyme in wheat bran media with all the moisture levels tested (Fig.4.18), although an initial moisture content of 60% showed maximal enzyme activity (681 U/gIDS). It supported a specific activity of 38 U/mg and a protein of 30mg/gIDS. Further increase in the moisture content led to relative decrease in the enzyme yield. The specific activity was maximal at moisture content of 80% (40 U/mg protein) and the protein content was maximal at 30% (21 mg/gIDS).

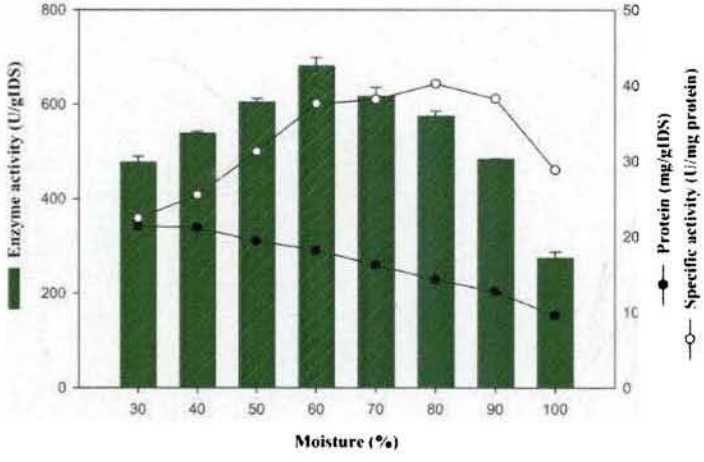

Fig. 4.18 Optimisation of moisture content for β-glucosidase production under SSF by *A. sydowii:* SSF was conducted using WB of particle size 425µ, moistened to varying levels with distilled water and incubated at 30°C for 72 h

4.3.2 Optimisation of incubation time

From the results presented in Fig.4.19, it is inferred that the fungus produced the enzyme after 48 h of incubation (353 U/gIDS) and there was a double fold increase in the enzyme yield after 72 h (696 U/gIDS). Hence, 72 h was selected as the optimal incubation time for the production of β-glucosidase for further studies. The maximal specific activity was recorded after 72 h of incubation as (56 U/mg protein and protein content of 20 mg/gIDS).

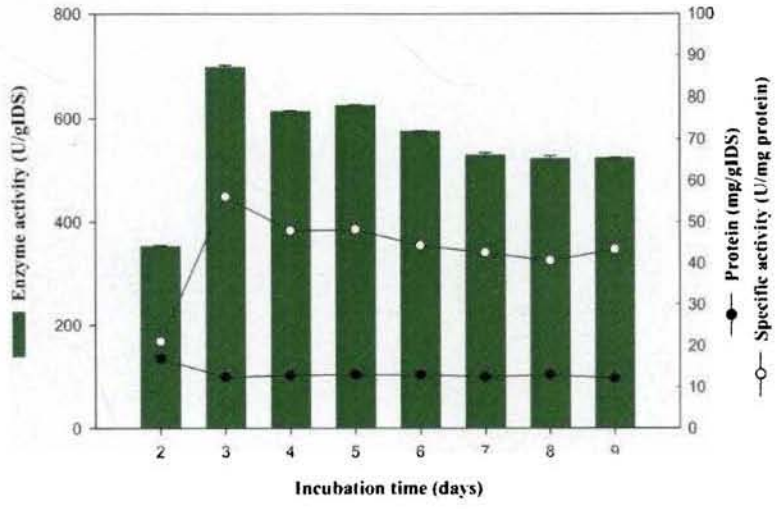

Fig. 4.19 Optimisation of incubation period for β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60% and incubated at 30°C for different incubation time.

4.3.3 Optimisation of particle size of Wheat bran

The importance of particle size on the growth and production of enzyme during SSF was evident from the data presented in Fig.4.20. WB particles of 425 μ supported maximal enzyme production (1027 U/gIDS) compared to others level of particle size tested. The particle size lesser than 425μ, 600 μ and mixed type could also support a good enzyme titre. However the enzyme activity gradually decreased along with increase in the particle size. The specific activity was maximal (792 U/mg protein) with the WB of particle size <425μ and the protein content was maximal at 600 μ (6 mg/gIDS).

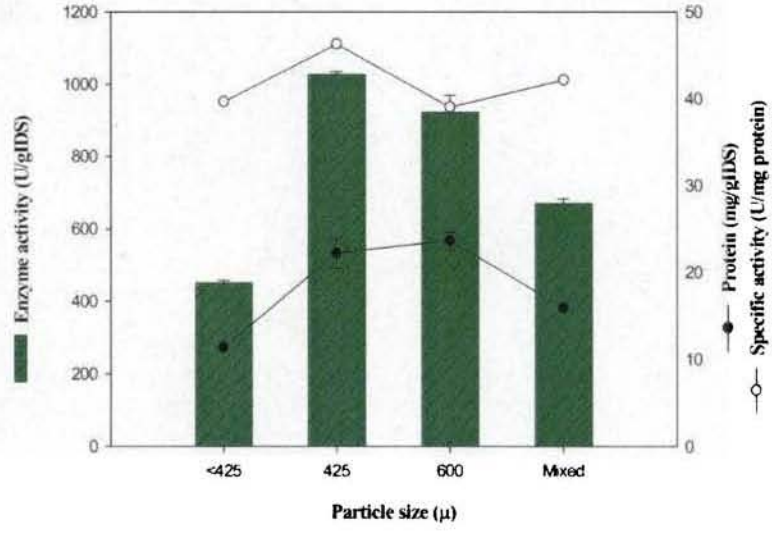

Fig. 4.20 Optimisation of particle size of the substrate (WB) for β-glucosidase production under SSF by *A. sydowii*:
SSF was conducted using WB of different particle sizes moistened with 60%, incubated at 30°C for 72 h.

4.3.4 Optimisation of initial pH of the medium

The results presented in Fig.4.21 clearly indicate that *A. sydowii* was able to grow at all the pH ranges tested and it exhibited maximal enzyme production at pH 8.0 (1139 U/gIDS), although the enzyme production was at comparable level at pH 7.0, 9.0 and 10.0. Nevertheless the maximal specific activity was recorded at pH 7.0 (49 U/mg protein) followed by 8.0. (46 U/mg protein). The protein content at pH 8.0 was recorded as 24 mg/gIDS.

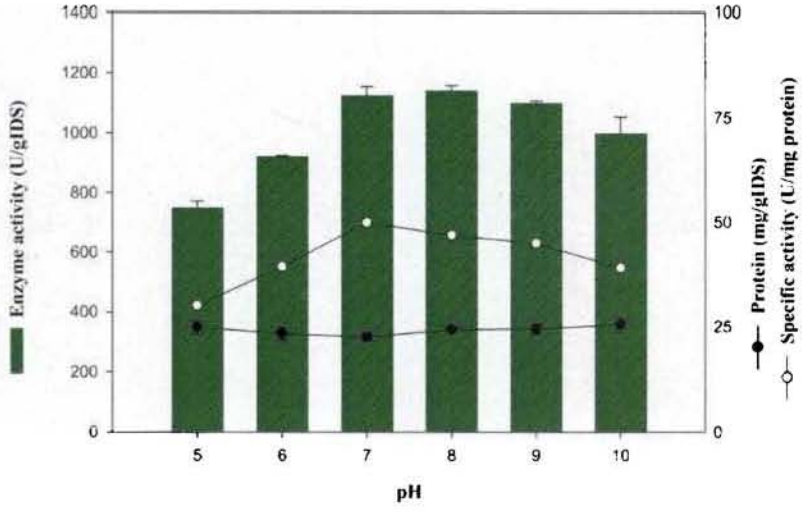

Fig. 4.21 Optimisation of pH for β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60% and incubated at 30°C for 72 h at different pH.

4.3.5 Optimisation of incubation temperature

Significant levels of enzyme production by the strain was observed during SSF at all the incubation temperatures tested (Fig.4.22). The yield was maximal at 30°C (1320 U/gIDS) with a specific activity of 49 U/mg protein and with a protein content of 26 mg/gIDS. Enzyme production at 25°C and 35°C was relatively very low.

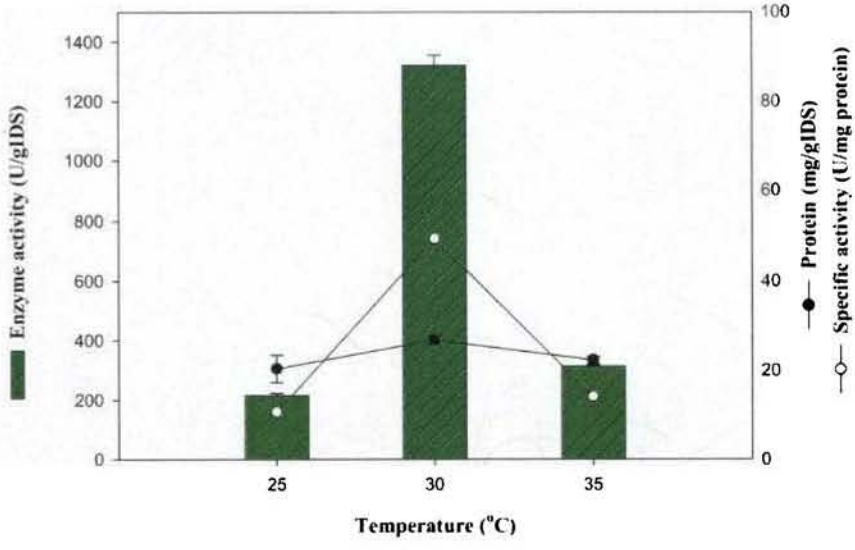

Fig. 4.22 Optimisation of incubation temperature for β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60%, pH 8.0, incubated at different temperatures for 72 h

4.3.6 Effect of additional Carbon source

Incorporation of different carbon sources as additional nutrients showed very marginal impact on enzyme production by *A. sydowii* compared to control with WB alone (1469 U/gIDS). Addition of CMC showed enhancement of enzyme activity (1651 U/gIDS) followed by glucose (1492 U/gIDS) and cellobiose (1471 U/gIDS) (Fig.4.23). All other carbon sources led to marginal decline in enzyme production compared to control.

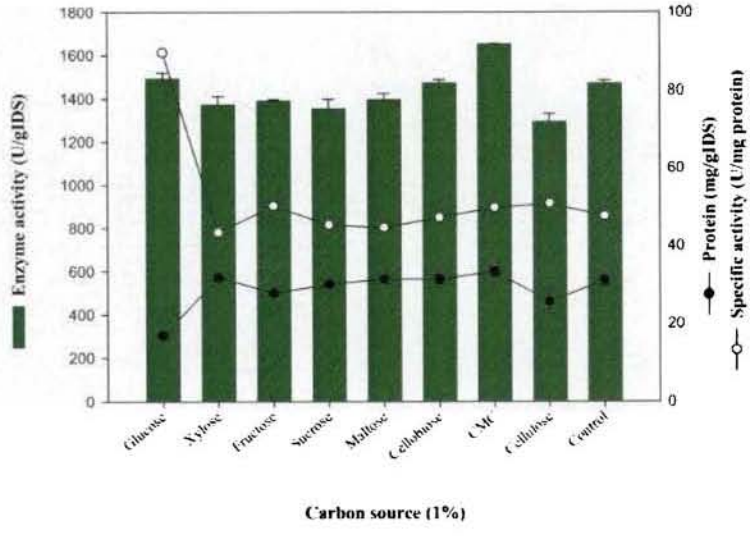

Fig. 4.23 Effect of additional Carbon sources on β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size of 425μ moistened to 60% containing different carbon sources at pH 8.0, incubated at 30°C for 72 h.

