P-3414

ISOLATION, PURIFICATION AND CHARACTERIZATION OF INDUSTRIALLY IMPORTANT ENZYMES

A Project Report

Submitted by

DEEPIKA R	**0710204009**
NANDHINI M	**0710204028**
SANDHYA R	**0710204041**
SOWMIYA K	**0710204048**

In partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIO TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An Autonomous Institution affiliated to Anna University of Technology, Coimbatore)

COIMBATORE-641 049

APRIL: 2011

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE-641 049

BONAFIDE CERTIFICATE

Certified that this project report entitled **"Isolation, purification and Characterization of Industrially important Enzymes"** is the bonafide work of

DEEPIKA R	-	**Register No.0710204009**
NANDHINI M	-	**Register No.0710204028**
SANDHYA R	-	**Register No.0710204041**
SOWMIYA K	-	**Register No.0710204048**

who carried out the project work under my supervision.

SUPERVISOR

Dr.P.RAMALINGAM

ASSISTANT PROFESSOR

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641 049

DEAN

DR.S.SADASIVAM

DEAN AND HEAD OF THE DEPARTMENT

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641 049

19/4/11

Internal Examiner

19/4/11

External Examiner

ACKNOWLEDGEMENT

We are happy to thank **Dr. S. Ramachandran**, principal , Kumaraguru college of Technology and the **Management**, Kumaraguru college of Technology for providing us all the facilities to carry out the project work.

Our sincere thanks to **Dr. S. Sadasivam**, Dean and Head of the Department, Department of Biotechnology, Kumaraguru college of Technology. His gracious and ungrudging guidance all through our project work is highly acknowledged with gratitude.

With our deepest sense of gratitude we extend our heartfelt thanks to **Dr.P.Ramalingam**, Associate Professor, Department of Biotechnology, Kumaraguru College of Technology, for his relentless support, masterly guidance, creative ideas and patient efforts for successful completion of the project.

We owe a lot to our committee members, **Dr.N.Saraswathy,** Associate professor and **Dr.V.Stephen Raphael,** Associate professor, Department of Biotechnology for helping us with their valuable ideas and suggestions.

We would like to express our thanks to **Mr.M.Shanmugaprakash,** our Project coordinator and Assistant Professor, Department of Biotechnology, Kumaraguru college of Technology, for his valuable suggestions throughout my project work.

We are happy to thank **Mr. S. Senthil Kumar**, our class advisor and Assistant Professo, Department of Biotechnology, Kumaraguru college of Technology, for his unsolicited and timely help and encouragement without any hesitation.

We wish to extend our thanks to all **Teaching** and **Non- Teaching staffs** of the Department of Biotechnology for their kind and patient help throughout the project work.

We thank all our friends who physically and emotionally helped us to bring out the work successfully.

Finally, we wish to express our deep sense of gratitude to our beloved parents and family members for their constant encouragement and love, without whose inspiration this study would not have seen the dawn of day.

R. Deep.
(DEEPIKA.R)

M. Nan
(NANDHINI.M)

R. Sandhya
(SANDHYA.R)

(SOWMIYA.K)

CONTENTS

ABSTRACT

ABSTRACT

α-Galactosidase and cellobiase were partially purified by acetone precipitation from fungal isolates while xylanase was partially piurified by ammonium sulphate fractional precipitation.

α-Galactosidase enzyme was optimally active at 60°C and pH 4.0. Heavy metal ions such as Ag^{+} and Hg^{2+} strongly inhibited enzyme activity to about 80.93 % and 51.3 %, respectively. Among the sugars tested, fructose and galactose inhibited α-galactosidase activity by 29.41% and 24%, respectively. In the reagents tested, N-bromosuccinimide inhibited the α-galactosidase activity to about 27%.

Cellobiase was optimally active at 70°C and pH 4.5. Heavy metal ions such as Fe^{2+} and Cu^{2+} strongly inhibited the enzyme activity to about 87.3% and 84.5%, respectively. Among the sugars tested, Glucose, galactose and raffinose inhibited β-glucosidase activity by 100%, 55.3% and 100%, respectively. In the reagents tested, N-bromosuccinimide completely inhibited the cellobiase activity..

Xylanase was optically active at 55°C and pH 5.0. Heavy metal ions such as Ag^{+}, Fe^{3+}, Hg^{2+} and Ca^{2+} showed complete inhibition of the xylanase activity. Among the sugars tested, fructose, arabinose, xylose and glucose exhibited total enzyme inhibition. In the reagents tested 1,10-phenanthroline completely inhibited the xylanase activity while EDTA to about 76.74% of its original enzyme activity indicating that it is a metalloenzyme.

Key words: α-Galactosidase, xylanase, cellobiase, raffinose

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

Abbrevations	Explanations
µg	Microgram
µl	Microlitre
Mg	Milligram
Ml	Millilitre
H	Hour
L	Litre
Min	Minutes
EDTA	Ethylene diamine tetra acetic acid
PNPG	p-Nitrophenyl-α-D-galacto-pyranoside
M	Molar
mM	Millimolar
Nm	Nanometer

INTRODUCTION

1. INTRODUCTION

1.1 α-Galactosidase

α-Galactosidase (EC.3.2.1.22) is an glycoside hydrolase enzyme that hydrolyzes (breaks) alpha-1,6-glycosidic bonds in galactosyl oligosaccharides (alpha-galactosides) of raffinose family of sugars such as raffinose, stachyose and polymeric galactomannans and guar gum (Dey & Pridham 1972); thus liberating simpler sugars (galactose, glucose, fructose, and sucrose). Alpha galactosidase is an enzyme that has several different functions. It is used by intestinal microorganisms to degrade the sugars in the complex carbohydrates found in vegetables and grains. This process often leads to the production of gases that can cause bloating and flatulence. This temporary condition may be treatable with dietary supplements containing alpha galactosidase. The absence of this enzyme can cause a genetic disorder known as Fabry's disease.It also has several industrial applications. (Zeilinger *et al.*, 1993) reported the use of this enzyme in the paper industry for the hydrolysis of galactomannose, one of the main wood hemicellulose components. α-Galactosidase has been used in the sugar beet refining, in the improvement of gellifying properties of thickeners, and galacto-oligosaccharide reduction in soybean milk and vegetable products (Kotwal *et al.*,1997). Moreover, it is used in enzyme replacement therapy for Fabry disease (Zang *et al.*, *2009*).

α-Galactosidases are widely distributed in nature, where it has been purified from a number of sources including plants, animals and microorganisms. The enzyme is ubiquitous in legume seeds. It is present in sweet almond (Malhotra & Dey 1967), melon fruit (Zhifang & Arthur 1999), and immature stalks of sugar cane (*Saccharum officinarum*) (Chinen *et al.*, 1981). Among microorganisms, α-galactosidase activity was first detected in brewers' yeast . Subsequently, it was also detected in *Aspergillus niger* (Ronald *et al.*, 1999), *Trichoderma reesei* (Zeilinger *et al.*, 1993), *Monascus pilosus* (Wong *et al.*, 1986), *Humicola* sp (Kotwal *et al.*,1997), *Lactobacillus plantarum* (Sanni *et al.*, 1995), *Aspergillus oryzae* (Ramalingam and Mulimani, 2004) and *Penicillium purpurogenum* (Ramalingam et al., 2009).

Humans lack the enzyme α-galactosidase, which is required to hydrolyze α-1, 6 bonds of raffinose and stachyose. The undigested raffinose and stachyose enter the colon and can cause intestinal flatulence when anaerobic microorganisms ferment these

galactosaccharides producing carbon dioxide, hydrogen and methane. This can result in discomfort, headaches, dizziness, and slight mental confusion, reduced ability to concentrate, slight retinal edema, diarrhoea, dyspepsia, constipation and painful contraction of the colon. The use of α-galactosidase is one of the method to reduce the flatulence factors. Although α-galactosidases have been extracted from microorganisms, only a few are available commercially, which are very expensive.

In plants and micro-organisms, the enzyme is involved in a variety of processes, importantly in the hydrolysis of oligosaccharides such as raffinose and stachyose during the early germinatiron period, resulting in the liberation of soluble sugars, which may serve as a ready energy source for the growing plant (Dey *et al.*, 1972). In animals this enzyme hydrolyses galactolipids. The reduced activity of α-galactosidase results in Fabry's disease in humans, a deficiency resulting from the progressive accumulation of globotriaosylceramide and related glycosphingolipids. Affected patients have microvascular disease of the kidneys, heart, and brain (Feldt-Rasmussenet *et al.*, 2002). The enzyme also occurs in brain tissues with possible involvement in the hydrolysis of monogalactosyldiglycerides and digalactosyl diglycerides (Subba Rao *et al.*, 1970).

The hydrolytic action of alpha-galactosidase on various sugars is shown as below:

Melibiose yields D-galactose and D-glucose,

Raffinose yields one molecule of galactose and sucrose,

Stachyose yields two molecules of galactose and sucrose,

Verbascose yields three molecules of galactose and sucrose.

1.1.1 Specificity

α-Galactosidase exhibited a broad range of specificity and removes the terminal α- D-galactose attached by α-1,2-, α-1,3-, α-1,4-, and α-1,6-linkages (Dey and Pridham, 1972, Varbanels *et al.*, 2001). α–Galactosidase exhibits two types of activity. They are as follows:

A. Hydrolase activity, and

B. Transgalactosylase activity.

A. Hydrolase activity:

The configuration of hydrogen and hydroxyl groups on any single carbon atom of a glycosidic substrate plays an important role on the hydrolytic action of particular hydrolases. The hydrolytic action of α–galactosidase on its substrate depends on two main factors, which are as follows:

i) The ring structure of the substrate must be pyranoid, and

ii) The configuration –H and –OH groups on carbon atoms 1, 2, 3, and 4 must be similar to that on α–D-galactose.

Like other carbohydrates, α-galactosidase can also tolerate at C-6 of the glycosyl moiety of the substrate. Hence, α–galactosidase from several sources has the capacity to hydrolyze β-L-arabinosides. However, α–galactosidase from *Streptococcus bovis*, *Diplococcus pneumoniae* and *Calvatia cyathiformis* cannot act on arabinosides (Dey and Pridham, 1972) have reported that p-nitrophenyl α–D-fucoside can be hydrolysed by α–galactosidase, because p-nitrophenyl α–D-fucoside has the similar configuration with that of D-galactose. α–Galactosidase from sweet almond and yeast are not able to hydrolyze the α-isomer of D-glycero-D-galactoheptoside.

B. Transgalactosylase Activity

Blanchard and Albon (1950) for the first time reported the transferase properties α–galactosidase from yeast. They found that galactose from one melibiose was transferred to a second melibiose acceptor molecule leading to the formation of manninotriose. This transgalactosylation property of α-galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity, donor and acceptor concentration, pH, temperature and the source of enzyme (Dey and Pridham, 1972). Apart from hydrolase activity, α–galactosidase also exhibits transglycosylase properties, but relatively at high substrate concentrations (Ohtakara *et al.* 1984).

In tranglycosylation reactions, glycogen moiety from donor is accepted by hydroxyl group of methionine present at the active site of α-galactosidase from *Trichoderma reesi* (Eneyskaya *et al.*, 1998). Van Laere et al., (1999) reported that α-galactosidase from *Bifidobacterium adolescentis* synthesized stachyose in the presence of raffinose, and from stachyose, verbascose was obtained.

1.1.2 Raffinose-family of sugars

The oligosaccharides belong to raffinose-family sugars are raffinose, stachyose and verbascose. These sugars contain one, two or three galactose units joined to sucrose by α–1,6-linkages. The raffinose-family sugars have been identified as one of the contributors of flatus (gas production) inhuman and experimental animals (Cristafaro *et al.*, 1973). The raffinose family of oligosaccharides (RFO) are almost ubiquitous in the plant kingdom,being found in a large variety of seeds. Humans and other monogastric animals do not possess the alpha-galactosidase enzyme to break down these RFO and the oligosaccharides pass undigested through the stomach and upper intestine.In the lower intestine they are fermented by gas-producing bacteria that produce carbon dioxide, methane or hydrogen.

1.1.3 Raffinose

Raffinose[O-α–D-galactopyranosyl-(1→6)-α–D-glucopyranosyl-(1→2)-β-D-fructo furanoside] is a complex carbohydrate.It can be found in beans, cabbage, broccoli, other vegetables and whole grains. Raffinose was first isolated in a crystalline form from *Eucalyptus mann* (Johnston, 1843). Raffinose and related oligosaccharides accumulates during seed maturation (Holl and Vose, 1980). Raffinose-family oligosaccharides are important sugar translocators in several plant species, such as cucurbits. The accumulation of raffinose is often linked to frost-hardiness of plants and their levels can be regulated by both temperature and photoperiod.

1.1.4 Stachyose

Stachyose[O-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D-gluco pyranosyl-(1→2)-β-D-fructofuranoside] was first isolated from the rhizomes of *Stachys tubifera* (Dey, 1985). In storage organs, for example tubers of *Stachys steboldi*, vacuoles are the site of storage of stachyose. The mechanisms of uptake of oligosaccharides and sucrose are quite similar. A steep concentration gradient of stachyose from cytosol to vacuole exists and the transport is antiport manner (Keller, 1992).

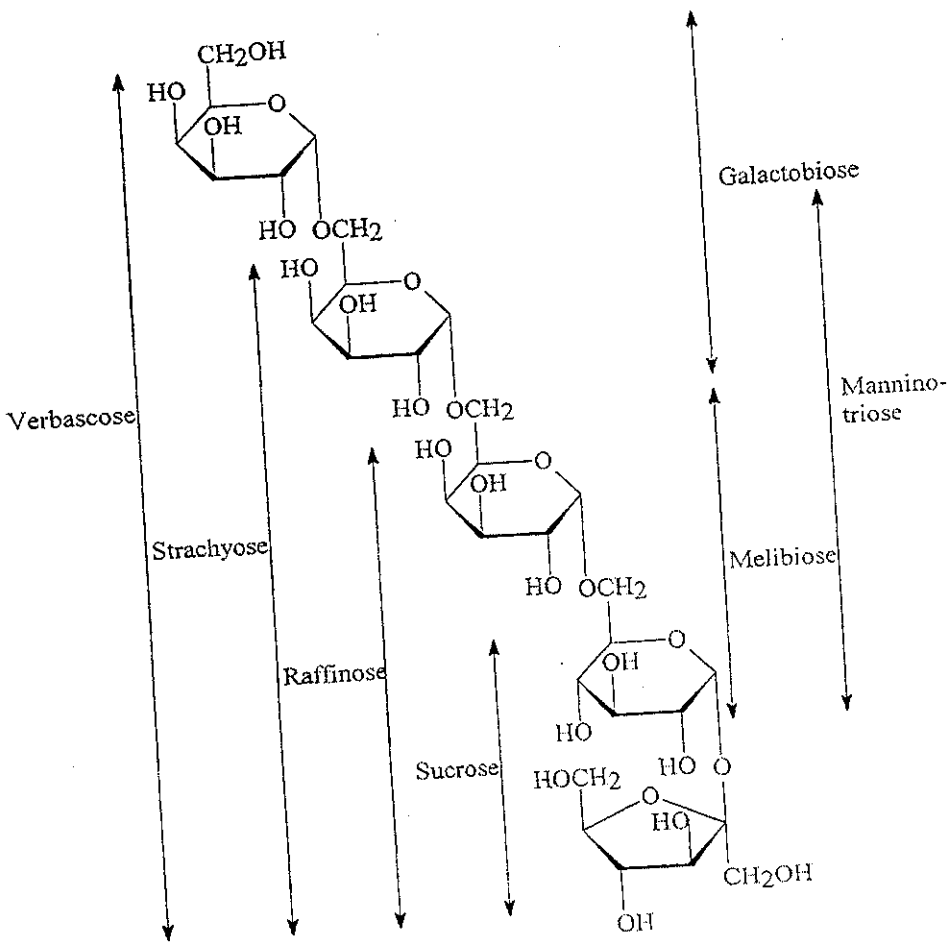


Figure 1: Structural relationship of raffinose family of sugars

1.1.5 Verbascose

Verbascose[O-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D-glucopyranosyl-(1→2)-β-D-fructofuranoside] was first isolated from the roots of *Verbascum thapus* by (Bourquelot and Bridel 1910). *In vitro* experiment using an enzyme preparation from the mature seeds of *Vicia faba* demonstrated that the transfer of galactinol to stachyose yielding verbascose.

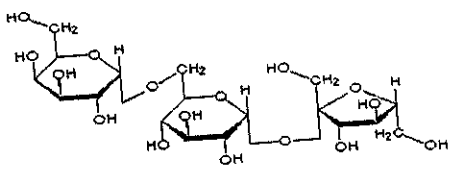


Figure 2: Chemical structure of Raffinose

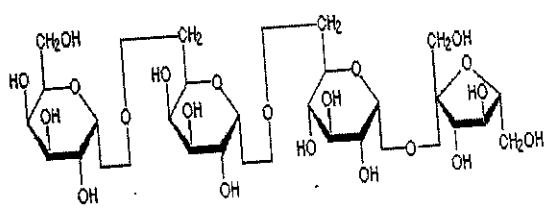


Figure 3: Chemical structure of stachyose

1.1.6 Applications of α-Galactosidase

α–Galactosidase is widely distributed in nature. α–Galactosidases are a group of exotype carbohydrases, which release α–D-galactose from melibiose, raffinose, stachyose, verbascose, galactomannans glycoproteins, ceramide trihexosides and the higher homologous as well as derivatives (Dey and Pridham, 1972)

α–Galactosidase has the following applications:

A. Beet sugar industry
B. Pulp and paper industry
C. Food processing industry
D. Medical application

E. Hydraulic fracturing of oil and gas wells

1.1.6.1 Applications of α–galactosidase in beet sugar industry:

The content of raffinose in the sugar beet is gradually increased during storage and usually it comes to 0.15%. In the beet sugar industry raffinose is known as an obstacle substance for the normal crystallization of beet sugar.The only alternative before the beet sugar industry is the use of crude α-galactosidase (Puchart *et al.*, 2000). Crude α-galactosidase from microbial sources is used to hydrolyze raffinose into galactose and sucrose.

1.1.6.2 Use of α-galactosidase in pulp and paper industry:

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature. The major constituents of hemicellulases are hetero-1,4-β-D-xylans and the hetero-1,4- β-D-mannans (galactoglucomannans and glucomannans). Heteroxylans are most abundant in softwoods. In the case of galactomannans (softwood), enzymatic hydrolysis occurs with the concerted action of the following hydrolytic enzymes: endo-β-1,4-mannases [EC 3.2.1.78], α-galactosidase [3.2.1.22], and β-glucosidase [3.2.1.21]. In the soft wood pulp bleaching, microorganisms, which could produce enzymes β-mannases and α-galactosidase without cellulose, are preferred to avoid the degradation of cellulose (Zeilinger *et al.*, 1993).

1.1.6.3 Application of α-galactosidase in food processing industry :

α-Galactosidase is potentially important in the hydrolysis of raffinose-family of oligosaccharides (raffinose, stachyose, verbascose and ajugose) in pulses. While pulses are good sources of protein, many common varieties of pulses are known to contain anti-nutritional factors including oligosaccharides of the raffinose-family, in which galactose is present in α-linkage are present in mature seeds (Shallenberger and Moyer, 1961). Raffinose and stachyose present in soymilk and pulses are responsible for intestinal discomfort and flatulence.

1.1.6.4 Medical applications of α-galactosidase:

The α-galactosidase is capable of hydrolyzing the non-reducing terminal α-D-galactopyranosyl residue of blood group–B erythrocytes, thus leading to blood group O - erythrocytes. α-Galactosidase from a number of plant and microbial sources is able to release D-galactose from blood group-B. However, hydrolysis of the terminal α-galactopyranosyl moieties of the blood group-B substances has been reported for a limited number of α-galactosidases, including those from coffee beans, soybeans, *Streptomyces sp.* and *Trichoderma foetus*. Fabry disease of humans is due to a deficiency of thermolabile lysosomal α-galactosidase. α-Galactosidase may be used in the near future for such medical purposes as enzyme therapy

1.2 Xylanase

xylanase (E.C.3.2.1.8) deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans also produce xylanase. Xylanase belong to the glucanase enzyme family and are characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Xylanase readily crystallizes in ammonium sulphate and sodium/potassium phosphate across pH 3.5 to 9.0. Xylanase can also be crystallized with other salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulphate. Xylanase solubility in phosphate buffer pH 9 decreases in the temperature range of 0°C to 10°C. But remains constant in the pH range of 10 through 37°C. Xylanase has been extracted from many different fungi, bacteria and actinomycetes. It is commonly used in animal feeds, paper production and food production.

Microbial xylanases have commercial applications in agriculture, industry and human food production. Xylanases are useful in bioconversion of lignocellulosics to fuel and chemicals, to improve silage for better digestion by ruminants, to improve quality of detergent, and also used for clarification of juices, in flour improvements for bakery products

and controlling environmental hazards through biopulping. Xylanases used in pulp pre-bleaching process remove the hemicelluloses, which bind to the pulp. The hydrolysis of pulp bound hemicelluloses releases the lignin in the pulp, reducing the amount of chlorine required for conventional chemical bleaching and minimizing the toxic, chloroorganic waste.

Therefore, xylanases from alkalophilic bacteria actinomycetes have been studied widely. The use of thermostable alkaline xylanases for enzyme assisted pulp bleaching could greatly reduce the need for pH and temperature adjustment, thus offering enormous technical and economic advantages. Thermostable xylanases active at alkaline pH are of great interest for application in the pulp and paper industry to decrease the consumption of chlorine chemicals.

1.2.1 Xylan

The substrate for xylanase is xylan, which is an important constituent of the plant cell wall. Xylan, a polymer with a β-1, 4- linked backbone of xylose, is the major carbohydrate in the hemicellulose portion of plant cell walls. Xylan is the most abundant noncellulosic polysaccharide present in both hardwoods and annual plants, and accounts for 20-35% of the total dry weight in tropical plant biomass. In temperate softwoods, xylans are less abundant and many comprise about 8% of the total dry weight. The principal monomers present in most of the hemicellulose are D-mannose, D-galactose and L-arabinose. The main chain of xylan is made up of β-xylopyranose residues that are linked by β-(1→4) linkages.

Xylan is considered to be forming an interphase between lignin and other polysaccharides. It is likely that xylan molecules covalently link with lignin phenolic residues, and also interact with polysaccharides, such as pectin and glucan. In simplest forms, xylans are linear homopolymers that contain D-xylose monomers linked through β-1, 4-glycosyl bonds.

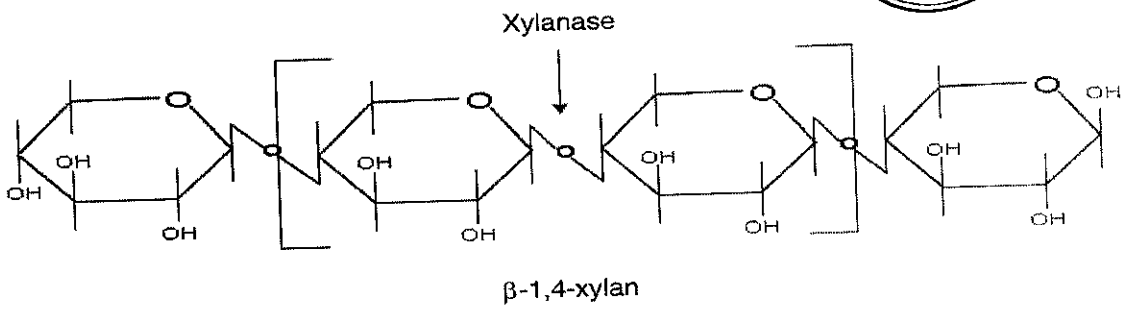


Figure 4: Structure of xylan, xylopyranosyl residues linked through β-1, 4 linkages; point of xylanase action is shown

1.2.2 Applications of xylanase

Xylanase has proven useful in many ways:

1 **Biobleaching paper pulp**: The use of xylanase leads to a reduction in organo-chlorine pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected.

2 **Improving animal feed**: Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal litter.It also changes hemicellulose to sugars so that nutrients formerly trapped within the cell walls are released.

3 **Improving silage** (or enhanced fermentative composting): Treatment of forages with xylanase (along with cellulase) results in better quality silage and improves the subsequent rate of plant cell wall digestion by ruminants. There is a considerable amount of sugar sequestered in the xylan of plant biomass. In addition to converting hemicellulose to nutritive sugar that the cow or other ruminant can digest, xylanase also produces compounds that may be a nutritive source for the ruminal microflora.

4. In Baking: Xylanase transforms water-insoluble hemicelluloses into soluble form, which binds water in the dough,therefore decreasing dough firmness, increasing volume and creating finer and more uniform crumbs. Thus it is used to improve dough handling

properties, to enhance bread quality, extend shelf life by reducing the staling rate, and they appear to be particularly effective in straight dough process.

1.3 Cellobiase or β-Glucosidase

β-Glucosidase (Cellobiase or β-D-glucoside glucohydrolases; EC 3.2.1.21) catalyze the hydrolysis of β-(1,4)-glucosidic linkages in the terminal non-reducing β-glucoside residues of cellobiose and lactose in to simple sugars (glucose and galctose) as well as the synthesis of oligosaccharides by transglycosylation (Chodi Masui *et al.*, 2009). Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition allowing endoglucanase and exoglucanase enzymes to function more efficiently (Christine Riou *et al.*, 1998).

In humans and other mammals the enzyme is involved in the hydrolysis of fatty substance glucosyl ceramides. The deficiency of the enzyme leads to Gauchers's disease. This can result in enlarged spleen and liver, liver malfunction, skeletal disorders and painful bone lesions, swelling of lymph nodes, distended abdomen, a brownish tint to the skin, anemia, low blood platelets and yellow fatty deposits on the white of the eye. Fungal β-glucosidases have broad substrate specificity and are used in a range of biotechnological processes. The most intensively studied area of their application is the saccharification of high cellulosic biomass in to fermentable sugars for fuel ethanol production (Ming Chen *et al* .,2006).

The β-glucosidase catalyse the hydrolysis of lactose (Korish M *et al* ., 2007), in addition to that they catalyse transgalactosylation reaction(. Both the hydrolase and transferase activity have recently attracted interest because of possible applications in food industries, such as production of low lactose milk which alleviates lactose maldigestion problems such as abdominal pain, flatulence or diarrhoea which may result from the fermentation of undigested lactose by colonic bacteria with production of H_2, CH_4, CO_2 and short chain organic acids. Also the hydrolysis of lactose is industrially beneficial to overcome lactose crystallisation in condensed milk and ice cream.

β–Glucosidase plays an important role for cellulose saccharification and transglycosylation reactions on an industrial scale. These types of reactions have great importance in wine industry because of its ability to improve the release of aromatic compounds like terpenol from the glucosidic precursor terpenylglucoside in mango and grapes. β-glucosidases are more effective and specific than acid hydrolysis process for liberating terpenol from terpenylglucoside. These glycosidically bound volatiles also have interest in the food, cosmetic and tobacco industries.

Oligosaccharides synthesized by enzymes are mainly used as food additives in beverage, infants milk powder, yoghurts, chewing gums, in the manufacture of candy, pastry, bread and jam .In the food industries, β-glucosidase is used as a tool for the hydrolysis of glucosidic linkages in various food materials, such as increase in starch recovery from potatoes, extraction of essential oils and the extraction of green tea components. In the flavour industry, β-glucosidase are key enzymes to release flavour compounds (Christine Riou *et al.*, 1998). from glucosidic precursors present in fruits and fermenting products. In the pulp and paper industry, cellulases having high ratio of β-glucosidase have been employed for modification of mechanical pulp, deinking of recycled fibres and preparation of biodegradable cardboard (Ashok Pandey *et al.*, 2005).

1.3.1 Ribbon structure of β- Glucosidase

X-ray structure of human acid-β-glucosidase, the defective enzyme in Gaucher disease. A cluster of mutations in α-helix 7 that cause Gaucher disease. Transparent ribbon diagram showing the three domains of acid-βglucosidase but rotated 90° around the *x* axis to look down helix 7, which is shown in red. The amino acids on this helix that are mutated in Gaucher disease (R359, Y363, S366, T369 and N370) are shown as red balls and sticks. E235 and E340 (the active-site residues) are shown with carbon atoms as yellow balls and oxygen atoms as red balls.

Figure 5: Ribbion structure of cellobiase in Gaucher disease.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 α- Galactosidase

The α - galactosidase (α-gal) (α-D-galactoside galactohydrolase, EC.3.2.1.22) is an exoglycosidase that catalyses hydrolysis of terminal α-1-6 galactosidic bonds present in oligosaccharides (α-galactosides) of raffinose family sugars such as melibiose, raffinose, stachyose and polymeric galactomannans and guar gum (Dey & Pridham, 1972; Naumoff 2004). Moreover, it also hydrolyses glycoproteins and glycosphingolipids. α-Galactosidases have many potential biotechnological applications. α-Galactosidases are widely distributed in nature, where it has been purified from a number of sources including plants, animals and microorganisms.