Hence concentration of CMC required for maximal enzyme was further optimised. The results presented in Fig.4.24 indicate that 0.5% CMC (1578 U/gIDS) is optimal for the β-glucosidase production by *A. sydowii* under SSF. The specific activity of 51 U/mg protein and a protein content of 31 mg/gIDS were also recorded.

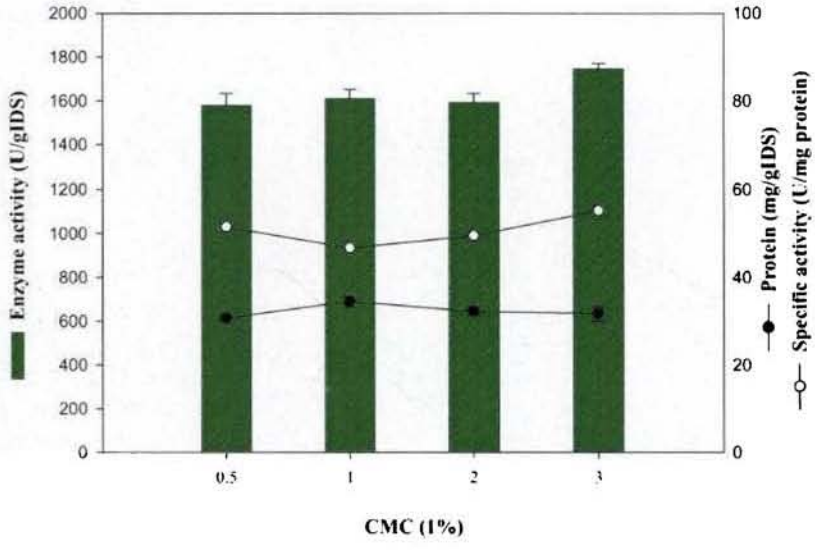

Fig. 4.24 Effect of additional Carbon sources on β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size of 425μ moistened to 60% containing different carbon sources at pH 8.0, incubated at 30°C for 72 h

4.3.7 Effect of additional Nitrogen source

Results presented in Fig.4.25 indicate that the addition of various nitrogen substrates showed both better growth and production of β-glucosidase enzyme compared to the control (WB + CMC). Among the nitrogen substrate tested, addition of peptone showed maximal β-glucosidase activity (1392 U/gIDS) followed by soybean meal (1350 U/gIDS) and ammonium nitrate (1317 U/gIDS). It was also noted that the optimal level of peptone was 0.5% for maximal enzyme production (1361 U/gIDS). The specific activity was 49 U/mg protein with the protein content of 28 mg/gIDS at 0.5% peptone in WB medium.

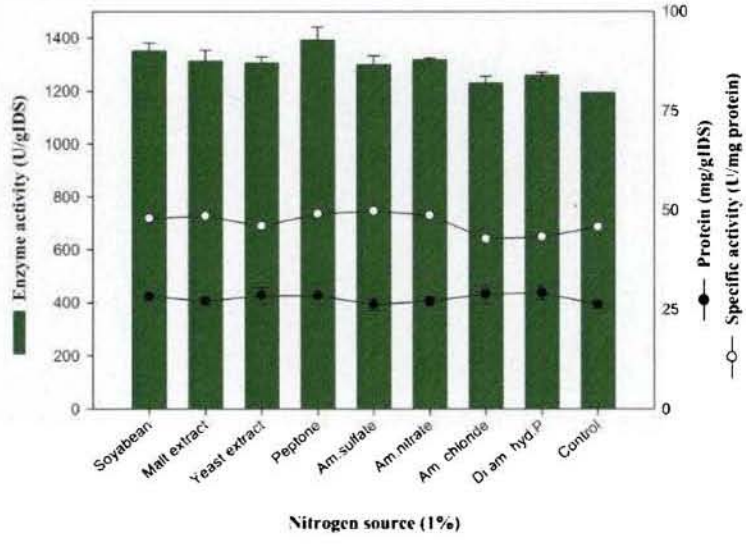

Fig. 4.25 Effect of additional nitrogen sources on β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60%, 0.5% CMC, pH 8.0, incubated at 30°C for 72 h with different nitrogen sources.

4.3.8 Effect of NaCl

Results presented in Fig.4.26 clearly indicate that the fungus produced β-glucosidase at all the NaCl concentration tested. Though the yield was recordable in WB media without additional NaCl (1337 U/gIDS), the production fairly increased in the presence of 0.5% concentration (1505 U/gIDS). The increase in NaCl concentration did not enhance the enzyme yield compared to that of 0.5% level of the additional salt. The maximal specific activity (67 U/mg protein) was also recorded at the 0.5% level with a protein content of 23 mg/gIDS.

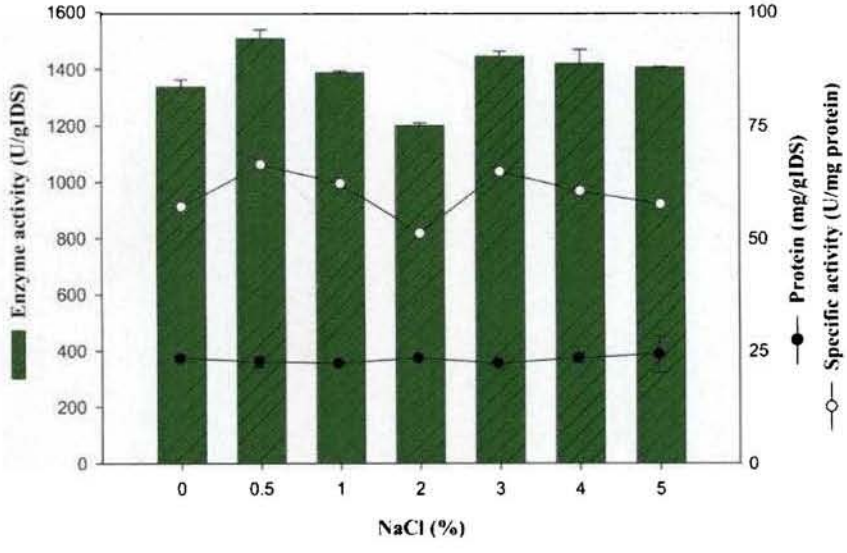

Fig. 4.26 Effect of NaCl on β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60%, 0.5% CMC, 0.5% peptone, pH 8.0, different concentrations of NaCl at 30°C for 72 h.

4.3.9 Optimisation of inoculum concentration

The β-glucosidase production showed a linear increase along with increase in inoculum concentrations (Fig.4.27). Though the production was maximal at 50% among the tested concentration, the inoculum concentration of 10% was taken as optimal (2411 U/gIDS with a specific activity of 94 U/mg protein and a protein content of 26 mg/gIDS), since the level of enhanced enzyme yield at inoculum concentration was 20%.

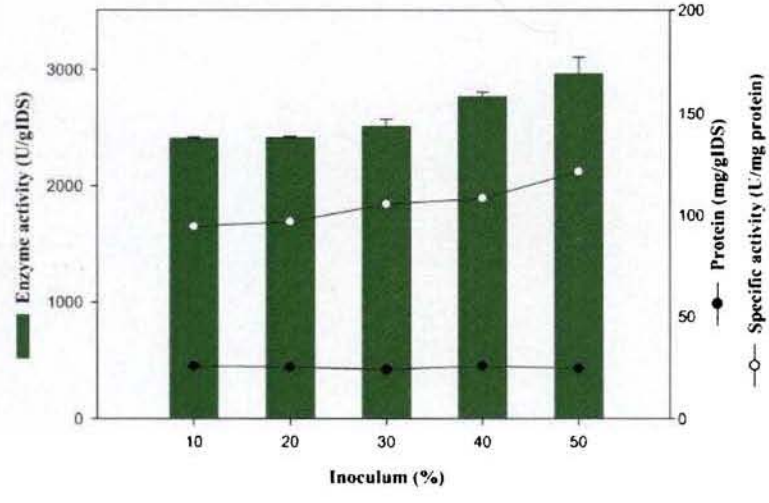

Fig. 4.27 Optimisation of inoculum concentration for β-glucosidase production under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60%, 0.5% CMC, 0.5% peptone, 0.5% NaCl, pH 8.0, incubated at 30°C for 72 h with different inoculum concentrations.

4.3.10 Time course experiment

The data obtained for the time course studies of β-glucosidase production by *A. sydowii* under solid-state fermentation is presented in Fig.4.28. From the results it is noted that the enzyme production commenced 2 days (127 U/gIDS) and there was a sharp increase on the 3rd day of incubation (949 U/gIDS). The maximal production was recorded on the 4th day (1401 U/gIDS). However it declined during the following days. The maximal specific activity was also noted on the 4th day of incubation (52 U/mg protein) with a protein of 27 mg/gIDS.

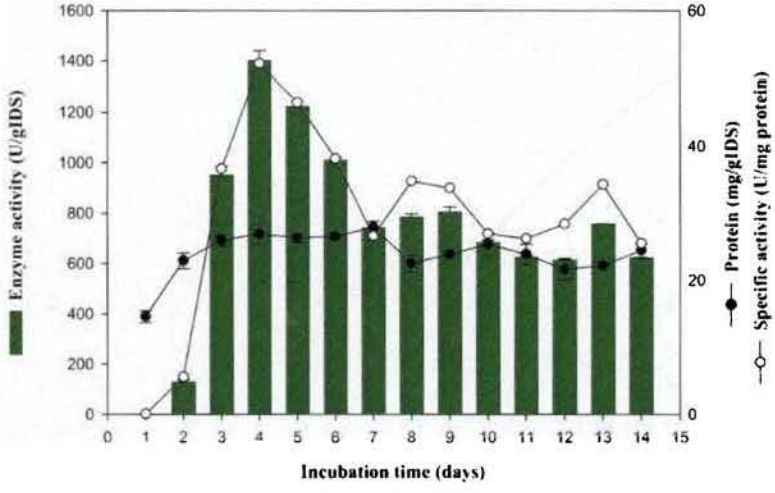

Fig. 4.28 Time course studies of β-glucosidase under SSF by *A. sydowii*: SSF was conducted using WB of particle size 425μ moistened to 60%, 0.5% CMC, 0.5% peptone, 0.5% NaCl, 10% inoculum, pH 8.0 at 30°C.

4.4 Optimisation of process variables for β- Glucosidase production by A. *sydowii* under SSF - Statistical approach

4.4.1 Plackett – Burman Experimental Design

According to the Plackett-Burman experimental design twelve different conditions were run for evaluating the effects of independent variables on the production of β-glucosidase under solid state fermentation by the marine fungus *A. sydowii.* Each row represents an experiment and each column represents an independent variable. The signs (+) and (−) represent the higher and lower levels of the independent variable under investigation. The predicted and experimental responses are presented in the Table 4.2.

The estimated effects of individual parameters are presented in Table 4.3. From this, it was inferred that the moisture content, incubation temperature, pH, concentrations of inoculum, CMC, peptone, Tween 80 and incubation time had positive influence on β-glucosidase production, whereas the particle size of the wheat bran, concentrations of NaCl and ammonium sulphate did not show any significant influence.