2.1.1 Specificity:

α-Galactosidase exhibited a broad range of specificity and removes the terminal α-D-galactose attached by α-1,2-, α-1,3-, α-1,4-, and α-1,6-linkages (Dey and Pridham, 1972, Varbanels *et al.*, 2001). α-Galactosidase exhibits two types of activity. They are as follows:

A. Hydrolase activity, and

B. Transgalactosylase activity.

A. Hydrolase activity :

α-Galactosidase from Streptococcus bovis, Diplococcus pneumonia and *Calvatia cyathifomis* cannot act on arabinosides (Dey and Pridham,1972). Dey and Pridham (1969) have reported that p-nitrophenyl-α-D-fucoside can be hydrolyzed by alpha-galactosidase,because p-nitrophenyl-α-D-fucoside has the similar configuration with that of D-galactose. α-D-Galactopyranosyl residues from galactomannan are released during seed getrmination and serves as energy source for the growing seedling(McCleary *et al.*,1981).

B.Transgalactosylase activity:

The galactose from one melibiose was transferred to a second acceptor molecule leading to the formation of mannanotriose.This trnsglycosylation property of α-galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity,donor and acceptor concentration,pH,temperature and source of enzyme(Dey and Pridham, 1972). Apart from hydrolase activity, alpha-galactosidase also exhibits transglycosylase properties, but relatively at high substrate concentrations (Ohtakara *et al.*, 1984; Van Laere *et al.*, 1999). In recent years, transglycosylation catalysed by α-galactosidase is receiving a considerable attraction form investigators (Eneyskaya *et al.*, 1998; Van Laere et *al.*, 1999; Spangenberg et al., 1999; Andre et al., 2001). At high substrate concentrations, 64% of the enzyme showed transglycosylation activity.Disaccharides is found to be good acceptors (Hinz *et al.*, 2005). Transglycosylation activity showed that the enzyme has two galactose binding sites and hydrophobic site in its active center (Eneyskaya *et al.*, 1997).

2.1.2 Raffinose family sugars:

The raffinose family sugars have been identified as one of the important contributors of flatus (gas production) in human and experimental animals (Cristafaro *et al.*, 1973).

2.1.2.1 Raffinose:

Raffinose was first isolated in a crystalline form from Eucalyptus mann (Johnston, 1843).Raffinose and related oligosaccharides accumulates during seed maturation (Holl and Vose, 1980).

2.1.3 Stachyose:

Stachyose was first isolated from the rhizomes of Stachys tubifera (Dey, 1985).

2.1.4 Verbascose:

Verbascose was first isolated from the roots of Verbascum thapus by Bourquelt and Bridel (1910).

2.2 Xylanases

Xylanases are genetically single chain glycoproteins, ranging from 6-80 kDa, active between pH 4.5-6.5, at 40-60°C. Xylanases from different sources differ in their requirements for temperature, pH, etc. for optimum functioning. The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a group of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Hazlewood and Gilbert, 1993; Cesar and Mrsa, 1996; Latif *et al.*, 2006). The most important enzyme is endo-1,4-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylooligosaccharides. Xylosidase, debranching enzymes (L-arabinofuranosidase and glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase) allow the complete degradation of the xylooligosaccharides to their monomeric constituents (Jeffries, 1996; Biely *et al.*, 1997; Subramaniyan and Prema, 1998).

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and structures. Wong *et al.* (1988) classified xylanases into two groups on the basis of their physicochemical properties: (*i*) having low molecular mass (<30 kDa) and basic pI, and (*ii*) having high molecular mass (>30 kDa) and acidic pI. However, many xylanases, in particular fungal xylanases, cannot be classified by this system. A more complete classification system has been introduced which allows the classification of not only xylanases, but also of glycosidases in general. This system has now become the standard means for the classification of these enzymes. It is based on primary structure comparison of the catalytic domains only and classifies the enzymes in families of related sequences (Henrissat and Coutinho, 2001).

Different enzymes may be more effective in the hydrolysis of xylobiose, substituted xylo-oligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues. The different forms may also have varying abilities to interact with xylanases in xylan hydrolysis (Reilly, 1981). Three apparent xylosidases have been classified as 'exoxylanases' because they have detectable activity on xylan. Two of these enzymes

apparently lack transferase activity and one enzyme causes configuration inversion (initially yield α-Dxylose during hydrolysis), which is the characteristic used to distinguish between β-glucosidases and exoglucanases (Reilly, 1981; Eriksson and Wood, 1985).

Furthermore, an exoglucanase from *Trichoderma viride* has been shown to attack xylan in an endwise fashion to initially yield xylobiose. Exoxylanases increase the rate of xylan hydrolysis by attacking large xylo-oligosaccharides that are released by endoxylanases and are ineffectively hydrolyzed by β-xylosidases. This form of cooperation would not be expected to increase the extent of hydrolysis unless other factors are involved *e.g.* accessibility of xylosidic linkages in short and/or branched xylo-oligosaccharides, reduction of product inhibitions, or amounts of extracellular β-xylosidases (Shikata and Nisizawa, 1975). Xylans are usually cleaved at unsubstituted regions to yield mixtures of unsubstituted xylo-oligomers, as well as short and long chain substituted xylooligomers. Removal of the substituents groups by auxiliary enzymes creates new substrates for endoxylanase (EC 3.2.1.8) action (Si, 1997; Maheshwari *et al.*, 2000).

2.2.1 Xylanase production

The various biotechnological techniques like submerged and solid state fermentation are employed for xylanase biosynthesis (Cai *et al.*, 1998; Gawande and Kamat, 1999; Kansoh and Gammel, 2001). The submerged fermentation is most beneficial as compared to other techniques due to more nutrients availability, sufficient oxygen supply and less time required for the fermentation (Hoq *et al.*, 1994; Gomes *et al.*, 1994; Veluz *et al.*, 1999; Bim and Franco, 2000; Gouda, 2000). The production of microbial xylanases is preferred over plant and animal sources because of their availability, structural stability and easy genetic manipulation (Bilgrami and Pandy, 1992).

Over the years, a number of organisms including the strains of *Penicillium* spp (Fadel and Fouda, 1993; Gasper *et al.*, 1997), *Trichoderma reesei* (Liu *et al.*, 1999), *Aspergillus nidulans* (Pinaga *et al.*, 1994; Ganga *et al.*, 1998), *Aspergillus Kawachii* (Ito *et al.*, 2000), *Streptomyces* (Patel *et al.*, 1994; Kansoh and Gammel, 2001) and *Bacilus pumilus* (Rashid, 1999) are being manipulted for xylanase biosynthesis. However, *Aspergillus niger* is

described as the most potent organism for xylanase biosynthesis (Wang *et al.*, 1998; Chen *et al.*, 1999; Wu *et al.*, 2000; Haq *et al.*, 2002b).

Aspergillus niger has been investigated for xylanase synthesis and Gawande and Kamat (1999) reported maximum xylanase activity (26.7 IU/ml) after 48 hours of incubation whereas, Chen *et al.* (1999) reported maximum enzyme recovery (357.2 U/ml) at 28-32°C after 60 hrs of incubation. Palma *et al.* (1996) and Kohli *et al.* (2001) observed maximum enzyme production after 96 hrs. However, in another study conducted by Ismail (1996), *Aspergillus niger* A-20 produced highly active enzyme system in shake flask culture when the fermentation was carried out for 5 days. Agricultural waste materials/by products like wheat bran, corn cobs, sugar cane bagasse , rice husk, rice straw and oat straw have been used by many scientists for xylanase synthesis (Siedenberg *et al.*, 1998; Christov *et al.*, 1999; Gawande and Kamat,1999; Haq *et al.*, 2002). When sugarcane bagasse was used as subsratse, the crude enzyme produced highest yields (0.26 g/g) of reducing sugar (Sawarachorn, 1999).

During the investigations on biosynthesis of xylanase from *Aspergillus niger* using pure xylan polymer, wheat bran, sugar cane bagasse and rice straw, it was observed that wheat bran gives maximum activity of xylanase as compared to other substrates (Ferriera *et al.*, 1999; Park *et al.*, 2002; Haq *et al.*, 2002a).Grajek and Gervais, (1987) produced D-xylanase by thermophillic fungi using different culture methods. Seven strains of fungi were examined for their ability to produce xylanase in liquid and solid state fermentation. It was found that the best organisms for xylanase synthesis were *H. lanuginosa* and *S. thermophile.*

Mutant strains of *Aspergillus niger* NCIM 1207 when tested for xylanase, CMCase and β-glucosidase production, it secreted low levels of all enzymes in the culture broth. However, mutants UV-10 and UVIII-39 showed almost two times enhanced productivity of xylanase over the wild strain in shake flask culture (Gokhale *et al.*, 1988). Biswas *et al.* (1990) produced xylanase from *Aspergillus ochraceus* employing both fermentation methods i.e. liquid broth and solid state fermentation. The enzyme was purified using ammonium sulphate precipitation and gel filtration. The optimum pH for the enzyme was found to be 6.0.

Chen *et al.* (1990) screened a strain of *Aspergillus niger* C-2 from the soil and treated with UV and EMS to obtain mutant colonies and the conditions for submerged fermentation were studied. The produced enzyme had weak thermal stability and when incubated at 55°C for one hour, it lost 60% of its stability. Higher levels of xylanase and β-glucosidase have been obtained when *A. niger* NCIM 1207 was cultivated by submerged fermentation. Ammonium sulphate, ammonium-di-hydrogen-orthophosphate and corn-steep liquor were found to be the best carbon source for the production of enzymes (Gokhale *et al.*, 1991).In another study, Haq *et al.* (1993) obtained higher yields of xylanase and cellulase when *A. niger* was cultured on wheat bran. Different culture conditions like enzyme synthesis rate, effect of diluents and nitrogen sources were optimized for the maximum recovery of cellulase and xylanase. Costa *et al.* (1994) investigated *A. niger* CCMI 850 for the production of xylanolytic enzymes in batch cultures with 4% xylan as carbon source. A maximum activity of 65 IU/ml of β-xylanase was observed during the experiment. Likewise, Siedenberg *et al.* (1997) produced xylanase from *A. niger* awamori on synthetic medium and investigated the role of stirred tank and airlift tower top reactors, stirrer speed and phosphate concentration for xylanase biosynthesis. The highest xylanase activity was obtained at intermediate stirrer speed and low phosphate concentration.

Veluz *et al.* (1999) screened 67 strains of *Rhizopus sp.* on the basis of their capability to produce xylanase in solid state and liquid fermentation method. The highest xylanase activity of 516 IU/ml for liquid culture was exhibited by strain *Rhizopus* peka from Philippine. For solid state fermentation, the highest activity of 7802 IU/ml was achieved by *Rhizopus sp.* MKU 32 from Thailand. The xylanase can be synthesized by *Trichoderma reesi* by providing proper fermentation conditions. The rate of synthesis depends on the nature of carbon source, its concentration and carbon to nitrogen ratio. It can be enhanced by reducing the carbon source and increasing C/N ratio (Liu *et al.*, 1999). Xiong *et al.* (2005) studied the effect of L-arabinose-rich plant hydrolysate for the synthesis of xylanase by *T. reesi* C-30. The researchers reported higher activities of xylanase in cultures containing oat husk and sugar beet pulp hydrolysate than on lactose. The xylanase activity was about 9 times higher with oat husk (510 IU/ml) than in lactose (60 IU/ml). In the case of batch

cultivations on sugar beet pulp hydrolysate and lactose even higher xylanase activity (630IU/ml) was obtained.

Gokhale *et al.* (1986) obtained higher yields of the enzyme when *A. niger* NCIM 1207 was grown on either xylan (3%) or wheat bran (4%). The optimum pH and temperature for β-xylosidase were 4.5 and 65oC respectively.Bailey *et al.* (1992) used *A. fumigatus* and *A. oryzae* for xylanase production using the xylan as carbon source. *A. fumigatus* produced higher levels of xylanase on insoluble xylan than on soluble oligosaccharides due to the presence of inhibitors. It produced high levels of xylanase at pH below 3.0. In case of birch wood xylan, *A. oryzae* produced less xylanase activities as compared to *A. fumigatus*. Cai *et al.* (1997) studied the potential of *A. niger* A3 for the production of xylanase in solid state fermentation. At the initial pH 4.6, temperature 28°C, 1.0 ml spore suspension inoculum, ratio of wheat bran to bagasse 1: 1.5 and fermentation for 72 hours; optimum activity of xylanase (5147IU/g) wasobserved.

In another study, Chen *et al.* (1999) investigated 150 fungal strains for xylanase synthesis. Eight strains produced mainly xylanase with activity more than 100 IU/ml. The fungus No 49 produced the highest activities of xylanase and was identified as *A. niger*. The most suitable medium for the xylanase synthesis was NaNO3 1% and wheat bran 1% that was prepared in Mandel's nutritional solution without NH4SO4 and Urea. After incubation by shake flask fermentation, the maximum activity (357.2 IU/ml) was obtained at 28-32°C and 60 hours of incubation period. The optimum pH for the xylanase activity was 4.6 and the enzyme was able to retain its stability between the pH values 3-11.

Gawande and Kamat (1999) separated xylanase using affinity precipitation and a commercial enteric polymer Eudragit S100 from crude culture filtrates of *Aspergillus* sp 5 and *Aspergillus* sp 44. The yields after precipitation were 85.3 and 82.7% and the purification folds in specific activity were 10.8 and 4.08 for *Aspergillus* sp 5 and *Aspergillus* sp 44, respectively. The analysis of zymograms revealed the recovery of three and two forms of xylanases from *Aspergillus* sp 5 and *Aspergillus* sp 44, respectively. Later, Gawande and Kamat (1999) studied *A. terreus* and *A. niger* for the production of xylanolytic enzymes. It was found that the both strains produced valuable amounts of xylanase with almost undetectable activities of cellulase under solid state fermentation conditions. The

medium of *A. terreus* consisted wheat bran moistened with Mandel's and Stenberg mineral solution containing 0.1 % tryptone at 35°C. *Aspergillus terreus* produced 68.9 IU/ml of xylanase and *A. niger* 74.5 IU/ml.

During another research work, Gouda (2000) evaluated *A. tamarii* for the biosynthesis of xylanase in both solid state and submerged fermentation methods. The fungus produced the maximum xylanase activity (124.83U/g) in solid state method when corncobs were used as carbon source. In case of solid state fermentation, the optimum temperature was 35°C while 30°C was found to be the best for maximum enzyme recovery during submerged fermentation.

The cellulase free, endo 1,4-β-xylanase production was carried out at 50°C and pH 8.5 by *Thermoactinomyces thalophilus*. The maximum xylanase activity was achieved in the fermentation medium using birch wood xylan as carbon source after 96 hours of growth (Kohli *et al.*, 2001).Kansoh and Gammal (2001) isolated 24 strains of *Streptomyces* from soil and evaluated their ability to produce xylanase. Among all the isolates, *Streptomyces lividans* was found the most potent to produce xylanase. The optimum temperature and time of incubation for xylanase activity were 5 days and 30°C, respectively. Park *et al.* (2002) optimized conditions in solid state fermentation for xylanase synthesis. The activity of xylanase obtained after 5 days of fermentation was 50171 IU/ml.