Table 4.3 Estimated effects of individual parameters

Code	Parameter	Estimated effects
A	Moisture	224.1
B	Temp.	70.5
C	pH	17.3
D	Inoculum	274.7
E	Particle size	-18.6
F	CMC	78.5
G	Peptone	387.3
H	$(NH_4)_2SO_4$	-35.5
J	NaCl	-40.8
K	Tween 80	0.4
L	Time	102.5

Table 4.2 The result of the Plackett–Burman experimental design for optimisation of process variables for β- glucosidase production by *A. sydowii* under SSF

Run	Coded variable level											Y_1 (U/gIDS)	Y_2 (U/gIDS)
	Moisture (%)	Temperature (oC)	pH	Inoculum (%)	Particle size (µ)	CMC (g/L)	Peptone (g/L)	$(NH_4)_2SO_4$ (g/L)	NaCl (g/L)	Tween 80 (%)	Incubation time (h)		
1	50	25	9	10	600	10	1	10	7.5	1	72	305.247	391.111
2	50	30	7	20	600	2	10	10	7.5	0.5	72	941.568	1053.17
3	70	25	9	20	425	10	10	10	2.5	0.5	72	1250.41	1277.26
4	50	25	7	10	425	2	1	1	2.5	0.5	72	303.916	391.111
5	50	30	9	10	600	10	10	1	2.5	0.5	120	941.568	778.494
6	50	25	7	20	425	10	10	1	7.5	1	120	1106.64	1053.17
7	70	30	7	10	425	10	1	10	7.5	0.5	120	703.28	615.198
8	70	25	9	20	600	2	1	1	7.5	0.5	120	863.026	889.872
9	70	25	7	10	600	2	10	10	2.5	1	120	964.198	1002.58
10	50	30	9	20	425	2	1	10	2.5	1	120	733.898	665.785
11	70	30	7	20	600	10	1	1	2.5	1	72	933.58	889.872
12	70	30	9	10	425	2	10	1	7.5	1	72	962.867	947.336

(Y_1, experimental responses; Y_2, predicted values calculated from the fitted first-order model)

Statistical analysis of the results indicate that although most of the parameters had a positive influence, the moisture content (*A*), inoculum (*D*) and peptone (*G*) had significant influence on β-glucosidase under SSF. A graphical representation of the positive (orange bars) and negative (blue bars) effects of the variables tested was presented as a Pareto Chart in Fig.4.29. The significant ones were labeled by respective codes and ranked based on their estimated effects.

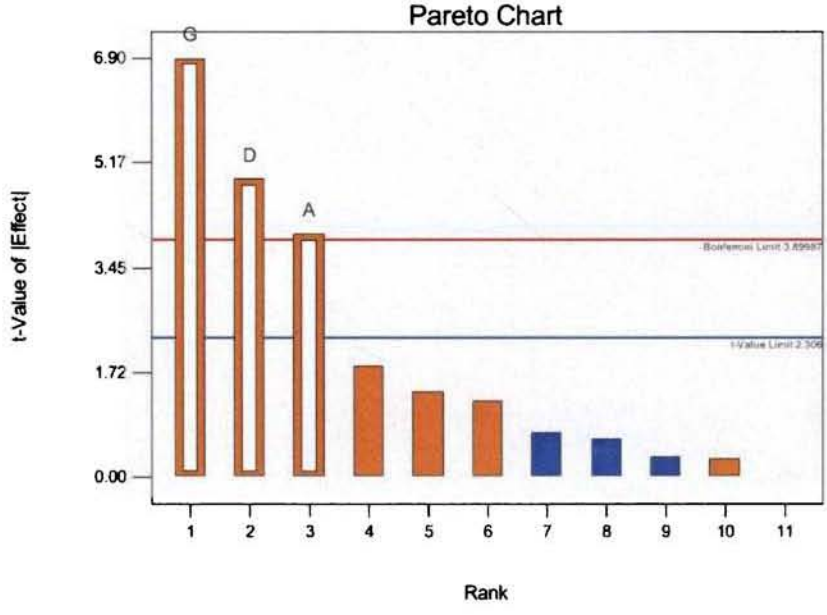

Fig. 4.29 Pareto Chart of standardized effects of the selected parameters

On the basis of the experimental values, statistical testing was carried out using Fisher's test for analysis of variance (ANOVA) (Table 4.4). The results were used to fit a first order polynomial equation and the model equation for β-glucosidase yield could be written as:

$$Y = 834.18 + 112.04\,A + 137.34\,D + 193.69\,G,$$

where Y is yield of β-glucosidase, A is moisture content (%), D is inoculum concentration (%) and G is peptone concentration (g/L).

Table 4.4 Analysis of Variance (ANOVA) for the factorial model on β-glucosidase production in SSF by *A. sydowii*

Source	Sum of Squares	DF[a]	Mean Square	F Value	p-Value Prob > F
Model	8.272E+005	3	2.757E+005	29.15	0.0001
A- Moisture	1.506E+005	1	1.506E+005	15.92	0.0040
D- Inoculum	2.263E+005	1	2.263E+005	23.92	0.0012
G- Peptone	4.502E+005	1	4.502E+005	47.59	0.0001
Residual[c]	75682.39	8	9460.30		
Cor. Total[d]	9.029E+005	11			

[a]Degree of freedom
[b]Significant at "Prob > F" less than 0.05
[c]Difference between experimental and predicted points
[d]Total of all information corrected for the mean

$R^2 = 0.9162$
Adjusted $R^2 = 0.8847$
Predicted $R^2 = 0.8114$
Adequate Precision = 15.780

The Model F-value of 29.15, and values of prob > F (<0.05) indicated that the model terms are significant. The test model was statistically significant with a confidence levels above 95%. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2), which equaled 0.9162, indicating that 91.62% of the variability in the response could be explained by the

model. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response (Haaland, 1989). The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. Adequate precision measures signal to noise ratio. An adequate precision of 15.78 for β-glucosidase production was recorded. The predicted R^2 of 0.8114 was in reasonable agreement with the adjusted R^2 of 0.8847. This indicated a good agreement between the experimental and predicted values for β-glucosidase production. The value of the adjusted determination coefficient (Adj. R^2 = 0.8847) was also very high to advocate for a high significance of the model. These results indicated that the response equation provided a suitable model for the Plackett–Burman design experiment.

The adequacy of the model was further examined at additional independent conditions that were not employed to generate the model. It was observed that the experimental and predicted values of β-glucosidase production showed good correlation (Table 4.5). The optimum conditions predicted for the production of 1257.09 U/gIDS β-glucosidase were as follows: moisture (X_1) – 68.6 (%), inoculum (X_2) – 19.93 (%), and peptone(X_3) – 9.94 (g/L). The actual experimental value obtained at these predicted conditions was 1335.61 U/gIDS, which was in good agreement with the predicted value.

Table 4.5 Validation of the model

X_1	X_2	X_3	Y_{exp}	Y_{pred}
68.6	19.93	9.94	1355.61	1257.09
69.9	19.65	9.92	1202.49	1262.83
69.5	19.55	9.92	1338.27	1255.65

4.5 Enzyme purification and characterization

The β-glucosidase enzyme isolated from *A. sydowii* BTMFS 55 was purified by ammonium sulphate fractionation followed by anion exchange chromatography on DEAE Sepharose. β-glucosidase could be precipitated with 60% ammonium sulphate saturation. Table 4.6 ssummarizes the data of the purification steps of the extracellular β-glucosidase.

Table 4.6 Purification of β-glucosidase isolated from *A. sydowii* BTMFS 55

Purification step	Volume (ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Yield of Protein (%)	Yield of Activity (%)	Fold of Purification
Crude extract	780	1560	134160	86	100	100	1
$(NH_4)_2SO_4$	5	35	5830	167	2	4	2
DEAE Sepharose	10	2	1270	635	0.1	0.9	7

During ion exchange chromatography on DEAE Sepharose, the β-glucosidase was eluted as a single active peak (Fig.4.30). The elution was carried out with 0.1 M NaCl in phosphate buffer of pH 8.0 at a flow rate of 10 ml/h. The active fractions were pooled and dialyzed overnight against phosphate buffer (0.1M; pH 8.0). This enzyme solution was the purified β-glucosidase preparation used for subsequent studies.

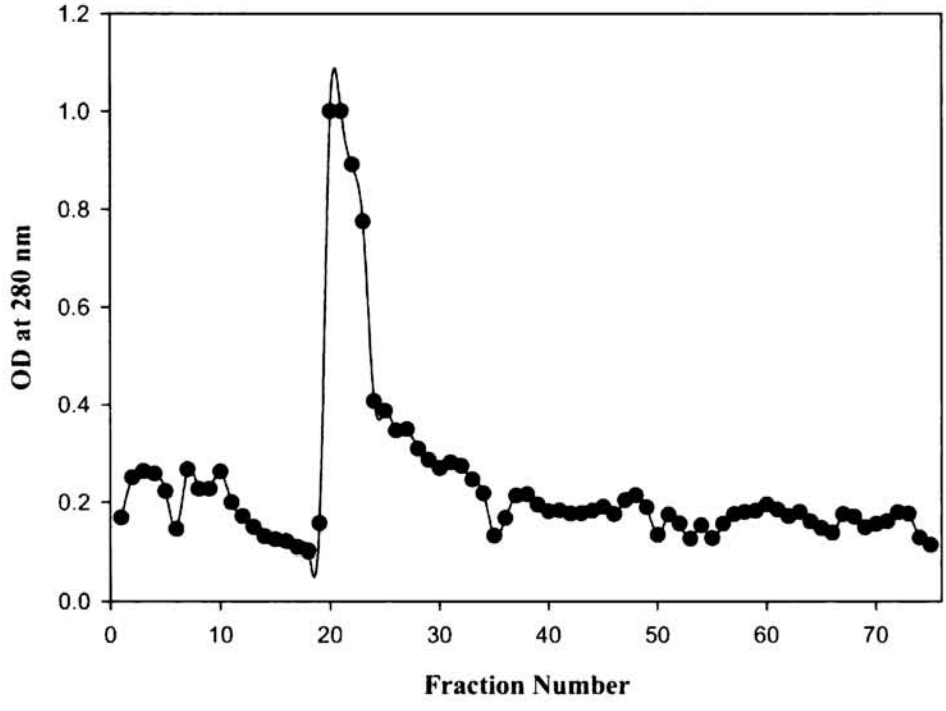

Fig. 4.30 Ion exchange chromatography profile of β-glucosidase on DEAE Sepharose

4.5.1 Properties of β-glucosidase enzyme

The various physico-chemical properties such as the molecular size of the protein, temperature and pH optima, substrate specificity and kinetics, effect of metal ions on β-glucosidase enzyme were evaluated and are in detail below.

4.5.1.1 Native polyacrylamide gel electrophoresis (Native PAGE)

The purified β-glucosidase enzyme showed a single band on native PAGE analysis (Fig.4.31). The zymographic analysis of the corresponding band exhibited β-glucosidase activity as fluorescence under UV light, after staining with 4-Methylumbelliferyl-β-D-glucopyranoside (4-MUG).

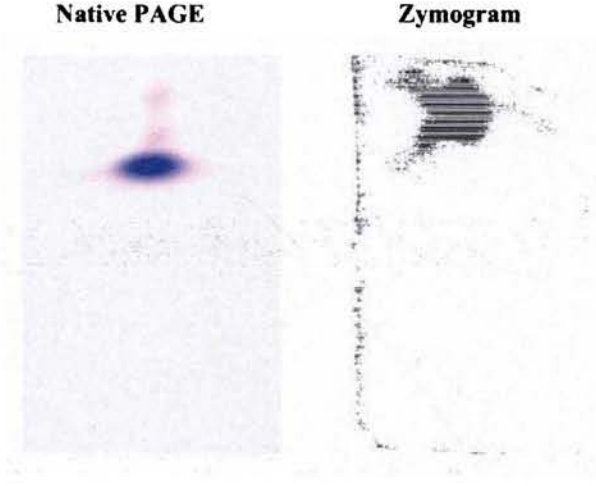

Fig. 4.31 Native PAGE analysis of A. sydowii β-glucosidase

4.5.1.2 Molecular weight determination

The purity and apparent molecular mass of the β-glucosidase preparation was determined by SDS–PAGE electrophoresis (Fig.4.32). The purified enzyme showed a single protein band on SDS-PAGE, having a molecular mass of ~ 95 kDa. The zymogram analysis of the gel confirmed the single polypeptide nature of the corresponding band by staining with 4-MUG.

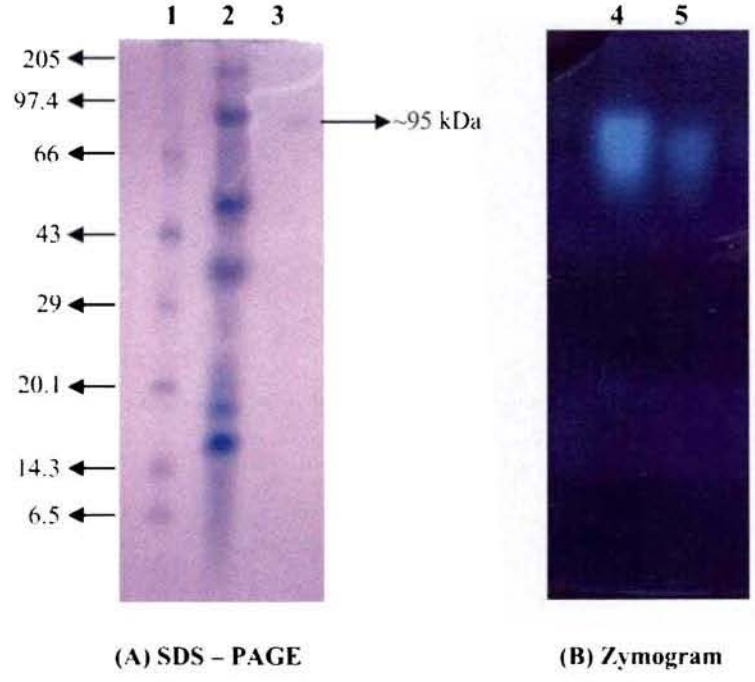

Fig. 4.32 SDS-PAGE analysis of β glucosidase showing (A) Coomassie blue staining and (B) Zymogram analysis

Lane 1 – Protein Marker (kDa)

Lane 2 & 4 - $(NH_4)_2SO_4$ precipitated fraction

Lane 3 & 5 - Ion exchange purified sample

4.5.1.3 Effect of Temperature on β-glucosidase activity and stability

The temperature optimum for maximal β-glucosidase activity was 50°C when the reaction mixture was incubated for 30 min. However, it demonstrated significant activity (up to 40% of the maximal activity) over a temperature range of 40-70°C (Fig.4.33).

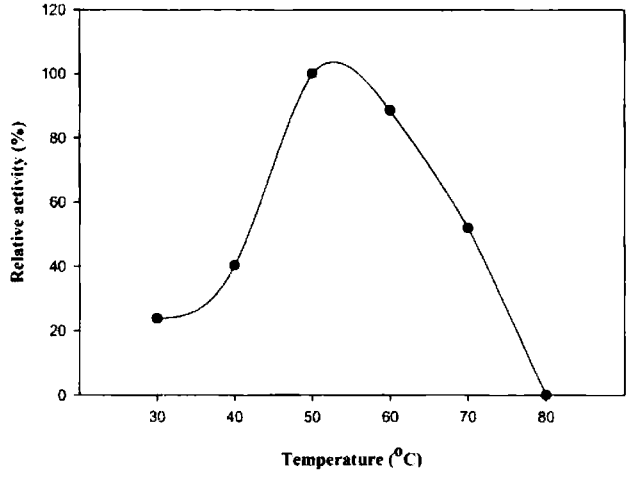

Fig. 4.33 Effect of temperature on activity of β-glucosidase by *A. sydowii*

4.5.1.4 Thermostability of β-glucosidase enzyme

The thermal stability of the β-glucosidase was investigated by measuring the residual activity after 30 min, 1h, 12h and 24 h of incubation at temperatures ranging from 30-80°C (Fig.4.34).

The enzyme showed 10% increase in residual activity at 50°C, compared to control (4°C). It could withstand the temperatures of 40, 50, and 60°C even after 24 h of incubation. The β-glucosidase was not stable at 80°C, whereas it showed a weaker stability pattern at 30 °C and 70°C.

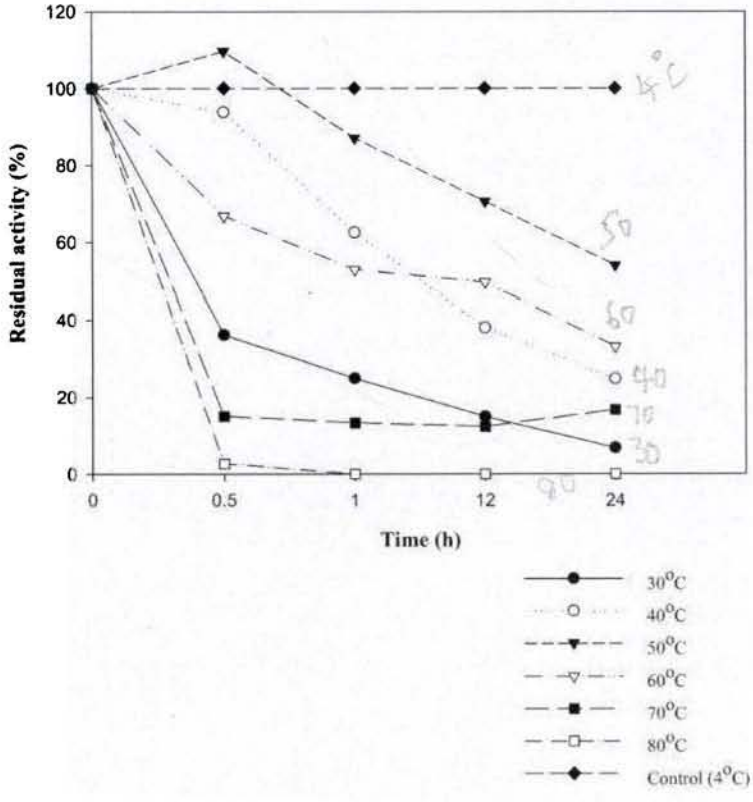

Fig.4.34 Thermostability of β-glucosidase by *A. sydowii* BTMFS 55

4.5.1.5 Effects of pH activity and stability

The β-glucosidase enzyme displayed a pH optimum of pH 5.0, under optimal temperature conditions. The enzyme was able to retain 65% activity at pH 6.0, followed by 45% at pH 4.0, 27% at 7.0, 18% at 8.0 and 13% at 9.0 and was completely inactivated at pH 10.0 (Fig.4.35). The enzyme was stable at pH 4.0-6.0. 86% of activity remained at pH 5.0 which 50% remained at pH 6.0, after 24 h at 50°C. Maximal enzyme stability was displayed at pH 4.0.

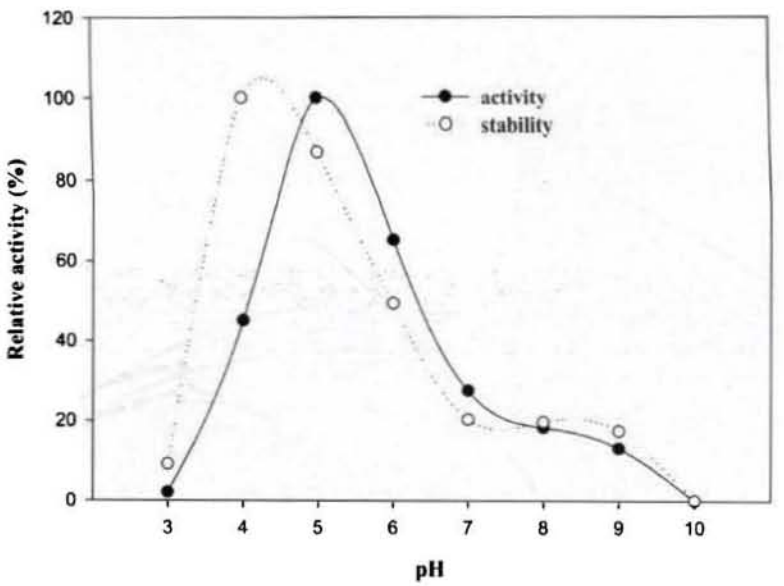

Fig. 4.35 Activity and stability profile of β-glucosidase of *A. sydowii* BTMFS 55 over different pH

4.5.1.6 Substrate specificity

Though the enzyme showed high reactivity towards *p*-nitrophenyl-β-D-glucoside (*p*NPG), it hydrolyzed natural oligosaccharides having (1-4)-β-glycosidic linkages, such as cellobiose and lactose (Table 4.7). The enzyme could also hydrolyze maltose, which have (1-4)-α-glycosidic linkage. But the enzyme was totally inactive on sucrose, trehalose and raffinose.

Table 4.7 Substrate specificity of β-glucosidase of *A. sydowii* BTMFS 55

Substrate (5 mM)	Linkage of glycosyl group	Relative initial rate of hydrolysis (%)
*p*NPG	β -Glc	100
Cellobiose	β (1-4) Glc	0.56
Lactose	β (1-4) Gal	2.14
Maltose	α (1-4) Glc	11.66

4.5.1.7 Enzyme Kinetics

The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plots (Fig.4.36) with *p*NPG under optimal conditions (30 min, pH 8.0, 50°C). The enzyme had an apparent K_m value of 0.67 mM, and a V_{max} value of 83.3 U/ml for the hydrolysis of *p*NPG. The specificity constant (V_{max} / K_m) for the *p*NPG hydrolysis was also determined as 123.37. The molar activity (k_{cat}) and catalytic efficiency (k_{cat}/ K_m) of the enzyme were calculated to be 2.14 x 10^{-2} s^{-1} and 3.19 x 10^{-2} mM^{-1} s^{-1}, respectively.