Similarly, Haq *et al.* (2002) used the mutant strain *A. niger* GCBMX-45 for enhanced xylanase production. The organism was found to produce high activities of xylanase (1845U/g). Later, it was selected for optimization of conditions for solid state fermentation. Ten gram wheat bran with 1: 1 dilution ratio was found to be the best for optimum xylanase synthesis. The xylanase production obtained from the mutant strain was 1.36 folds higher than the parental *A. niger*. Senthilkumar *et al.* (2005) used *A. fischeri* to produce alkali-stable xylanase at pH 9.0 using wheat bran as carbon source in solid state fermentation.

2.2.2 Xylan

Xylan is the major type of hemicellulose and its hydrolysis depends on two classes of enzymes. The Endoxylanases (EC 3.2.1.8) cleave the xylan backbone into smaller parts like oligosaccharides that are further degraded to xylose by xylosidases (EC 3.2.1.37).

During the last few decades, great interest has been developed in xylan and its hydrolytic enzymatic complex, for application in bread production, supplement in animal feed, preparation of drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production.

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely joined together. Xylan is the second most abundant polysaccharide and major component in plant cell wall that consists of β-1,4-linked xylopyranosyl residues (Puls, 1997). The structure of xylans found in cell walls of plants can differ greatly depending on their origin and different structures attached to the xylan backbone. Although most of the xylans have branched structures, however some linear polysaccharides have been isolated (De Vries and Visser, 2001). Lignin is bound to xylans by an ester linkage to 4-*O*-methyl-D-glucuronic acid residues. These linkages in lignocellulose can be disrupted by using various pretreatment methods that expose most of the polysaccharide components to enzymatic hydrolysis (Chang *et al.*, 1981).

Selective hydrolysis of xylan has been observed when purified (Paice and Jurasek, 1984) and crude enzyme was applied in which cellulases were inhibited (Mora *et al.*, 1986). In all of these cases, complete removal of the xylosyl residues from the fibers was not achieved. The residual xylosyl residues may be inaccessible to xylanolytic enzymes due to the presence of substituents; modification of fiber synthesis and occurrence of xylans enclosed by other polysaccharides. There are observations which suggest that cellulose is protected from cellulases due to xylan and mannan (Sinner *et al.*, 1979). When xylan or mannan is selectively removed from delignified fiber, the residual cellulose becomes accessible to cellulase hydrolysis. However, a similar prehydrolysis of cellulose or mannan does not improve accessibility of xylan to xylanases. Selective removal of xylans increases accessibility of other polysaccharides due to increased fiber porosity that is positively correlated with cellulose hydrolysis in pretreated fibers (Grethlein, 1985; Wong *et al.*, 1988).

In cereals, arabinoxylans are among the major non-starch polysaccharides. They constitute 4-8% of barley kernel and represent 25 and 70% of the cell wall polysaccharides of endosperm and aleurone layer, respectively. The arabinoxylans are partly water-soluble and result in highly viscous aqueous solution (Dervilly Pinel *et al.*, 2001). Cereal xylans contain large quantities of L-arabinose and therefore, often referred as arabinoxylans

whereas, hardwood xylans are often referred to as glucuronoxylans due to large amount of D-glucoronic acid attached to the backbone. Arabinose is connected to the backbone of xylan *via* α-1,2 or α-1,3 linkage either as single residues or as short side chains. The side chains also contain xylose α-1,2-linked to arabinose, and galactose, which can be either β-1,5-linked to arabinose or α-1,4-linked to xylose (De Vries and Visser, 2001).

2.2.3 Purification and characterization

During enzyme production, in addition to the required enzyme, growth medium may have some undesirable metabolites of the micro-organisms which lead to lower enzyme activity. Purified enzymes exhibit higher activity, lesser risk of harmful substances and better application for the specific product. Moreover, characterization of enzyme is vital to achieve better performance in a particular application; as it provides information regarding suitable conditions for enzyme functioning.

Kavita *et al.* (2002) observed higher yields of xylanase (40 IU/ml) when grew *A. nidulans* KK-99 on a basal medium supplemented with wheat bran (2% w/v) and KNO3 (at 0.15% N) at pH 10.0 and 37°C. The enzyme was alkaline, thermostable and the optimum activity of partially purified xylanase was found at pH 8.0 and 55°C temperature. It retained more than 80% of its activity at 55°C over a broad pH range of 4.0–9.5. In another study, Taneja *et al.* (2002) used *A. nidulans* KK-99 to produce xylanase and reported an activity of 40IU/ml when incubated in basal medium containing 2% wheat bran (w/v) and KNO3 (0.15% N) at pH 10.0 and 37°C. The partially purified enzyme showed maximum activity at 55°C and pH 8.0. The xylanase was active in a wide range of pH i.e. 4.0-9.5 for 1 hour at 55°C and retained almost 80% of its activity.

Later, Anthony *et al.* (2005) used *A. niger* BRFM281 in shake flask fermentation for overproduction of XynB and reported a yield of 900 mg/L. The recombinant enzyme was purified to 1.5-fold by immobilized metal affinity chromatography with enzyme recovery of 71%. During characterization of the enzyme, it was found that it had molecular weight 23 kDa, optimum pH 5.5 and optimum temperature 50°C. The enzyme showed stability over a pH range of 4.0 to 7.0 and temperature up to 50°C. The thermophilic fungus *Humicola grisea* var. *thermoidea* has potential to produce several extracellular enzymes (Chaves *et al.*,

1989; Peralta *et al.*, 1990; Tosil *et al.*, 1993; Zimmermann *et al.*, 1990). Monti *et al.* (2003) used this fungus to produce two forms of extracellular xylanase. After the production, a fraction of crude enzyme was purified by electroelution method. The molecular mass of the purified enzyme was found to be 61.8 kDa.

An alkaline xylanase was purified from crude xylanase fermentation broth extracted in aqueous two phase system (ATPS) composed of 16% polyethylene glycol (PEG 6000) and 6.0% phosphate salt. A purification factor 57 and 41% yield of the enzyme activity were calculated for the system containing 16% PEG 6000, 8% K2HPO4 and 12% NaCl (Duarte *et al.*, 1999).

Coelho and Carmona (2003) stated that xylanase exhibited high thermalstability in the pH range 4.5 to 10.5. Earlier Christakopoulos *et al.* (1996) demonstrated that xylanase II from the fungus *Fusarium oxysporum* F3 was stable at a temperature 44-55°C. Damasco *et al.* (2002) produced cellulase free xylanase from *Thermomyces lanuginosus* in shake cultures by using corn cobs as carbon source and found that crude xylanase exhibited appreciable thermostability, retaining almost 50% of activity during 24 hours of incubation at 50oC and about 50% of activity was present at 60oC even after 4 hours of incubation.

Damasco *et al.* (2000) reported that xyalanase was active in the broad range of pH and temperature, however the optimum pH and temperature were found as 6.0 and 75°C respectively. Carmona *et al.* (1998) described the temperature 55oC for the optimum activity of xylanase from *Aspergillus versicolor* and 70oC from *A. niger*. However according to Uhlig (1998), a temperature range of 50-55oC seems the best for optimum xylanase activity.Coral *et al.* (2002) determined the molecular weight of xylanase produced by an *Aspergillus niger* strain as 36KDa. These results are also supported by the findings of Kulkarni *et al.* (1999); according to them, xylanase from the microbial origin are single sub unit proteins with the molecular mass ranging from 8-145 KDa.

Camacho and Aguillar (2003) estimated the molecular weight of xylanase from *Aspergillus sp* as 22 KDa. A study conducted by Sardar *et al.* (2000) indicated that when the purified xylanase was subjected to SDS-PAGE, the molecular weight of xylanase was found to be 24 KDa.

2.3 Cellobiase or β-Glucosidase

The β-Glucosidase (β-D-glucoside glucohydrolase EC.3.2.1.22) catalyze the hydrolysis of β-(1,4)-glucosidic linkages in the terminal non-reducing β-glucoside residues of cellobiose and lactose in to simple sugars (glucose and galctose) as well as the synthesis of oligosaccharides by transglycosylation (Chodi Masui *et al.*,2009). The exocollobiohydrolase and the endoglucanases act together to hydrolyze cellulose into small oligosaccharides. β-Glucosidase have many potential biotechnological applications. β-Glucosidase are widely distributed in nature, where it has been purified from a number of sources including plants, animals and microorganisms.

2.3.1. Mechanism of Action of β-glucosidase

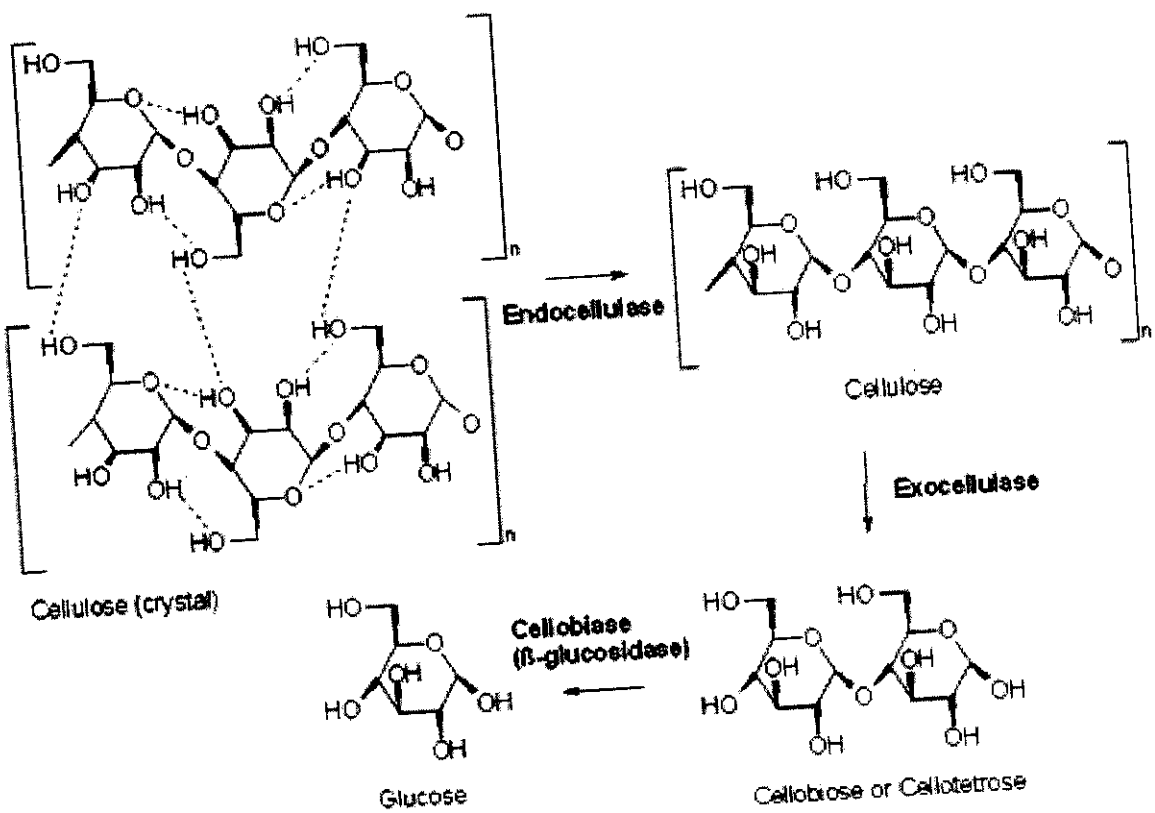
β-Glucosidases are retaining enzymes since their products retain the same anomeric configuration as the substrate. Their reaction follows a double-displacement mechanism.

2.3.1.1 Hydrolysis

β-Glucosidases normally catalyze the hydrolysis of β 1,4-glucosidic bonds in aryl- and alkyl β-D-glucosides from the non-reducing termini. In the first step the enzyme's nucleophile in the active centre attacks the substrate and an α-glycosyl enzyme intermediate is formed. In the second step the intermediate is hydrolyzed by H2O and β-glucose is released as the product . The nucleophile residue is in many cases Asp or Glu. In *Rhizomucor miehei* the analysis of the β-glucosidase identified that the residue Asp254 acts as the catalytic nucleophile, situated in a conserved motif SDW. For the release of the aglycon, another amino acid residue in the catalytic domain acts as a H+ donor to the glycosidic oxygen, resulting in the departure of the aglycon in R-OH form. The potential H+ donor can be His. The β-glucosidase from *R. miehei*, the residue His177 was proposed as H+ donor in the motif KHY.

2.3.1.2 Reverse hydrolysis or transglycosylation

In the synthetic reactions, the reactive molecule in the second step is an R'-OH instead of H2O, yielding oligosaccharides or other glycosides. In reverse hydrolysis, the substrate is a sugar, mainly glucose, yielding a disaccharide product. In ransglycosylation process, formation of the product is the result of competition between water and the acceptor molecule. In many cases, lowering the water activity would shift hydrolysis to transglycosylation.



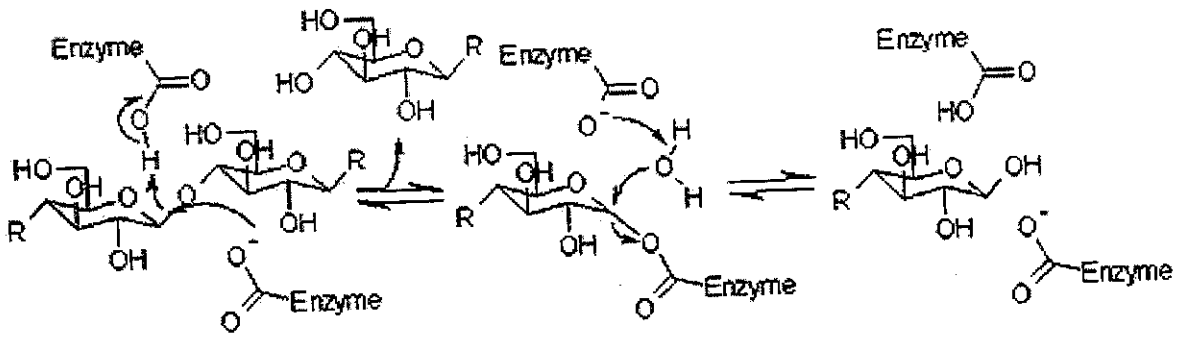


Figure 6: Mechanism of action of β- Glucosidase

2.3.2 Hydrolysis of lactose:

The β-glucosidase catalyse the hydrolysis of β-1,4 glucosidic linkages in lactose (Korish *et al* ., 2007). In food industries , β-glucosidase used in the production of low lactose milk which alleviates lactose maldigestion problems such as abdominal pain, flatulence or diarrhoea which may result from the fermentation of undigested lactose by colonic bacteria with production of H_2, CH_4, CO_2 and short chain organic acids.