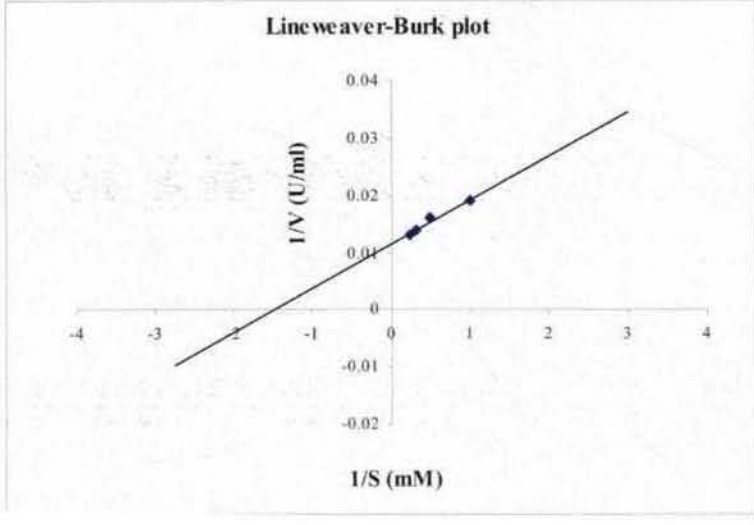

Fig. 4.36 Lineweaver – Burk plot of purified β-glucosidase of *A. sydowii*

4.5.1.8 Effects of glucose on *p*NPG hydrolysis

The inhibition constant, K_i, is an indication of how potent an inhibitor is. It is the concentration required to produce half maximum inhibition. The plotting of 1/V against concentration of inhibitor (Glucose) at each concentration of substrate (*p*NPG) yielded a family of intersecting lines and the convergence of lines above the X-axis showed the Glucose was a competitive inhibitor (Fig.4.37). The K_i value of Glucose was obtained as 17 mM from the intersection of the line of the Dixon plot.

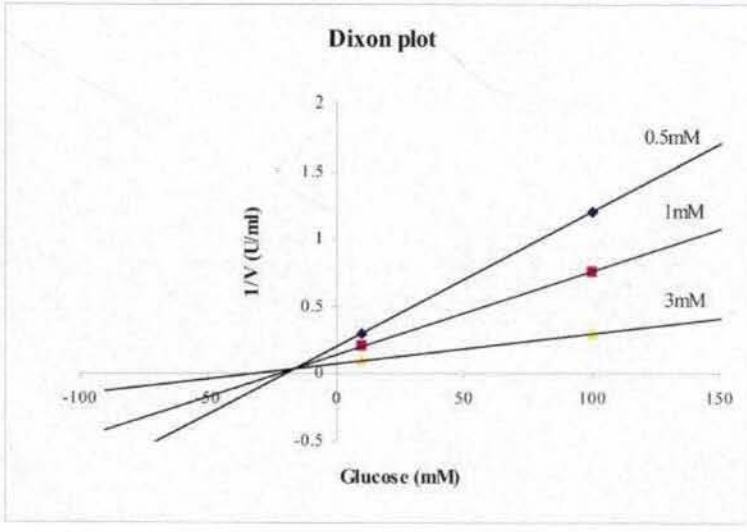

Fig. 4.37 Dixon plot of inhibitory effects of glucose on *p*NPG hydrolysis by β-glucosidase of *A. sydowii*

4.5.1.9 Effect of metal ions and compounds

The effect of various metal ions on β-glucosidase activity was investigated and the results are shown in Table 4.8. Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+}, K^{+} and Zn^{2+} showed significant increase in enzyme activity at a concentration of 1 mM tested. Whereas, the presence of Mg^{2} and Ag^{2+} completely inhibited the enzyme activity at all the concentrations tested. The Fe^{3+} and Hg^{2+} led to increase in activity along with increased concentration. The metal ions Co^{2+} and Zn^{2+} showed 52% and 28% enhancement of enzyme activity at a concentration of 1mM, but completely inhibited enzyme activity at the higher concentrations of 5mM and 10 mM tested. Cu^{2+} ions could enhance the activity at 1mM concentration only and Mn^{2+} ions could increase the activity both at 1 and 5mM, but completely inhibited activity at 10mM concentration.

Table 4.8 Effect of various metal ions on β-glucosidase activity of A. *sydowii*

Compound	Residual activity (%)		
	1 mM	5 mM	10mM
Calcium chloride (Ca^{2+})	103	39	53
Cobalt chloride (Co^{2+})	152	0	0
Cupric sulphate (Cu^{2+})	211	61	0
Ferric chloride (Fe^{3+})	414	141	54
Magnesium chloride (Mg^{2+})	70	59	63
Manganese chloride (Mn^{2+})	322	205	0
Mercury chloride (Hg^{2+})	187	109	87
Potassium chloride (K^{+})	115	71	78
Silver sulphate (Ag^{2+})	28	24	18
Zinc sulphate (Zn^{2+})	128	0	0

4.5.1.10 Potential inhibitors and activators

Significant enzyme inhibition was observed with group-specific potential inhibitors, such as β-mercaptoethanol, SDS, DEPC, EDTA and DMSO at all the concentrations tested (Table 4.9). They did not completely inhibit, but strongly slowed down the β-glucosidase activity. But in the case of DMSO, though the low concentrations decreased the activity, the addition of 10mM showed 47% increase in enzyme activity.

Table 4.9 Effect of various inhibitors on β-glucosidase activity of A. *sydowii*

Inhibitor	Residual activity (%)		
	1 mM	5 mM	10 mM
β-mercaptoethanol	78	74	76
DEPC	74	78	73
DMSO	74	77	74
EDTA	64	36	16
SDS	92	92	67

4.5.1.11 Effect of organic solvents

It was observed that organic solvents had a stimulating effect on β-glucosidase activity especially alcohols at lower concentrations (Table 4.10). Among the solvents tested, ethanol was the most effective one which could enhance the activity 41% at a concentration of 10% (v/v) followed by methanol (30%) and n-butanol (21%) except for propanol. This activation decreased with increasing alcohol concentration. Propanol did not inhibit the enzyme activity but it reduced all the concentrations tested. Methanol could enhance the enzyme activity at lower concentrations of 10 and 20 % whereas all the others, butanol and ethanol led to an increase in the activity only at 10% level.

Table 4.10 Effect of organic solvents on β-glucosidase activity of A. *sydowii*

Concentration (%, v/v)	Relative activity (%)					
	0	10	20	30	40	50
Methanol	100	130	107	29	2	7
Ethanol	100	141	73	18	8	7
Propan-2-ol	100	90	44	44	7	2
n-Butanol	100	122	80	57	43	33

4.6 Application studies

4.6.1 Ethanol production from Wheat bran

From the results presented in Fig.4.38, it is inferred that the ethanol production commenced after 24 h of incubation (9.98 g/L) and attained a maximum at 48 h (11.22 g/L). The production then gradually decreased during further incubation from 72 to 120 h. The level of glucose in the fermentation medium at zero h was 2.65 g/L and the level got increased to 3.643 g/L after 24 h, which later gradually decreased.

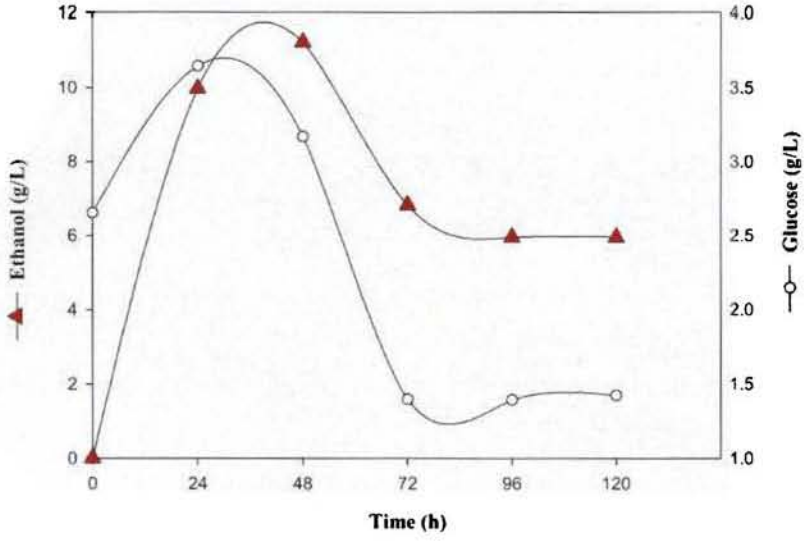

Fig. 4.38 Production of ethanol from Wheat bran

4.6.2 Ethanol production from Rice straw

Data presented in Fig.4.39 evidence that a maximum yield of ethanol from Rice straw by the β-glucosidase enzyme and yeast (10.32 g/L) 24 h could be obtained after of incubation and the glucose level was reduced from 2.443 (zero hour) to 1.258 g/L. The yield of ethanol dropped down during subsequent days of incubation in the fermentation medium at 37°C, except at 120 h (7.89 g/L) with a glucose level of 0.495 g/L.

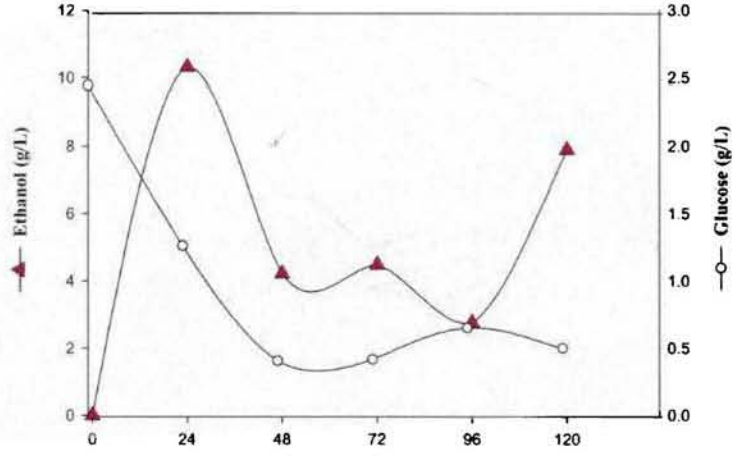

Fig. 4.39 Production of ethanol from Rice straw

Chapter 5

DISCUSSION

Marine environments, in general, are unique by virtue of their salinity, wide range of mineral content and well knitted ecosystem when compared to terrestrial environments which is constantly disturbed by human activities. The marine fungi have not been experimentally tried for their potential in many of the human endeavours in which they would have a major role to play as a biocatalyst, through their enzymes or whole cell systems.

Aspergillus sydowii BTMFS 55 isolated from sea water was found as a potential strain, that produced extracellular β-glucosidase among other fungal isolates obtained from marine environments. *Aspergillus sydowii* BTMFS 55 isolated from sea water produced extracellular β-glucosidase in Czapek-Dox medium and the process optimization for the maximal production of the enzyme was done under submerged and solid state fermentations.

5.1 Molecular identification of the fungal strain

Traditional identification methods of fungi such as morphological and cultural characteristics may be tedious, time consuming and require a great deal of skill. The PCR amplification of rDNA sequences using taxon-specific primers which were derived from sequence data and were checked for cross reaction with related fungi is thought to be the most powerful molecular tool for fungal diagnosis which has been developed so far (Sugita and Nishikawa, 2003). A portion of the 28S rRNA gene

(~700 bp) was amplified from the genomic DNA using a primer pair for 28S rDNA (NL1F and NL4R) and the partial nucleotide sequence obtained after sequencing was of 494 bp. The identity of the sequence was determined by BLAST software (Altschul et al., 1990) and the resultant sequence showed 93% identity with the already available sequences of *Aspergillus sydowii* in the GenBank. A phylogenetic tree was constructed using nucleotide evolutionary model based on synonymous and non-synonymous nucleotide substitutions. *A. sydowii* shared a close affinity with most of the terrestrial species of *Aspergillus* suggesting that *A. sydowii* could have migrated to marine sediments through surface drain from terrestrial environments and could have adapted to marine environments in due course of time.

Aspergillus sydowii BTMFS 55 isolated from sea water produced extracellular β-glucosidase in Czapek-Dox medium and the process optimization for the maximal production of the enzyme was done under submerged and solid state fermentations.

5.2 Submerged Fermentation (SmF)

There are so many reports regarding the production of extracellular β-glucosidase enzyme from different fungal species and among the group *Aspergillus* sp. is considered as the best since it produce more β-glucosidase than the others (Asquieri and Park, 1992; Gupte and Madamwar, 1997). Among the genus, *A. niger* (Galas and Romanowska, 1997; Yan and Lin, 1997) , *A. aculeatus* (Takada et al., 1999), *A. japonicus* (Sanyal et al., 1988), *A. nidulans* (Bagga et al., 1990), *A. oryzae* (Riou et al., 1998), *A. kawachii* (Iwashita et al., 1999), and *A. terreus* (Tavolibov et al., 1988) are the mostly reported ones. But there are no reports available about the

production, purification or characterization of the β-glucosidase from *Aspergillus sydowii* either from terrestrial or marine source.

A. sydowii BTMFS 55 produced extracellular β-glucosidase in CD medium utilizing all the substrates evaluated and the enzyme production was maximum with glucose (9.86 U/ml) as the sole source of carbon, thus seemed as a constitutive enzyme. Generally, most of the microbial cellulases are inducible enzymes and they are secreted when the microorganisms grow in cellulose (Kubicek et al., 1993; Mach and Zeilinger, 2003). According to Kang et al (2004) the production of the β-glucosidase enzyme is affected by the nature of the substrate used in fermentation, hence the choice of an appropriate inducing substrate is highly important. But, in the case of *Candida peltata*, the extracellular β-glucosidase enzyme found as constitutive and produced regardless of the presence or absence of cellulosic substrate (Saha & Bothast, 1996). The β-glucosidase from *Pyrococcus furiosus* was isolated from a maltose based medium (Bauer et al., 1996) and *Aureobasidium pullulans* produced β-glucosidase activity constitutively when grown in liquid medium containing lactose as the carbon source (Saha et al., 1994). It has been reported that the presence of glucose in the fermentation medium was the most effective for promoting activity of the glucanase enzyme (Sharma et al. 1996). There are so many reports regarding the growth and production of β-glucosidase in presence of glucose as carbon source (Riou et al., 1998; Saha et al., 1996; Parry et al., 2001).

Although there was growth and enzyme production in the presence of additional carbon sources, the enzyme activity was comparatively lesser than the activity recorded with glucose (9.86 U/ml) as the only carbon source. Hence, it is inferred that there is no need for any additional carbon sources for enzyme production. Results obtained in the present study were in agreement with the earlier reports that glucose acts as a good growth substrate for production of β-glucosidase by yeasts

(Saha & Bothast 1996). *Candida molischiana* 35 produced an exocellular β-glucosidase with a wide substrate spectrum (Vasserot et al. 1995). The high level of enzyme production on glucose and the glucose tolerance observed in the present study are one of the most important characteristics of the *A. sydowii* and could have an application in the enzymatic hydrolysis of cellulose to glucose.

Among the various nitrogen sources, both organic and inorganic, tested as additional nitrogen sources, ammonium sulphate supported maximal enzyme activity (12.8 U/ml). Both peptone (11.2 U/ml) and malt extract (11 U/ml) were also equally good in enhancing enzyme production. May be the marine fungus preferred ammonium sulphate as source of additional nitrogen as well as sulphate for enhanced enzyme production compared to peptone and malt extract which are complex organic source of nitrogen. Further studies on this aspect may reveal the physiology of this marine fungus with particular reference to the role of ammonium sulphate in enzyme synthesis.

The requirement of NaCl for the production of β-glucosidase was tested by incorporating different concentrations of NaCl (0-5%) in the CD medium along with the already optimized parameters. Even though there was good growth in the presence of NaCl at all the concentrations tested, β-glucosidase production was maximal only at 0.5% of NaCl (13.9 U/ml), which was higher than that observed with 1% NaCl (10.5 U/ml) which was normally used in the CD medium. From the results it is inferred that this fungus though exist in sea water, where the NaCl concentration is around 3%, it does not secrete maximal enzyme at 3% NaCl as the organism may not require production of copious amount of this enzyme in its natural environment. Whereas, under controlled conditions at lesser levels of NaCl the enzyme synthesis machinery might have been induced for enhanced levels of enzyme production. May be it is possible that at higher concentration of NaCl above 1%, the glucose induction

of β-glucosidase production could have been repressed and hence level of enzyme production decreased along with increase in concentration of NaCl, in spite of the presence of optimal level of glucose in the medium. Since this is a new marine fungus this aspect needs to be investigated further particularly with respect to the relationship between glucose induction of enzyme and NaCl concentration at molecular level.

The surfactants play a role in the secretion of cell membrane bound exoenzyme in microbes by increasing the cell permeability of the micro organisms (Reese et.al., 1969). The effect of Tween 80 as surfactant was tested and it was found that low concentration of Tween 80 (0.1%) could enhance the release of the enzyme (24.3 U/ml) in the medium. The present results were in agreement with an earlier observation where the production of cellobiase from *A. niger* A20 was enhanced by the addition of a low concentration (0.2%) of Tween 80 as optimal concentration (Abdel-Fattah et al., 1997).

After optimization of all the parameters a time course experiment was performed and it was noted that the considerable levels of β-glucosidase could be recorded after 4 days of incubation (9.3 U/ml) and the enzyme activity gradually increased to a maximum after 7 days (14.3 U/ml). The maximal biomass (dry weight) was recorded on the 3rd day of incubation (11.21 mg/ml), which decreased later and remained in a steady state from the 6th day on wards (5-9 mg/ml). Whereas, maximal enzyme production at considerable level was recorded from the 6th day onwards, this remained more or less steady with slight variations during the rest of the incubation period. These observations testify that the β-glucosidase production by *A. sydowii* is not growth associated and enzyme synthesis took place only during the stationary phase. A similar observation was made with *Aspergillus oryzae* for β-glucosidase production where enzyme production increased from 6th day to 14 days of incubation and remained steady (Gunata & Vallier, 1999).

5.3 Solid State Fermentation (SSF)

In the production of microbial exoenzymes, solid state fermentation (SSF) has several economic advantages over conventional submerged fermentation such as use of agro industrial wastes as simpler substrates, minimal requirement of water, production of metabolites in a more concentrated form and making the downstream processing less time consuming and less expensive (Sreeja et al., 2006). Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited owing to their ability to grow and produce a wide range of extracellular enzymes on complex solid substrates (Moo-young, 1983). Among processes used for enzyme production, SSF is an attractive one because it presents many advantages, especially for fungal cultivations (Weiland, 1988). In SSF, the productivity per reactor volume is much higher compared with that of submerged culture (Grajek, 1987). Also, the operation cost lower, because simpler plant, machinery and energy are required (Roche and Durand 1996).

In recent years, SSF has received more attention by the investigators, since several studies for enzymes, flavours (Ferron et al., 1996), colourants (Johns & Stuart, 1991) and other substances of interest to the food industry have shown that SSF can support higher yields (Tsuchiya et al., 1994) or better product characteristics than submerged fermentation. In addition, costs are much lower due to the efficient utilization and value-addition of wastes (Robinson& Nigam, 2003).

The marine fungi *A. sydowii* BTMFS 55 produced β-glucosidase as extracellular enzyme even without the addition of any nutrients to the wheat bran (WB). Wheat bran is considered as a complete medium for producing various industrially important enzymes (Smits et al 1996). It was observed that the fungus started to produce the enzyme after 48 h of incubation (353 U/gIDS) and there was a

double fold increase in the yield after 72 h with an enzyme activity of 696 U/gIDS. There are reports which supported the low activity during the first 24 h since the time needed for enzyme production was found to be longer when original substrate contained no compounds stimulating enzyme production. The action of both endoglucanase and exoglucanase during the early stages of growth resulted in cellobiose production that can induce β-glucosidase biosynthesis at later stages (Godden et al., 1989).

Though there were good growth and production of enzyme in wheat bran media with all the moisture level tested, the maximal enzyme activity was observed with 60% moisture content (681 U/gIDS). In solid state fermentation, the demands of moisture level differ according to the nature of enzyme to be produced, substrate, microorganisms as well as the particle size of the substrate (Muniswaran and Charyulu, 1994; Nandakumar et al, 1994; Krishna and Chandrasekaran, 1996; Fadel, 1999). Besides, the moisture content and relative humidity of the medium are the key factors, which determine the outcome of the process and moisture content affects both aeration and nutrients solubility and suitability to be utilized by microorganisms (Nigam, 1990). The moisture content, incubation temperature and oxygen supply are considered as the most critical parameters for the growth and enzyme production in SSF (Barrios-Gonzalez et al., 1993).

Incorporation of different carbon sources as additional nutrients showed an increased enzyme yield compared to SSF with Wheat Bran alone. The addition of CMC showed maximal enzyme activity (1651 U/gIDS) followed by glucose (1492 U/gIDS) and cellobiose (1472 U/gIDS). CMC, glucose and cellobiose could have played the role of an inducer for β-glucosidase. Apparently it seems this fungus is induced for it β-glucosidase production by its own metabolites rather external carbon sources for enhanced enzyme production

The addition of various nitrogen substrates showed both better growth and production of β-glucosidase enzyme compared to the control (WB + CMC). Among the substrates evaluated, addition of peptone showed maximal β-glucosidase activity (1392 U/gIDS). Fang and Zhong (2002) reported that organic nitrogen sources were efficient in *Ganoderma lucidum* fermentations for mycelial growth and polysaccharide production.

The β-glucosidase production showed a linear increase along with increase in inoculum concentration. The inoculum concentration of 10% was taken as optimal (2411 U/gIDS). The same was also reported with *A. niger* grown under SSF (Fadel, 2000). Inoculum size controls and shortens the initial lag phase, and while a smaller inoculum size led to extended lag phase, larger inoculum size contributed to increase in the moisture content to a significant extent (Sharma et al., 1996).

The time course experiment presented an interesting picture on the potential of this *A.sydowii* for β-glucosidase production. In SSF, the solid substrate Wheat bran not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells. The addition of 0.5% CMC as the additional carbon source and 0.5% peptone as the additional nitrogen source along with 0.5% NaCl, at pH of 8.0 and at room temperature, supported enhanced β-glucosidase production. The optimal size of the wheat bran particle size was found to be 425μ with an optimum of 60% moisture content and 10% inoculum for the enzyme production. It has been reported that the particle size, moisture content and the substrate used are the most critical factors in solid state fermentations (Liu & Tzeng, 1999; Roussos et al., 1993; Sarrette et al., 1992; Smail et al., 1995; Zadrazil & Punia, 1995). During the course of incubation for 14 days, under optimized conditions, maximal enzyme production occurred only on the 4th day (1401 U/gIDS) at 30°C at a pH of 8.0. As per Godden et al (1989) and

Fadel (2000), the low activity for β-glucosidase during first 24 h may be due to the fact that original substrate contained no compounds stimulating enzyme production. The enzyme production declined after 4 days. It was noted during the course of the present study that *A. sydowii* took 7 days for maximal enzyme production under SmF conditions compared to the 4 days under SSF. It is reported literature that the incubation time needed for enzyme production was shorter on SSF compared to SmF (Illanes etal 1992, Jiafa et al 1993).