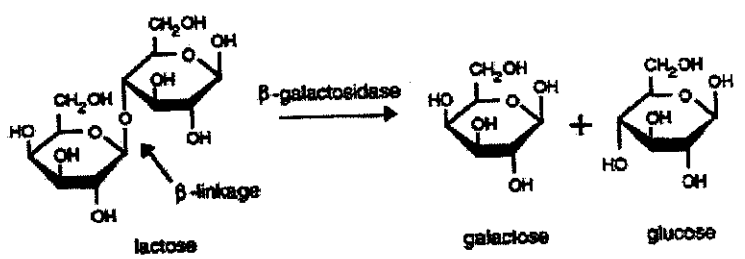


Figure 7: Hydrolysis reaction of lactose

2.3.3 Biotechnological Applications of β-glucosidase

β-glucosidase is widely distributed in nature. β-glucosidase are β-D glucohydrolases , which release β–D-glucose from cellobiose and lactose (Chodi Masui *et al.*,2009).

β-Glucosidase has the following biotechnological applications:

A. Pulp and paper industry

B. Food processing industry

C. Bioethanol production, &

D. Medicinal applications

A. Use of β-glucosidase in pulp and paper industry:

In the pulp and paper industry, cellulases have been employed for biomechanical pulping for modification of the coarse mechanical pulp (Ashok Pandey *et al.*, 2005) and hand sheet strength properties, de-inking of recycled fibres, for improving drainage and runnability of paper mills. The enzyme is used in the manufacture of soft paper including towels and sanitary paper.

B. Applications of β-glucosidase in food processing industry :

In the flavor industry, β-glucosidases are key enzymes in the enzymatic release of aromatic compounds like terpenol from the glucosidic precursor terpenylglucoside in mango and grapes(Christine Riou *et al.*,1998). Finally, β-glucosidases can also improve the organoleptic properties of citrus fruit juices, in which the bitterness is in part due to a glucosidic compound, naringin is removed. Unlike acidic hydrolysis, enzymatic hydrolysis is highly efficient and does not result in modifications of the aromatic character.

C. Applications of β-glucosidase in bio ethanol production:

β- glucosidases as part of the cellulase enzyme complex hydrolyze cellobiose and cello-oligosaccharides to yield glucose which is fermentable by yeasts into fuel ethanol. The conversion of cellulosic biomass into fermentable sugars is a very attractive way of ethanol production (Ming Chena *et al.*, 2006).

D. Medicinal applications of cellobiase

Oligosaccharides can be used as therapeutic agents, diagnostic tools, and growth promoting agents for probiotic bacteria. They have important functions in biological systems including fertilization, embryogenesis, and cell proliferation. Galacto-oligosaccharides, the transgalactosylation products from lactose, were found to be good growth factors for intestinal *Bifidobacteria.* Alkyl-glycosides are non-ionic surfactants with high biodegradability, and have good antimicrobial properties.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

1. Fungal isolates.
2. Raffinose, stachyose and p-nitrophenyl-α-D-galactopyranoside (PNPG) were purchased from Sigma Chemical Co.,USA.
3. Galactose, fructose, sucrose, maltose, and PMSF were obtained from Hi-Media, Mumbai.
4. Molecular weight markers (Genei, Bangalore)

3.2. Methods

3.2.1. Preparation of enzyme extract

Fungal isolates 1, 2 and 3 were isolated from soil samples collected from KCT campus. They were sub-cultured periodically on Czapak-Dox broth and maintained at 4°C. Batch, submerged fermentations were carried in 250 ml Erlenmeyer flasks containing 50 ml of Czapak-Dox broth with maltose as source of carbonThe flasks were kept on an orbital shaker for 5 days at 30°C and 120 rpm. At the end of the fifth day the contents were filtered through Whatman filter paper no.1, the filtrate was dialyzed and the dialyzate was used as source of enzyme.

3.2.2. Assay of α-galactosidase

Table 1: Assay of α-galactosidase

Sl. no.	Acetate buffer (ml)	Enzyme (ml)	PNPG (ml)	Incubate the tubes at 37°C for 15 min.	0.2 M Na_2CO_3	Optical Density at 405 nm
Blank	0.9	0	0.1		3	
1	0.8	0.1	0.1		3	
2	0.8	0.1	0.1		3	

Assay of α-galactosidase was carried out by incubating 100 µl of appropriately diluted enzyme, 800 µl of acetate buffer (0.2M, pH 5) and 100µl of 10mM *p*-nitrophenyl-α-galactopyranoside PNPG for 15 min at 37°C. The reaction was stopped by the addition of 3 ml of 0.2 M Na_2CO_3 solution and the absorbance of the liberated *p*-nitrophenol liberated was measured at 405 nm (Mulimani and Ramalingam, 1995). One unit of enzyme

activity was defined as the amount of enzyme required to liberate one micromole of the p-nitrophenol per minute under assay conditions.

3.2.3 Assay of Xylanase

Xylanase assay was carried out using 1% (w/v) xylan as substrate and 1 ml of enzyme, the mixture was kept at 40°C for 15 minutes. Then, 1 ml of DNS was added. The tubes were kept in a boiling water bath exactly for 5 minutes and the optical density was measured at 540 nm. One unit of xylanase activity was described as the amount of enzyme producing 1 µmol of reducing sugar per min per ml of enzyme under assay conditions.

Preparation of 1% xylan solution:

1 g of xylan is dissolved in 100 ml of distilled water and it is kept in a boiling water bath for 5 minutes. The contents were filtered through Whatman filter paper no.1 and the filtrate was made upto 100 ml with distilled water.

Dinitrosalicylic acid reagent (DNSA):

NaOH : 8.0 g

DNSA : 5.0 g

Sodium potassium tartarate: 150 g

Distilled water : 500 ml

Sodium hydroxide was dissolved in 300 ml of distilled water. DNS was slowly added to this with continuous stirring on a magnetic stirrer for complete solubilisation. Sodium potassium tartarate was then added slowly with stirring till all of it was solubilised. The volume of the solution was then made up to 500 ml.

3.2.4 Assay of β- glucosidase (cellobiase)

Table 2 Assay of β- glucosidase

Sl. no.	Acetate buffer (ml)	Enzyme (ml)	PNPG (ml)	Incubate the tubes at 45°C for 15 min.	0.2 M Na_2CO_3	Optical Density at 405 nm
Blank	0.9	0	0.1		3	
1	0.8	0.1	0.1		3	
2	0.8	0.1	0.1		3	

Assay of β-Glucosidase was carried out by incubating 100 µl of appropriately diluted enzyme, 800 µl of acetate buffer (0.2M, pH 4.5) and 100µl of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (PNPG) for 15 min at 45°C. The reaction was stopped by the addition of 3 ml of 0.2 M Na_2CO_3 solution and the absorbance of the liberated *p*-nitrophenol liberated was measured at 405 nm (Xin-Liang Li *et al*, 2004). One unit of enzyme activity was defined as the amount of enzyme required to liberate one micromole of the p-nitrophenol per minute under assay conditions.

3.2.5 Protein estimation

A standard protein curve was constructed by the method described by Lowry *et al* (1951). Bovine serum albumin (BSA) 1mg/ml was used as standard protein. Different concentrations of BSA solutions were pipetted out in a series of test tubes and the total volume was made up to 1ml with distilled water. About 2.1 ml of alkaline copper reagent was added to each test tube, mixed thoroughly and allowed to stand at room temperature for 10 min. Then, 0.2 ml of freshly prepared 1 N Folin-Ciocalteau's reagent was added to each tube and the solution was shaken well. The extinction was read after 20 min incubation at 660nm in ELICO spectrophotometer. A standard graph was constructed.

3.2.5.1 Alkaline copper reagent

- Reagent A was prepared by dissolving 2% sodium carbonate in 0.1N sodium hydroxide solution.
- Reagent B was prepared by dissolving 0.5% copper sulfate in 1% sodium potassium tartarate solution.
- Reagent C was prepared by mixing 50ml of reagent A and 1ml of reagent B just before use.

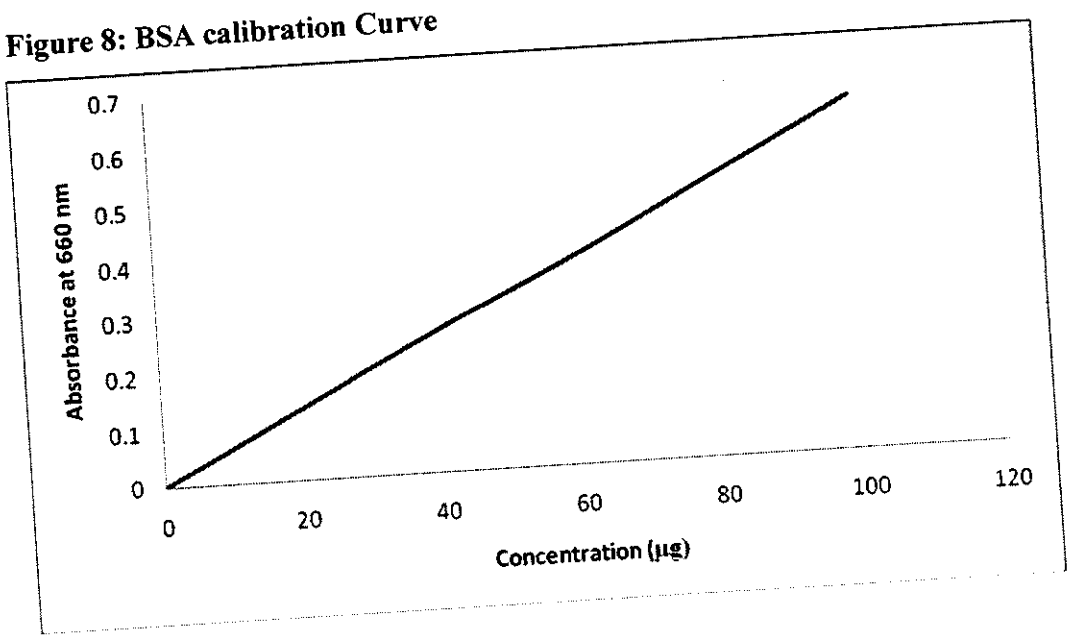
3.2.5.2 Folin-Ciocalteau's reagent

2N Folin-Ciocalteau reagent commercially available was diluted with distilled water in the ratio 1:1 (v/v) just before use.

Table 3: BSA calibration table:

Reagents	B	S1	S2	S3	S4	S5
Volume of working standard (µl)	0	20	40	60	80	100
Concentration of protein (µg)	0	20	40	60	80	100
Volume of distilled water (µl)	100	80	60	40	20	0
Volume of alkaline copper reagent (ml)	2.1	2.1	2.1	2.1	2.1	2.1
Incubate at room temperature for 10 minutes						
Volume of folin's reagent (ml)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate at room temperature for 20 minutes						
Absorbance at 660 nm	0.00	0.13	0.26	0.38	0.51	0.64

Figure 8: BSA calibration Curve



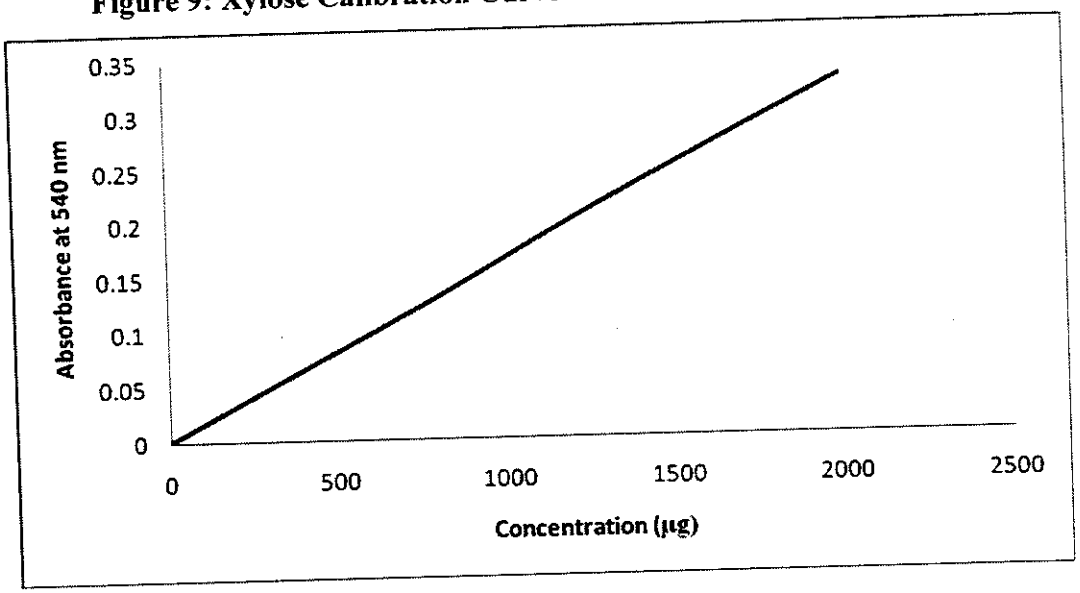
3.2.6 Xylose Calibration Curve:

A standard calibration curve of xylose was constructed. A standard xylose solution was prepared with a concentration of 1 mg/ml. Different volume of xylose solution 0.4,0.8,1.2,1.6 and 2 ml was taken in a series of test tubes and the volume was made up to 2 ml with distilled water to each tube. One ml of DNS was added and the tubes were kept in a boiling water bath exactly for 5 minutes. The optical density was measured at 540 nm against blank in spectrophotometer. A standard graph was constructed.