5.4 Media optimisation by statistical analysis under Solid State Fermentation

Medium optimization by the one-factor-at-a-time method involves changing one independent variable (i.e., nutrients, temperature, pH, etc.) while fixing others at certain levels. This method is not only time-consuming, but also often leads to an incomplete understanding of the behaviour of the system, resulting in confusion and a lack of predictive ability (Xu and Yun, 2003). Hence statistical approach was attempted towards optimization of bioprocess variables for β-glucosidase production.

Optimization through factorial design and response surface methodology is a common practice in biotechnology for the optimization of media components and culture conditions (Chen, 1996). In order to obtain optimum yield of an enzyme, development of a suitable medium and cultural conditions is obligatory. Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components. Basically this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Using the mathematical model, the levels of variables giving maximum response can then be determined.

There are a number of reports in which the Plackett-Burman design has been used to screen the factors in a fermentation medium to be optimized in subsequent experiments (Krishnan et al., 1998; Reddy et al., 1999; Son et al., 1998; Srinivas et al., 1994; Yu et al., 1997).

In the present study, the Plackett-Burman experimental design was used to optimize the solid state culture conditions for the production of β-glucosidase by *A. sydowii*. The experiment was done according to the Plackett-Burman experimental design and the statistical analysis was carried out using fisher's test for ANOVA. Among the eleven parameters tested, moisture content, concentration of peptone and inoculum only showed positive significance for the β-glucosidase enzyme production. The test model was statistically significant with a confidence levels above 95%, and by the model, 91.62% of the variability in the response could be explained. A validation run was carried out under the conditions predicted by the model. The experimental values (1355.6, 1202.5, and 1338.3) were found to be close to the predicted values (1257.1, 1262.8, and 1255.7 respectively), and hence, the model was successfully validated. The results obtained from the statistical optimizations studies confirm the observations made during the conventional optimization studies conducted under SmF and SSF, particularly in the case of peptone more evidencingly.

5.5 Enzyme purification and characterization

The native PAGE analysis of the ion exchange purified β-glucosidase enzyme from *A. sydowii* showed a single band and the molecular weight and purity was determined by SDS-PAGE electrophoresis. The electrophoresis showed a single polypeptide band having a mass of ~95 kDa. The β-glucosidase activity of the corresponding band was confirmed as monomer by zymogram analysis. It should be

noted that the molecular mass of most fungal β-glucosidases are often greater than 80 kDa (Woodward and Wiseman, 1982). Extracellular β-glucosidases which have molecular masses of 96 kDa (Witte and Wartenberg, 1989), 100 kDa (Galas and Romanowska, 1997) and 105 kDa (Yan and Lin, 1997) have been purified from three different strains of *A. niger* . The β-glucosidases with molecular masses of 106 kDa, 114 kDa and 85 kDa from *Neurospora crassa* (Yazdi et al., 2003), *Phanerochaete chrysosporium* (Lymar et al., 1995) and *Stachybotrys* sp.(Amouri and Gargouri, 2006), have also been reported, respectively.

The temperature and pH optima for the activity and stability of the extracellular β-glucosidase of *A. sydowii* BTMFS 55 were comparable with respective values of other fungal β-glucosidases. The maximal enzyme activity was recorded at 50°C and it was active over a range of 30-70°C. The optimal pH and temperatures of β-glucosidases from various microbial sources range between 3.0-7.0 and 40-105°C, respectively (Coughlan, 1985; Kengen et al., 1993; Saha et al., 1995; Woodward and Wiseman, 1982). The optimum temperature (50°C) of the purified enzyme is similar to that of *A. oryzae* (Riou et al., 1998), *A. nidulans* (Kwon et al., 1992), *Candida peltata* (Saha and Bothast, 1996), *Stachybotrys* sp. (Amouri and Gargouri, 2006), *Volvarialla volvacea* (Li et al., 2005) and *T. reesei* (Cascalheira and Queiroz, 1999). The thermal stability (30-70°C for 12 h at pH 8.0) of the purified enzyme is sharing some what similar pattern reported for this enzyme from other fungal strains. The β-glucosidase of *Thermoasus auranticus* showed a thermal stability of 40-70°C (Parry et al., 2001) whereas, *Aureobasidium* sp. showed a range of 30-80°C (Hayashi et al., 1999), with an optimum at 80°C.

The optimal pH (5.0) activity of the purified enzyme was seemed to be acidic, ranging from 4.0-7.0. It is similar to that of *A. oryzae* (Zhang et al., 2007), *A. niger*

(Yan and Lin, 1997), *Neurospora crassa* (Yazdi et al., 2003), *Candida peltata* (Saha and Bothast, 1996), *A. fumigatus* (Ximenes et al., 1996), and *Stachybotrys* sp. (Amouri and Gargouri, 2006). The pH stability of the enzyme (4.0-6.0) supported with that from *Aureobasidium pullulans* (Saha et al., 1994) and *Candida peltata*(Saha and Bothast, 1996). It was observed that the β-glucosidase of Thermomyces lanuginosus (Lin et al., 1999) and *Aureobasidium* sp. (Hayashi et al., 1999) exhibited enzyme stability in a wide range of pH such as 5.0-12.0 and 2.2-9.8, respectively.

On the basis of substrate specificity, β-Glucosidases may be divided into three groups: (i) aryl-β-glucosidases, which have a strong affinity for aryl-β-glucosidases; (ii) cellobiases, which hydrolyze only oligosaccharides; and (iii) broad-specificity β-glucosidases, which exhibit activity on many substrate types and are the most commonly observed β-glucosidases. The purified β-glucosidase from *A. sydowii* hydrolysed both oligosaccharides such as cellobiose, maltose and lactose and glucosides such as *p*NPG. The enzyme showed high affinity towards the *p*NPG than the cellobiose and other disaccharides tested. Plant et al (1988) suggested that the preference of β-glucosidases for aryl glycosides is due to the high electrophilicity of the aglycone moiety, which enhances the stability of the *ortho* or *para* nitophenoxide anion generated during the first step of catalysis. The β-glucosidase from thermophilic fungus, *Talaromyces thermophilus* showed an affinity of lactose and maltose than cellobiose with a relative activities of 75, 61 and 6%, respectively (Nakkharat and Haltrich, 2006). The β-glucosidase of *A. sydowii* also showed such a result that it hydrolyses maltose and lactose more efficiently than cellobiose. β-glucosidases with very broad specificity have been isolated from many fungi (Gueguen et al., 1995; Park et al., 2005; Pitson et al., 1997; Watanabe et al., 1992; Yan and Lin, 1997).

The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plots under optimal conditions (30 min, pH 8.0, 50°C). The enzyme had an apparent K_m value of 0.67 mM and a V_{max} value of 83.3 μmol min^{-1} mg $protein^{-1}$ for the hydrolysis of *p*NPG. The K_m value of *A. sydowii* β-glucosidase (0.67 mM) supported with that of *A. oryzae* and *A. phoenicis* which have a lower K_m of 0.55 mM and 0.58 mM, respectively. *Thermomyces lanuginosus*, *A. fumigatus* (0.075 mM each) and *Phanerochaete chrysosporium* (0.096 mM) were also exhibited a much lower K_m.

Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases (Gueguen et al., 1995; Saha and Bothast, 1996; Saha et al., 1995; Yan and Lin, 1997) and most microbial enzymes show inhibition constants of 0.6 to 8 mM for glucose (Ozaki and Yamada, 1992). The *Ki* values of *Aspergillus* sp. have been reported to range from 3 to 14 mM (Yan and Lin, 1997). The β-glucosidase of *A. sydowii* BTMFS 55 showed an inhibition constant (K_i) of 17 mM. The availability of β-glucosidase insensitive to glucose have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol. Glucose tolerant fungal β-glucosidases were reported from *A. oryzae* (136 mM), *A. niger* (543 mM), *Candida peltata* (140 mM), *Pyrococcus furiosus* (300 mM) and *A. tubingensis* (470 & 600 mM) by various authors (Decker et al., 2001; Kengen et al., 1993; Riou et al., 1998; Saha and Bothast, 1996; Yan and Lin, 1997).

The β-glucosidases from *Sporotrichum thermophile*, *Monilia* sp., *Fusarium oxysporum*, *Neocallimastrix frontalis*, *Botrytis cinerea*, and *Streptomyces* sp. strain QM-B814 were competitively inhibited by glucose, with *Ki*s of 0.5, 0.67, 2.05, 5.5, 10.5, 65 mM, respectively (Bhat et al., 1993; Christakopoulos et al., 1994; Dekker, 1981; Gueguen et al., 1995; Li and Calza, 1991; Perez-Pons, 1994). Glucose inhibited

the β-glucosidase-catalyzed reaction of *T.viride* cellulase in a mixed inhibition pattern with a competitive character (Montero and Romeu, 1992). The inhibition of β-glucosidase from *Pyrococcus furiosus* by glucose was almost negligible, with a *Ki* of 300 mM (Kengen et al., 1993). Aryl-β-glucosidase of *Trichoderma* spp. was totally inhibited by 1% glucose, and *Microbispora bispora* aryl-β-glucosidase was 35, 66, and 79% inhibited by 10, 20, and 30% glucose, respectively (Waldron et al., 1986). A cloned β-glucosidase from *Microbispora bispora* was activated two- to threefold in the presence of 2-5% glucose and tolerate up to 40% concentration (Wright et al., 1992). β-glucosidase from a *Streptomyces* sp. was activated twofold by 1.8% glucose (Ozaki and Yamada, 1992). In *Streptomyces* sp., the rate of *p*NPG hydrolysis has been enhanced the glucose at a concentration of 25 to 200 mM (Perez-Pons et al. 1995).

It was observed in the present study that organic solvents had a stimulating effect on β-glucosidase activity especially alcohols at lower concentrations. Among the solvents tested, ethanol was the most effective one which could enhance the activity 41% at a concentration of 10% (v/v) followed by methanol (30%), and n-butanol (21%) except for propanol. This activation decreased with increasing alcohol concentration. The enzyme activity was decreased with an increase of propanol concentrations. Methanol could enhance the enzyme activity at lower concentrations of 10 and 20 % while butanol and ethanol led to increased enzyme activity only at 10% level. The activation increased with the chain length of alcohols and the branched alcohols gave the more activation fold than the normal alcohols. The alcohol tolerant enzymes were reported to have some transglucosylation activities (Yan and Lin, 1997). The β-glucosidase could preferentially utilize alcohols rather than water as acceptors for the glycosyl moiety during catalysis of *p*NPG, resulting in elevated reaction rates, which suggests that ethanol increases the hydrolysis rate of *p*NPG by

acting as an acceptor molecule for glucose (Saha et al., 1994). Therefore, it is inferred that the purified β-glucosidase from *A. sydowii* may have transglucosylation activity.

Activation of enzyme by ethanol was observed from *A. tubingensis* (Decker et al., 2001), *A. oryzae* (Riou et al., 1998), *Candida peltata* (Saha and Bothast, 1996) *Fusarium oxysporum* (Christakopoulos et al., 1994), *Dekkera intermedia* (Blondin et al., 1983), and *Aurebasidium pullulans* (Saha et al., 1994). This activation may be due to glucosyltransferase activity of the enzyme (Pemberton et al., 1980). In *F. oxysporum* the presence of ethanol increased the β-glucosidase activity 1.5 fold (Christakopoulos et al., 1994). The initial β-glucosidase activity of *C. peltata* was stimulated 11% by ethanol at a concentration of 0.75%.