Table 4: Xylose Calibration Table:

Reagents	**B**	**S1**	**S2**	**S3**	**S4**	**S5**
Volume of working standard (ml)	0.0	0.4	0.8	1.2	1.6	2.0
Concentration of protein (µg)	0	400	800	1200	1600	2000
Volume of distilled water (ml)	2.0	1.6	1.2	0.8	0.4	0.0
Volume of DNS reagent (ml)	1	1	1	1	1	1
Keep the tubes in boiling water bath for 5 minutes						
Absorbance at 660 nm	0.00	0.065	0.130	0.200	0.265	0.328

Figure 9: Xylose Calibration Curve



3.2.7 Purification of enzyme

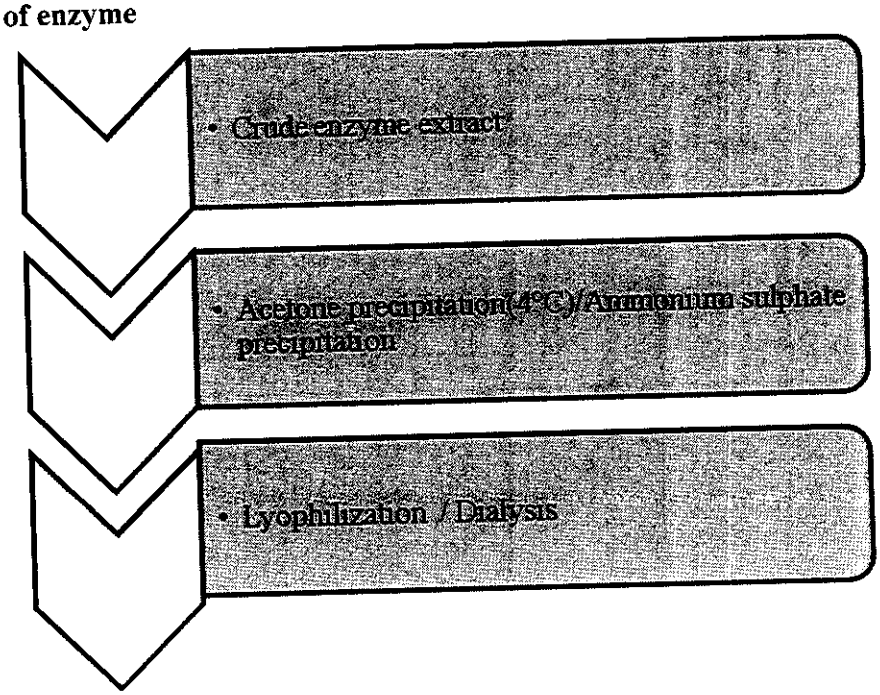


Figure 10: Purification of α-galactosidase, xylanase and cellobiose from fungal isolates.

3.2.7.1 Acetone precipitation

Chilled acetone (-20°C) in ratio of 1:1 (v/v) was added to the culture filtrate (1260 ml) of fungal isolate 3 with mild stirring and the mixture was kept aside at 4°C for 12 h. At the end of the 12th h, the mixture was centrifuged at 10,000 rpm for 10 min and the pellet was collected. The pellet was dissolved in minimum amount of acetate buffer (0.1 M, pH 4.0). The supernatant was discarded and the pellet containing centrifuge tubes kept in an inverted position till there is no acetone odour. Then, the pellet was dissolved in minimum amount of buffer (0.2 M, pH 4.0). The mixture was centrifuged and the the supernatant was used as source of enzyme for characterization studies.

3.2.7.2 Ammonium sulphate fractional precipitation:

Ammonium sulphate (35%, w/v) was added to the culture filtrate (1490 ml) with stirring and the mixture was kept aside at 4°C for 12 h. At the end of the 12 h, the mixture was centrifuged at 10000 rpm for 10 min and the supernatant was collected. The pellet thus obtained was dissolved in a minimum amount of acetate buffer (25 mM, pH 6.0). The above procedure was repeated with supernatant for 75% ammonium sulphate fractionation.

3.2.7.3 Dialysis

Dialysis bag was filled with ammonium sulphate precipitated enzyme. Dialysis bag was immersed in 25 mM acetate buffer (pH 4.0). The dialysis bag was kept at 4°C with intermittent change in buffer for every 12 hours. The content of dialysis bag was centrifuged and the enzyme activity was carried out in the supernatant by standard assay procedure.

3.2.7.4 Molecular weight determination

Native-PAGE was carried out with 10% polyacrylamide slab gel and the protein bands were stained with Coomassive brilliant blue R250 (Medium range 14.3-97.4 kDa) and the molecular weight of the enzyme was calculated from standard molecular weight markers (Genei, Bangaluru).

3.2.7.5 Polyacrylamide gel elctrophoresis

PAGE was used to analyze the proteins present in the samples according to the method of Lammli (1970). Non-denaturating PAGE (native-PAGE) was carried out without SDS.

3.2.7.6 Effect of temperature on enzyme activity

The effect of temperature on α-glactosidase activity was measured in the range of 10–90°C at pH 4, pH 3.5 and pH 4 for fungal isolates 1, 2 and 3, respectively. The effect of temperature on xylanase activity was determined by incubating the assay mixture for 15 minutes at temperature from 10-80°C for fugal isolates 1, 2 and 3, respectively. The effect of temperature on cellobiase activity was measured in the range of 30–90°C at pH 4.5 for fugal isolates 2 and 3, respectively.

3.2.7.7. Effect of metal ions, sugars and reagents concentration:

α-Glactosidase assays were performed with various metal ions (concentrations 1 mM and 5 mM) such as K^+, Ca^{2+}, Hg^{2+}, and Fe^{3+} as chloride salts; Ag^+ as nitrate salt; Cu^{2+}, Mg^{2+}, and Fe^{2+}as sulphate salts, Sugars such as glucose, galactose, fructose, lactose, sucrose, raffinose, ribose, maltose, arabinose and melibiose (final concentrations 1Mm and 5mM) and some reagents (EDTA, 1,10-phenanthroline, N-bromosuccinimide, iodoacetate, iodoacetamide and PMSF, final concentrations - 1mM and 5 mM) were incubated for 15 minutes at 55°C, prior to addition of substrate PNPαG / PNPβG. The residual activity of the enzyme was calculated by comparison of its activity in the presence and absence of the reagent or metal ions or sugars.

The reaction mixture consisting of 0.5 ml of xylanase enzyme solution, 2 ml of 0.2 M acetate buffer and 50 µl of metal ions (K^+, Ca^{2+}, Hg^{2+} and Fe^{3+} as chloride salts, Ag^+ as nitrate salt, Cu^{2+}, Mg^{2+} and Fe^{2+} as sulphate salts) or sugars (glucose, galactose, fructose, lactose, sucrose, raffinose, ribose, maltose, arabinose and melibiose) or Some reagents (EDTA, 1,10-phenanthroline, N-bromosuccinimide, iodoacetate, iodoacetamide and PMSF) were incubated for 5 minutes at room temperature. About 1 ml of xylan was added to

the reaction mixture and the test tubes were incubated for 15 minutes at optimum temperature. Then 1 ml of DNS was added to arrest the reaction and the tubes kept it in a boiling water bath exactly for 5 minutes and the absorbance was measured at 540 nm spectrophotometrically.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

For convenient clarity, result and discussion is classified into 3 categories:

α-Galactosidase

Xylanase, &

Cellobiase

4.1 α-Galactosidase

4.1.1 Partial Purification of α-galactosidase from fungal isolate 3

Acetone precipitation (ratio 1:1) increased the specific activity from 0.017 to 0.479 U/mg. The enzyme was purified to 29-fold with 70% yield (Table 5).

Table 5: Purification chart of α-galactosidase from fungal isolate 3

Enzyme fraction	Volume (ml)	Protein conc. (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (Fold)	Yield (%)
Crude extract	1260	0.92	19.353	0.017	1	100
Acetone precipitation	36	0.78	13.46	0.479	29	69.5

4.1.2 Optimum pH

α-Galactosidase showed its activity in a pH range of 3-5.5 and the optimum activity was observed at pH 4.0 for fungal isolate 3, with PNPG as substrate (Figure 14). α-Galactosidase from *Gibberella fujikuroi* showed optimum activity at pH 5.8 (Thippeswamy and Mulimani, 2002). α-Galactosidase from *Lactobacillus fermenti* and *Aspergillus flavipes* exhibited their optimal activity at pH 5.0 and 4.5-5.0 (Mudgett *et al.*,1993; Ozsoy *et al.*, 2003). α-Galactosidase from *Humicola sp.* (Kotwal *et al.*, 1999) showed enzyme stability in the pH range of 4.5-6.5. The enzyme lost only 45% and 53% of its original activity at pH 5.5, after 60 and 90 minutes While it lost 19% and 26% of its original activity in 60 and 90 minutes at pH 6.0.

4.1.3 Optimum Temperature

α-Galactosidase from fungal isolate 3 had an optimum temperature at 55°C. The enzyme activity increased with increase in temperature up to 55°C and then decreased (Figure 14).

4.1.4 Effect of metal ions, sugars and reagents

Among the cations tested, heavy metal ions such as Ag^{+} and Hg^{2+} strongly inhibited the alpha galactosidase activity to about 80.93% and 51.3%, respectively (Figure 19) for concentration 5 mM where as for 1 mM it was 72.27% and 55%, respectively (Figure 18) for fungal isolate 3. The inhibition of α-galactosidase activity by Hg^{2+} shows the presence of tryptophan residues at or near the active site of the enzyme. These ions also inhibited α-galactosidase from plant *Cucurbita pepo* (Richard *et al.*,1983), sugarcane (Chinen *et al.*, 1981) and bacterial sources *L. fermentum* (Garro *et al.*,1993), alkalophilic bacteria (Akiba *et al.*,1976).

Among the sugars tested, fructose and galactose (5 mM) inhibited α-galactosidase activity by 29.41% and 24.1%, respectively (Figure 20) while for concentration 10 mM its 28% and 28%, respectively (Figure 21) for fungal isolate 3. Glucose, lactose, melibiose and raffinose inhibited the enzyme activity to varying extent. Galactose also strongly inhibited α-galactosidase from plant, sugar cane (Chinen *et al.*, 1981)and fungal sources sources such as *G.lucidum* (Sripaun *et al.*, 2003), *Pycnoporus cinnabarinus* (Ohtakara *et al.*, 1984) and *Aspergillus oryzae* (Ramalingam and Mulimani, 2004) but did not inhibit α-galactosidase from alkalophilic bacteria such as *Micrococcus sp. and Bacillus sp.* (Akiba and Horikoshi, 1976).

Among the reagents tested N-bromosuccinimide inhibited the enzyme activity to about 35.5% at 1mM (Figure 22) and 27.4% at 5 mM (Figure 23) and for fungal isolate 3. Inhibition of enzyme activity by N-bromosuccinimide indicated the presence of tryptophan at or near the active site of an enzyme. The reagents such as EDTA and 1,10-phenanthroline did inhibit the enzyme activity suggesting that the metal ion is required for enzyme activity.

4.2 Cellobiase.

4.2.1 Partial Purification of cellobiase from fungal isolate 2

Acetone precipitation (ratio 1:1) increased the specific activity from 0.41 to 1.22 U/mg. The enzyme was purified 3-fold with 7.93% yield. (Table 6).

Table 6: Purification chart of cellobiase from fungal isolate 2

Enzyme Fraction	Volume (ml)	Activity (U)	Total Activity (U)	Protein conc. (mg/ml)	Total protein conc.(mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude filtrate	1250	0.197	246.25	0.48	600	0.41	1	100
Acetone precipitate	80	0.244	19.52	0.2	16	1.22	2.98	7.93

4.2.2 Optimum pH

The enzyme exhibited cellobiase activity in a pH range of 2-7.5 and the optimum activity was observed at pH 4.5 for fungal isolate 2, with PNPG as substrate (Figure 24). This result was of much importance since the optimum pH of β-glucosidase from *Candida peltata* (Badal *et al.*, 1996) was 5.0. β- Glucosidase from *Aspergillus niger* (Kiong *et al.*, 1992) showed an optimum pH 4.6 and for *Melanocarpus sp.* (Badhan *et al.*, 2006) the optimum pH was 6.0.

4.2.3 Optimum Temperature

Cellobiase from fungal isolate 2 had an optimum temperature at 70°C. The enzyme activity increased with increase in temperature up to 70°C and then decreased (Figure 25).

4.2.4 Effect of metal ions, sugars and reagents

Among the cations tested, heavy metal ions such as Fe^{3+} and Cu^{2+} strongly inhibited the enzyme activity to about 87.3% and 84.5 % respectively(Figure 26) for concentration 1mM .The inhibition of activity by Cu^{2+} shows the presence of tryptophan residues at or near the active site of the enzyme. These ions inhibited β-glucosidase from fungi *Melanocarpus sp* (Chada *et al.*, 2006).

Among the sugars tested, glucose and galactose at 5 mM completely inhibited cellobiase activity and raffinose at 5 mM concentration inhibited cellobiase activity of 55.3% (Figure 28). On the contrary glucose,galactose and raffinose at 10 mM concentration inhibited cellobiase activity to about 78%, 42.6 % and 50.4 %, respectively (Figure 29).

Among the reagents tested N-bromosuccinimide completely inhibited the enzyme activity to about 100% at 5mM and 1 mM concentration (Figure 30 and 31). Inhibition of enzyme activity by N-bromosuccinimide indicated the presence of tryptophan at or near the active site of an enzyme. The reagents such as EDTA and 1, 10-phenanthroline did inhibit the enzyme activity suggesting that the metal ion is required for enzyme activity

4.3 Xylanase

4.3.1 Partial Purification of xylanase from fungal isolate 3

Ammonium sulphate was used to precipitate the enzyme. The enzyme was purified to 0.67 fold with 4.84% yield. The enzyme obtained by 75% ammonium sulfate was preferred to 35% because of less protein with more enzyme activity (Table 7).

Table 7: Purification chart of xylanase from fungal isolate 3

Enzyme Fraction	Volume(ml)	Protein conc.(mg)	Total Activity(U)	Specific activity(U/mg)	Purificatin (fold)	Yied (%)
Crude filtrate	1490	447	449.98	1.01	1	100
NH_4SO_4precipitation (35%)	30	15	17.58	1.17	1.16	3.91
NH_4SO_4precipitation (75%)	35	32.2	21.77	0.68	0.67	4.84

4.3.2 Optimum pH

The effect of pH on activity of xylanase was examined in the pH range of 4-6 for fungal isolate 3. The xylanase showed optimum pH at 5.0 for fungal isolate 1 (Figure 32) and 5.5 for fungal isolate 2 and 3 (Figure 33 and 34). The optimum pH is similar to *A. niger* BRFM281 as reported by Anthony *et al.* (2005). The optimum pH for the enzyme xylanase from *Aspergillus ochraceus* was found to be 6.0 (Biswas *et al.*, 1990). In *A. awamori* K-1 the optimum pH for xylanase was found to be 3.5-4.0 (Ilieva et al., 1995). For *Trichoderma harzianum* the optimum pH for xylanase was found to be 4.0-4.5 (Tan et al., 1985).

4.3.3 Optimum Temperature

Xylanase from fungal isolate 3 had an optimum temperature at 55°C. The enzyme activity increased with increase in temperature up to 55°C and then decreased (Figure 36).

4.3.4 Effect of metal ions, sugars and reagents

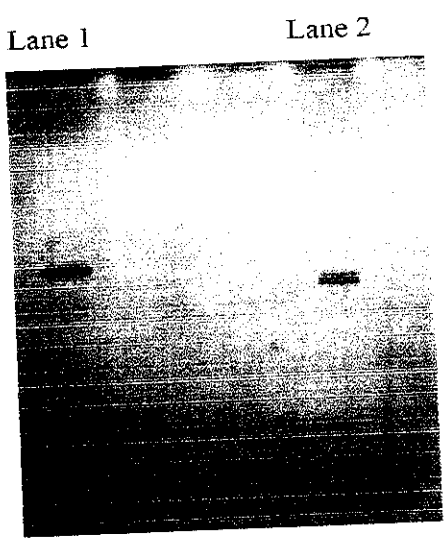
Among the cations tested, heavy metal ions such as Ag^{+} and Hg^{2+} at 1 mM concentration cmpletley inhibited the xylanase activity while Fe^{3+} also totally inactivated the enzyme (Figutre 37). Similarly Ag^{+} and Ca^{2+} at 5 mM level complexly inhibited the xylanase activity (Figure 38).

Among the sugars tested, fructose, arabinose, xylose and glucose at 5 mM concentration inhibited xylanase activity completely (Figure 39) while sucrose and raffinose at 10 mM level inhibited enzyme activity to about 89.71% and 82.72 % (Figure 40).

Among the reagents tested, 1, 10-phenanthroline at 5 mM concentration inhibited the xylanase activity completely and for EDTA it inhibited to about 76.74 % (Figure 41).

4.4 Characterization of the purified enzymes

Figure 11: Native PAGE of purified enzyme



Lane 1: Cellobiase

Lane 2: Xylanase

Figure 12: The effect of pH on α-galactosidase from fungal isolate 1

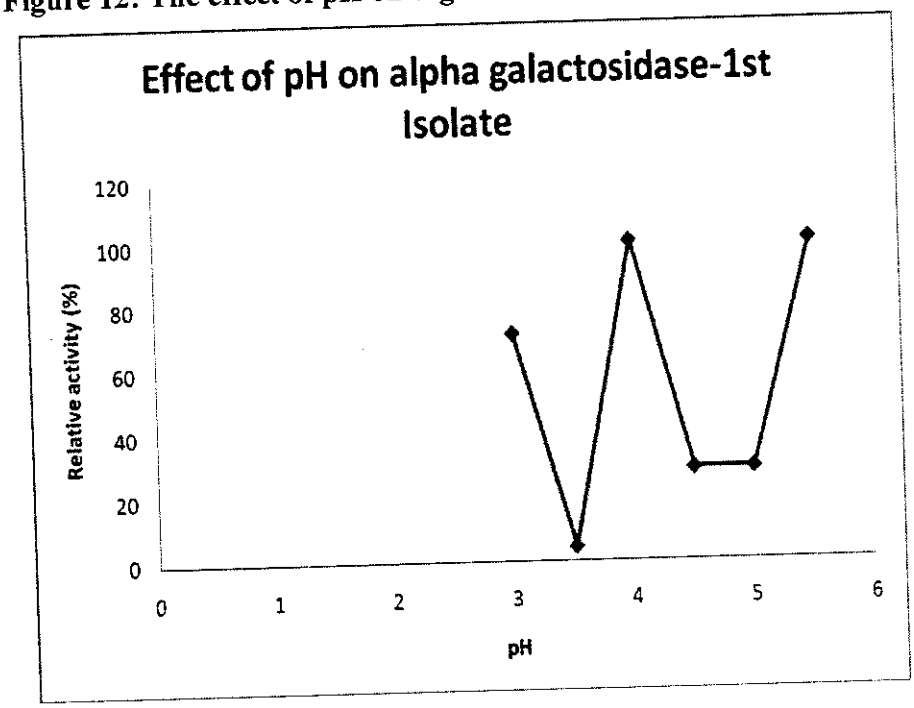


Figure 13: The effect of pH on α-galactosidase from fungal isolate 2

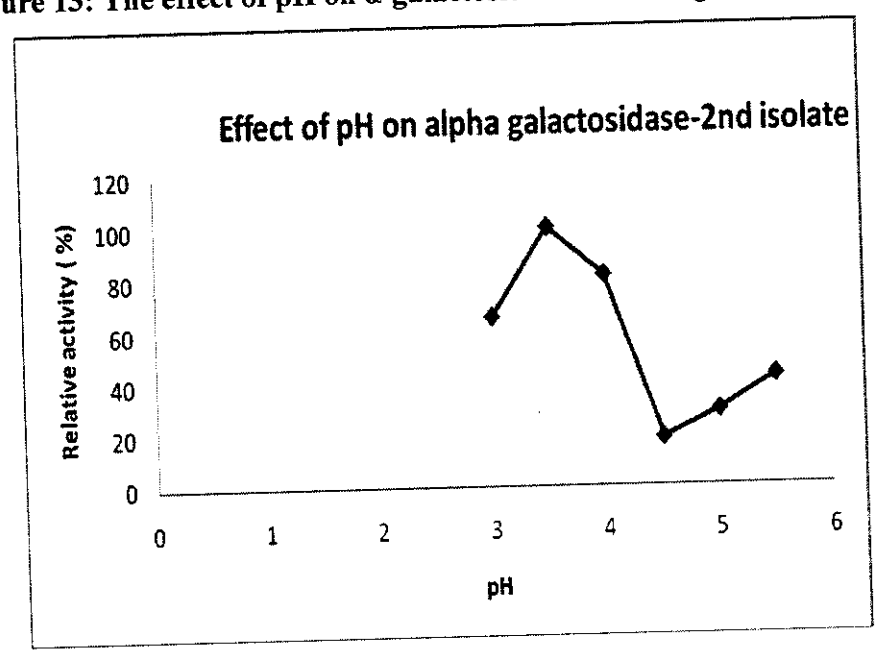


Figure 14: The effect of pH on α-galactosidase from fungal isolate 3

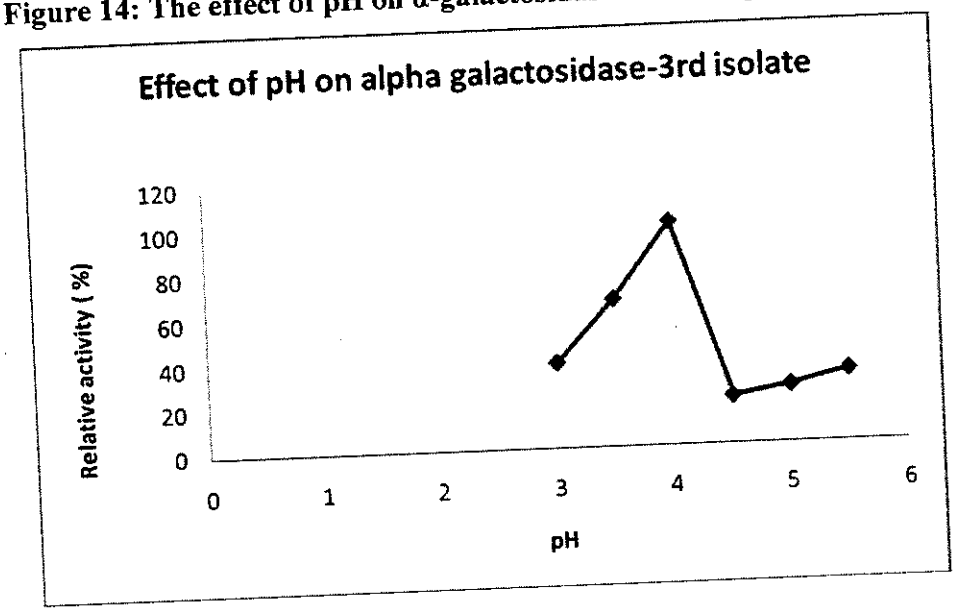


Figure 15: The effect of temperature on α-galactosidase from fungal isolate 1

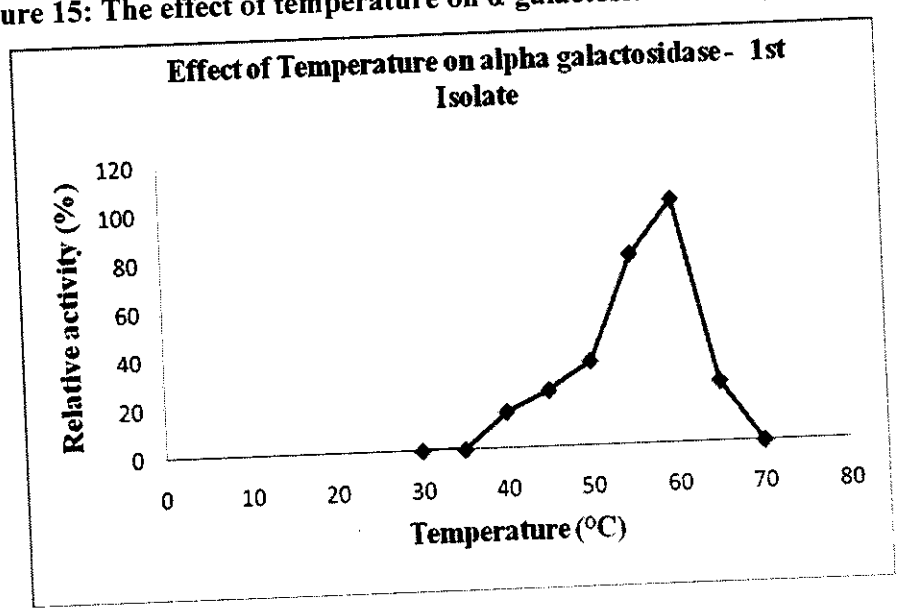


Figure16: The effect of temperature on α-galactosidase from fungal isolate 2

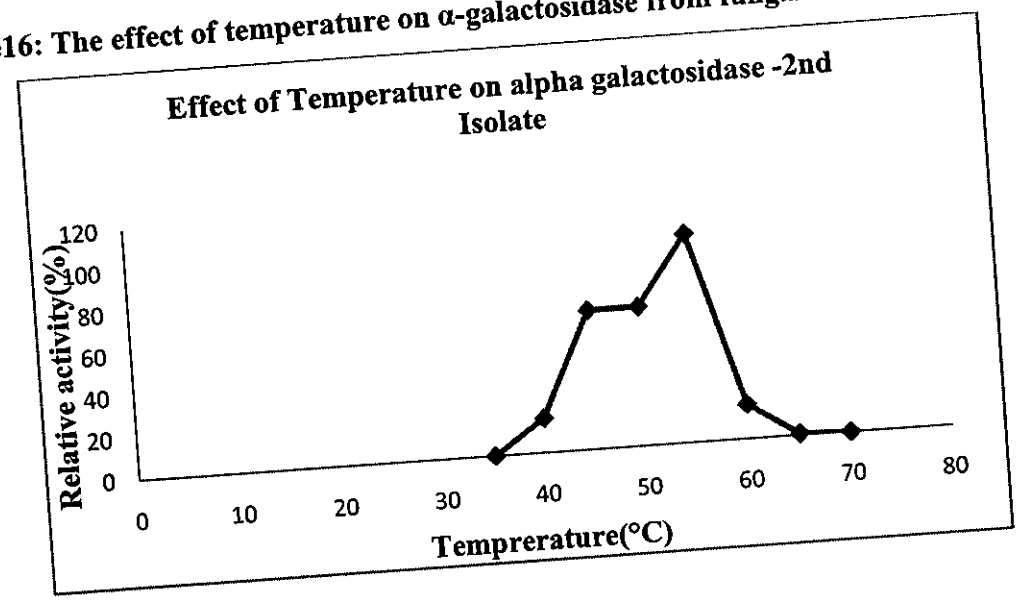


Figure 17: The effect of temperature on α-galactosidase from fungal isolate 3

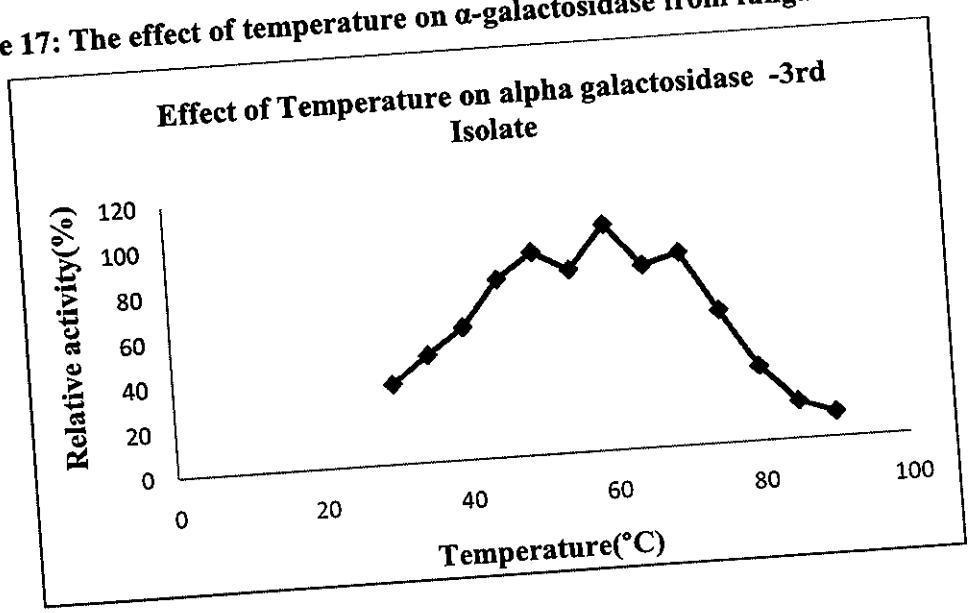


Table 8: Effect of metal ions on α-galactosidase from fungal isolate 3

Metal ions	Conc. (mM)	Residual activity (%)
Control	Nil	100
$FeSO_4$	1	95.5
KCl	1	68.2
$CaCl_2$	1	72.7
$FeCl_3$	1	54.55
$CuSO_4$	1	34.5
$HgCl_2$	1	45
$MgSO_4$	1	54.6
$AgNO_3$	1	27.7

Table 9: Effect of metal ions on α-galactosidase from fungal isolate 3

Metal ions	Conc.(mM)	Residual activity(%)
Control	Nil	100
$FeSO_4$	5	81.4
KCl	5	105
$CaCl_2$	5	118
$FeCl_3$	5	22.4
$CuSO_4$	5	79.6
$HgCl_2$	5	48.7
$MgSO_4$	5	8.3
$AgNO_3$	5	19.07

Table 10: Effect of sugars on α-galactosidase from fungal isolate 3

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
Fructose	5	70.59
Sucrose	5	70.59
Galactose	5	75.88
Arabinose	5	98.82
Glucose	5	95
Lactose	5	102.94
Raffinose	5	105.88
Maltose	5	111.76
Xylose	5	111.76

Table 11: Effect of sugars on α-galactosidase from fungal isolate 3

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
Fructose	10	72
Sucrose	10	125
Galactose	10	72
Arabinose	10	59.7
Glucose	10	72
Lactose	10	143
Raffinose	10	237.5
Maltose	10	72
Xylose	10	59.7