Various metal ions and potential inhibitors modified the activity of the purified enzyme. The enzyme was indeed greatly inhibited by Mg^{2} and Ag^{2-} . This may indicate that thiol groups are involved in the active catalytic site. However, Mn^{2-} did significantly stimulate enzyme activity at 1 and 5mM and completely inhibited at 10mM concentration. Since Mn^{2-} is not involved in the stability of the enzyme, this specific cation could play a role in the enzyme function (e.g., by modulating its activity according to environmental conditions). In the case of metal ion effects, Cu^{2-} has been generally reported as a strong inhibitor for fungal β-glucosidases (Cao and Crawford, 1993; Gueguen et al., 1995; Li and Calza, 1991; Sasaki and Nagayama, 1995) , but here, this metal ion activates the enzyme at a concentration of 1 mM. Cu^{2-} ions are generally involved in the enzyme reaction sequence. Yazdi et al. (2003) reported the activation of β-glucosidase of *Neurospora crassa* by Cu^{2-} ions at 1 mM level. The Ca^{2-} activated the enzyme at 1 mM concentration. The Ca^{2-} appears to play a role in maintaining the structure required for catalytic activity and enzymes requiring Ca^{2-} for activation are mainly extracellular one.

The enzyme exhibited moderate inhibition with reducing reagents such as β-mercaptoethanol, chelating reagent (EDTA), and detergent (SDS). The inactivation of DEPC observed indicates that tryptophan and histidine residues are important in the catalytic action of the enzyme. The chelating agent EDTA did not inhibit β-glucosidase activity, indicating that divalent cations are not required for enzyme activation.

5.6 Application studies

Bioethanol has significant environmental advantages over petroleum as a liquid fuel (Duff and Murray, 1996) if produced from cheap, renewable lignocellulosic feedstocks. Among the systems for ethanol production from cellulose, the simultaneous saccharification and fermentation (SSF) process has attracted many investigators (Hari Krishna et al., 1998). The SSF process offers benefits such as improved ethanol yields by reducing the product inhibition exerted by saccharification products and also eliminates the need for separate reactors for saccharification and fermentation, which results in cost reductions.

The production of ethanol from wheat bran and rice straw were performed after a pretreatment with 0.5% H_2SO_4 followed by autoclaving for 30 min. Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment mainly because the lignin in plant cell walls forms a barrier against enzyme attack (Sewalt et al., 1997). An ideal pretreatment reduces the lignin content and crystallinity of the cellulose and increases surface area (Takagi et al., 1977).

The production of ethanol from wheat bran and rice straw with help of baker's yeast in the presence of the purified β-glucosidase from *Aspergillus sydowii* BTMFS

55 showed a considerable yield of ethanol production in range of 24-48 h of incubation.

A considerable level of yield of ethanol from wheat bran was obtained after 48 h (11.2 g/L) at 37°C. Whereas, in the fermentation medium containing rice straw, the production was maximal at 24 h with an ethanol yield of 10.32 g/L. It was reported that the ethanol production rate was much higher at the initial stage of the fermentation such as 22 h (Saha et al., 2005). An incubation period of 24 h has been found to be optimum for production of ethanol by *S. cerevisiae* from acid and enzymatic hydrolysate of agricultural residues (Tewari et al., 1987).

The ethanol production from wheat bran with an enzyme yield of 17 g/L by simultaneous saccharification and fermentation with a recombinant *E. coli* strain, after 112 h was reported (Saha et al., 2005). Ethanol has been produced from a variety of substrates such as sunflower hull (Sharma et al., 2004), raw corn flour (Wang et al, 2007), wheat bran (Saha et al., 2005), lignocellulose from a weedy creeper, *Antigonum leptopus* and sugar cane leaves (Harikrishna et al., 2000,1998), barley husk (Adrados et al., 2005) and water hyacinth (Nigam, 2007).

Temperature is a crucial factor for Simultaneous Saccharification and Fermentation because of the differences in saccharification optima (50°C) and that of the yeast (35 °C). A temperature range of 39-40 °C was observed to be optimum for maximum ethanol yields. Slininger et al. (1987) reported that optimum fermentation rates with *Pachysolen tannophilus* were obtained at 32°C. They suggested that at high temperatures either the enzyme is not induced and/or once formed the enzyme degrades rapidly. According to Philippidis (1995), the optimal temperature for simultaneous saccharification and fermentation is around 38°C, which is a compromise between the optimal temperatures for hydrolysis (45-50°C) and

fermentation (30°C). The effect of initial pH value ranging from 3.5 to 5.5 on fermentation of enzymatic hydrolysate of sunflower hulls revealed maximum ethanol yield of 0:455 g/g at a pH of 5.0. A pH value lesser than 4.0 and higher than 5.0 resulted in sharp decrease in the ethanol yield (Sharma et al., 2004). The temperature, pH, substrate concentration, pretreatment of the lignocellulosic substrates etc are the significant factors that govern the ethanol production in large scale for the industrial use.

It is concluded that the production of ethanol from wheat bran and rice straw facilitated by the action of β-glucosidase from *A. sydowii* has potential for utilization of the process as a preliminary step for the ethanol production in large scale for industrial purposes. Further work is warranted for further optimization of various process parameters for increasing the yield of ethanol from these substrates.

Chapter 6

SUMMARY AND CONCLUSION

A potential fungal strain producing β-glucosidase enzyme from the marine environment was screened among 100 isolates, and identified as *A. sydowii* BTMFS 55 by MTCC, IMTECH, Chandigarh. Later, it was confirmed by a molecular approach based on 28S rDNA sequence homology which showed 93% identity with already reported sequences of *Aspergillus sydowii* in the GenBank.

A. sydowii BTMFS 55 isolated from seawater was observed to produce all the three types of cellulase enzyme complex, with a higher yield of β-glucosidase enzyme.

The process optimisation of the optimal media components which supported the maximal β-glucosidase production under submerged fermentation in Czapek-Dox minimal media was performed. A concentration of 2% glucose as the sole carbon source, along with 0.5% ammonium sulphate, 0.5% NaCl and 4% inoculum at a pH of 9.0 at 30°C supported maximal enzyme production. The time course experiment with the optimised conditions preferred with the fungus indicated that maximal β-glucosidase enzyme with a maximal activity of 14.1 U/ml could be obtained after 7 days.

The β-glucosidase enzyme production under solid state fermentation was optimised using wheat bran as the substrate for the growth and enzyme production. The optimised levels of different parameters for the maximal production were observed with wheat bran of particle size 425μ, moisture content- 60%, CMC- 0.5%,

peptone- 0.5%, NaCl- 0.5%, spore inoculum-10%, at pH 8.0, and an incubation temperature of 30°C. The maximal enzyme production was recorded after 96 h of incubation (1401 U/gIDS) under the above said conditions. It was observed that the marine fungus *A. sydowii* produced much higher yield of β-glucosidase enzyme under solid state fermentation than submerged condition.

The optimisation of various bioprocess variables that influenced maximal β-glucosidase production employing statistical approach under SSF was also done using Plackett-Burman experimental design. The results indicated that most of the parameters tested have positive effects on the enzyme production. Moisture content, concentration of peptone and inoculum had significant effects on β-glucosidase production under SSF. The test model was statistically significant with a confidence levels above 95%.

The β-glucosidase enzyme was purified by ammonium sulphate precipitation followed by ion exchange chromatography on DEAE Sepharose. The enzyme fraction was eluted as a single active peak with 7 fold of purification. The native PAGE and SDS-PAGE analysis evidenced the single polypeptide nature of the enzyme, and the activity was again confirmed with a zymogram staining using 4-methylumbelliferyl-β-D-glucopyranoside.

The purified β-glucosidase was noted to have 50°C as optimal temperature and pH 5.0 as optimum respectively for maximal activity. It was stable at pH 4.0-6.0 after 24 h at 50°C. The enzyme at 50°C showed thermal stability with a 10% increase in residual activity, and was stable over a range of temperature varying from 40°C to 60°C, after 24 h of incubation.

The purified β-glucosidase showed high affinity towards *p*NPG compared to cellobiose, lactose and maltose. It hydrolysed maltose, which have a (1-4)-α-glycosdic bond. Since the enzyme showed more affinity towards *p*NPG, it is considered as an aryl-β-glucosidase. The reaction kinetics of the enzyme exhibited a K_m and V_{max} of 0.67 mM and 83.3 U/ml, respectively. The specificity constant (V_{max} / K_m) for the *p*NPG hydrolysis was determined as 123.37. The molar activity (k_{cat}) and catalytic efficiency (k_{cat}/ K_m) of the enzyme was determined to be 2.14 x 10^{-2} s^{-1} and 3.19 x 10^{-2} mM^{-1} s^{-1}, respectively.

Glucose acted as a competitive inhibitor with an inhibition constant of 17 mM. Thus the β-glucosidase enzyme of *A. sydowii* is considered as glucose tolerant. This particular property is one of the important aspects of the enzyme since glucose tolerant β-glucosidases reduce the cellobiose inhibition and allow the enzyme to function more efficiently. Glucose insensitive β-glucosidases are highly useful for the enzymatic conversion of cellulosic biomass to glucose for the production of ethanol.

The enzyme activation by the presence of low concentration of alcohols, especially ethanol, is considered as another important feature of this enzyme. The activation by ethanol (41% increase of activity at 10% concentration) makes the enzyme very attractive for application in flavour release as well as in the simultaneous saccharification and fermentation of cellulosic materials to ethanol. Metal ions such as Mg^{2+} and Ag^{2+} inhibited the enzyme activity, whereas, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Hg^{2+}, K^{+} and Zn^{2+} activated the enzyme activity at 1mM level. Cu^{2+} supported enhancement of β-glucosidase activity at 1 mM concentration, inspite of the reports that it is a strong inhibitor of β-glucosidases. Various inhibitors and activators such as β-mercaptoethanol, SDS, DEPC, EDTA, and DMSO did not activate the enzyme.

The production of bio-ethanol from lignocellulosic materials is considered to be one of the major applications of β-glucosidases enzyme. The β-glucosidase by *A. sydowii* BTMFS 55 could produce ethanol from wheat bran and rice straw with the help of yeast, *S. cerevisiae*. Maximal ethanol production from wheat bran (11.2 g/L) was observed at 48 h, while in the case of rice straw it was at 24 h (10.32 g/L), facilitated by β-glucosidase.

Conclusion

The β-glucosidase enzyme purified from the marine fungus, *Aspergillus sydowii* BTMFS 55 showed a good yield of enzyme production under solid state fermentation. The statistical optimization of the media components revealed that moisture content, concentration of peptone and inoculum are the major parameters which supported the maximal enzyme production. The purified enzyme showed low pH activity and stability, glucose tolerance and activation by ethanol. It could produce ethanol from wheat bran and rice straw by simultaneous saccharification and fermentation with yeast.

In conclusion, the β-glucosidase purified from *Aspergillus sydowii* BTMFS 55 shows great potential for several biotechnological applications such as the production of bio-ethanol from agricultural biomass and improvement in the aromatic character of wines and fruit juices through the hydrolysis of flavour glucosidic precursors. There is immense scope for the application of this marine fungus in the biofuel production besides in other industries provided further studies are pursued in exploiting this enzyme and the organism particularly scale up studies with respect to application. There is also ample scope for cloning of the gene encoding β-glucosidase in domesticated hosts such as *Pichia pastoris* or *S. cerevisiae* that can produce ethanol directly from cellulosic biomass.

Chapter 7

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