Table 12: Effect of reagents on α-galactosidase from fungal isolate 3

Reagents	Conc.(mM)	Residual activity (%)
Control	Nil	100
1,10-Phenanthroline	1	62.3
EDTA	1	89.1
N-Bromosuccinimide	1	64.5
PMSF	1	73.7
Iodoacetatamide	1	82.4
Iodoacetate	1	84.6

Table 13: Effect of reagents on α-galactosidase from fungal isolate 3

Reagents	Conc.(mM)	Residual activity (%)
Control	Nil	100
1,10-Phenanthroline	5	62.5
EDTA	5	96.4
N-Bromosuccinimide	5	72.7
PMSF	5	78.6
Iodoacetatamide	5	95.5
Iodoacetate	5	92.3

Figure 18: Effect of pH on cellobiase from fungal isolate 2

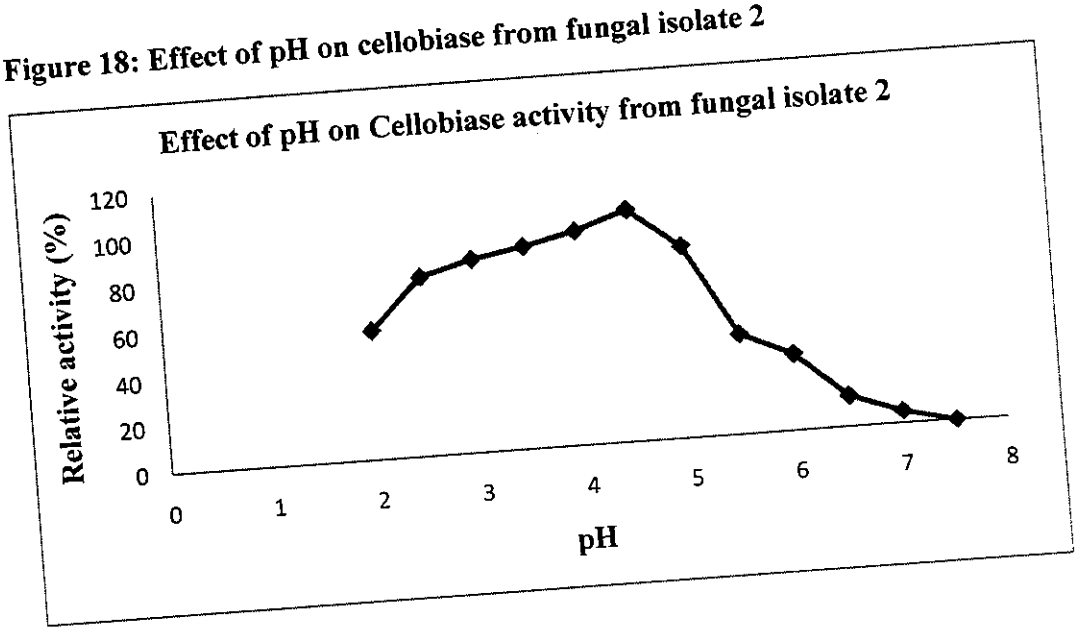


Figure 19: Effect of temperature on cellobiase from fungal isolate 2

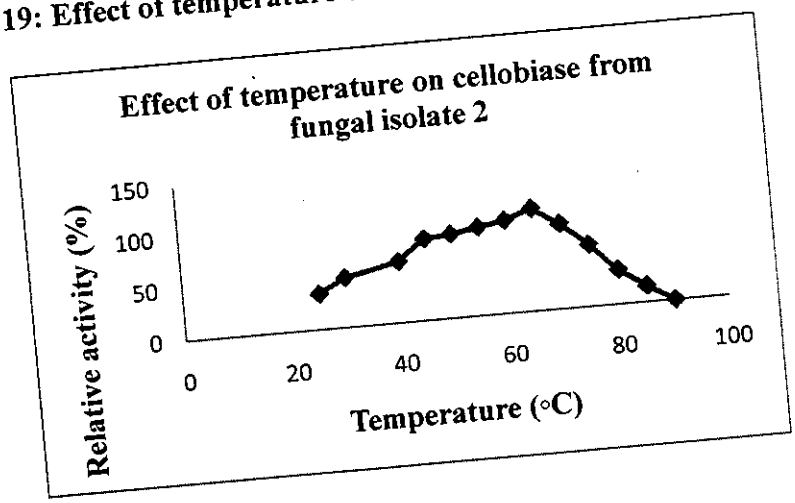


Table 14: Effect of metal ions on cellobiase from fungal isolate 2

Metal ions	Conc.(mM)	Residual activity (%)
Control	Nil	100
$FeSO_4$	1	44.4
KCl	1	88.8
$CaCl_2$	1	50
$FeCl_3$	1	12.7
$CuSO_4$	1	15.5
$HgCl_2$	1	52.2
$MgSO_4$	1	105.5
$AgNO_3$	1	39.4

Table 15: Effect of metals ions on cellobiase from fungal isolate 2

Metal ions	Conc. (mM)	Residual activity (%)
Control	Nil	100
$FeSO_4$	5	0
KCl	5	42.8
$CaCl_2$	5	52.94
$FeCl_3$	5	0
$CuSO_4$	5	0
$HgCl_2$	5	35.3
$MgSO_4$	5	76.4
$AgNO_3$	5	20.9

Table 16: Effect of sugars on cellobiase from fungal isolate 2

Sugars	Concentration (mM)	Residual activity (%)
Control	Nil	100
Arabinose	5	77.7
Galactose	5	44.7
Lactose	5	100
Maltose	5	0
Fructose	5	52.8
Sucrose	5	45
Glucose	5	16.7
Xylose	5	63.9
Raffinose	5	0
Melibiose	5	47.2

Table 17: Effect of sugars on cellobiase from fungal isolate 2

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
Arabinose	10	95.8
Galactose	10	57.4
Lactose	10	88
Maltose	10	68
Fructose	10	57.6
Sucrose	10	86.4
Glucose	10	22
Raffinose	10	49.6
Xylose	10	82.4
Melibiose	10	94.4

Table 18: Effect of reagents on cellobiase from fungal isolate 2

Sugars	Conc.(mM)	Residual activity(%)
Control	Nil	100
N – bromo succinimide	1	0
Phenanthroline	1	35.96
Iodo acetamide	1	38.42
Iodo acetate	1	30.04
EDTA	1	27.58

Table 19: Effect of reagents on cellobiase from fungal isolate 2

Reagents	Conc.(mM)	Residual activity(%)
Control	Nil	100
N – bromo succinimide	5	0
Phenanthroline	5	0
Iodo acetamide	5	53.7
Iodo acetate	5	52.21
EDTA	5	47.3

Figure 20: Effect of pH on xylanase from fungal isolate 1

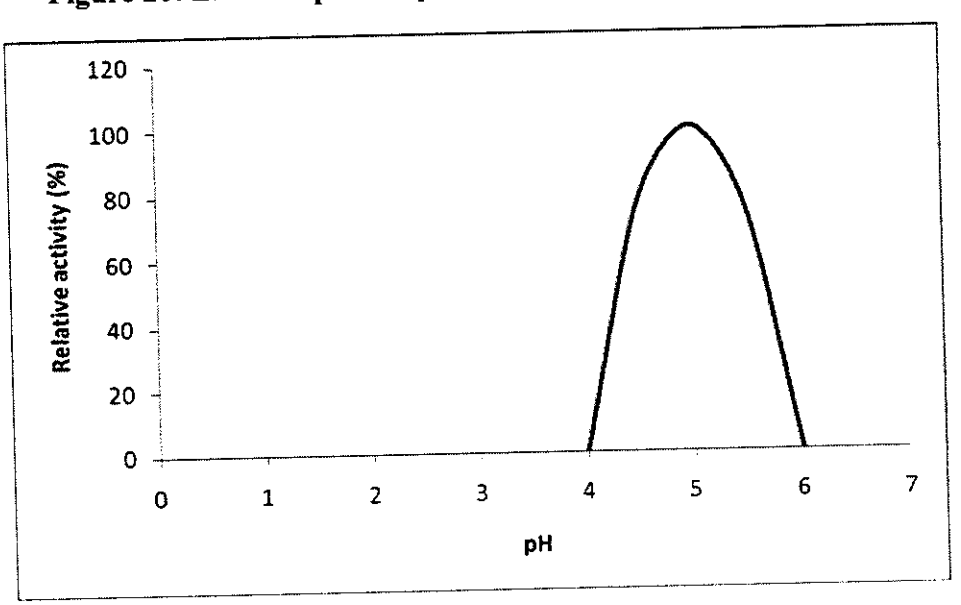


Figure 21: Effect of pH on xylanase from fungal isolate 2

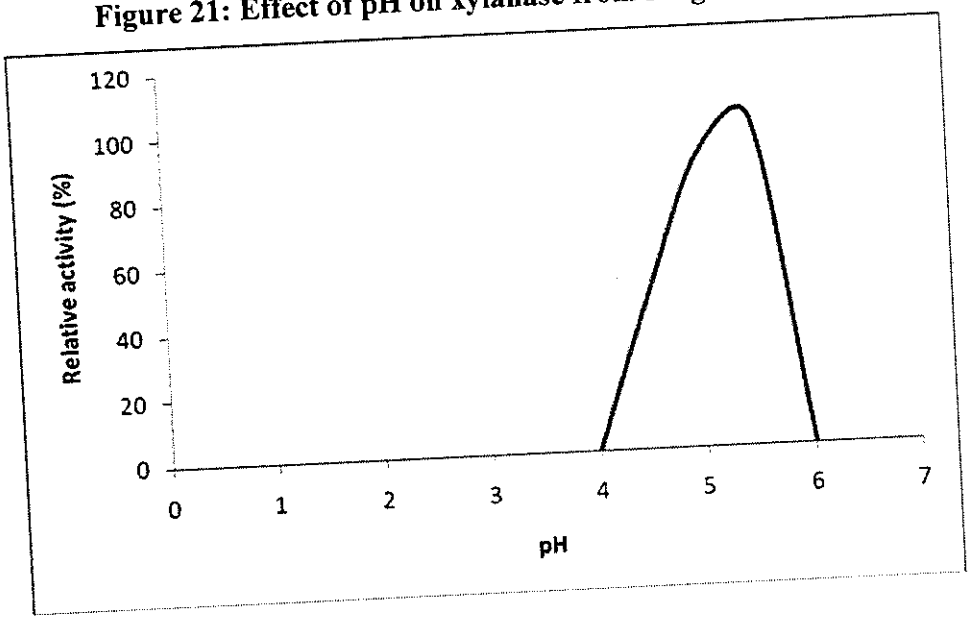


Figure 22: Effect of pH on xylanase from fungal isolate 3

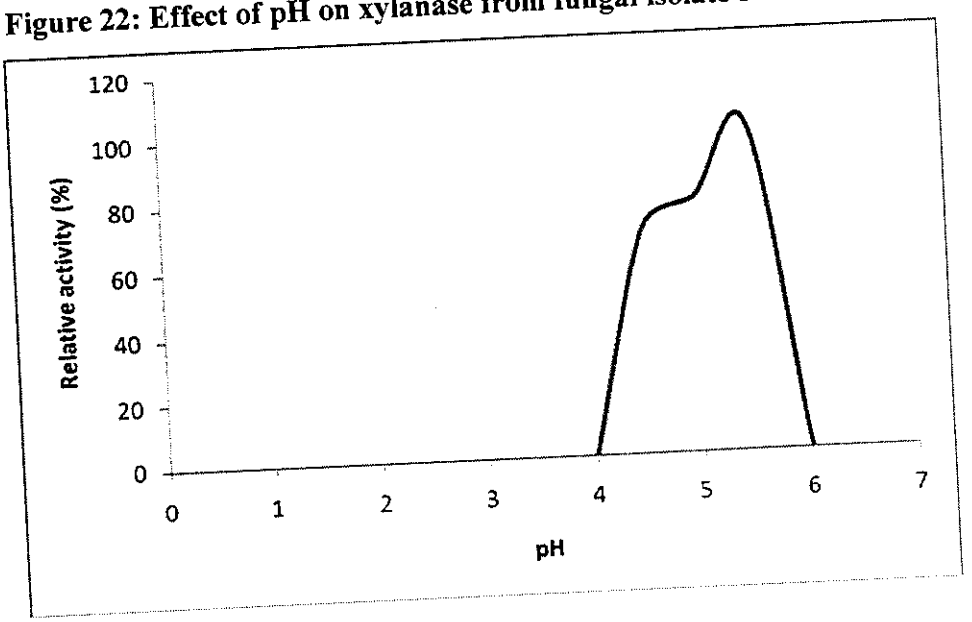


Figure 23: Effect of temperature on xylanase from fungal isolate 1

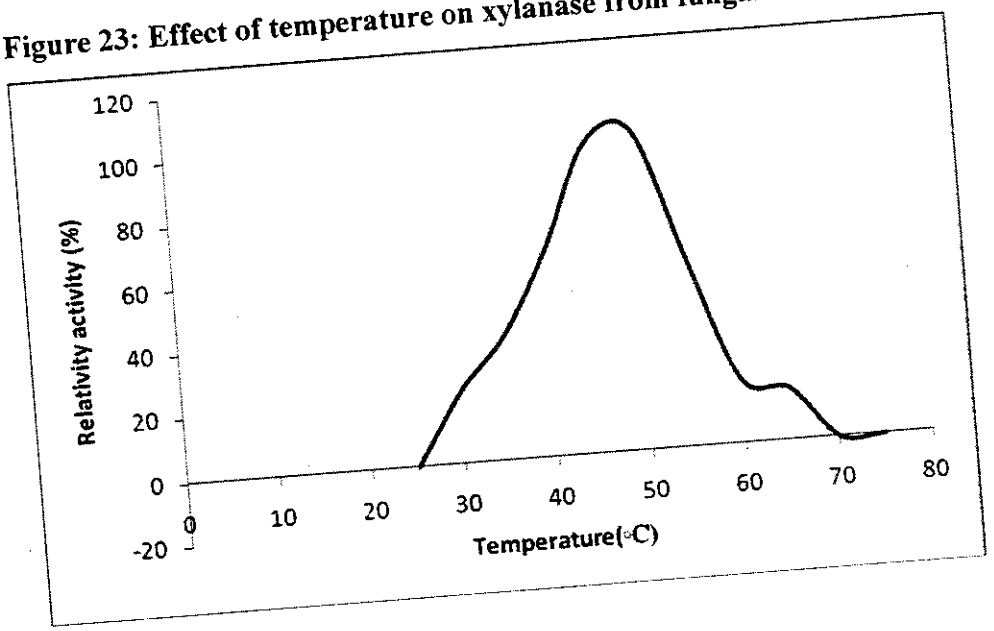


Figure 24: Effect of temperature on xylanase from fungal isolate 2

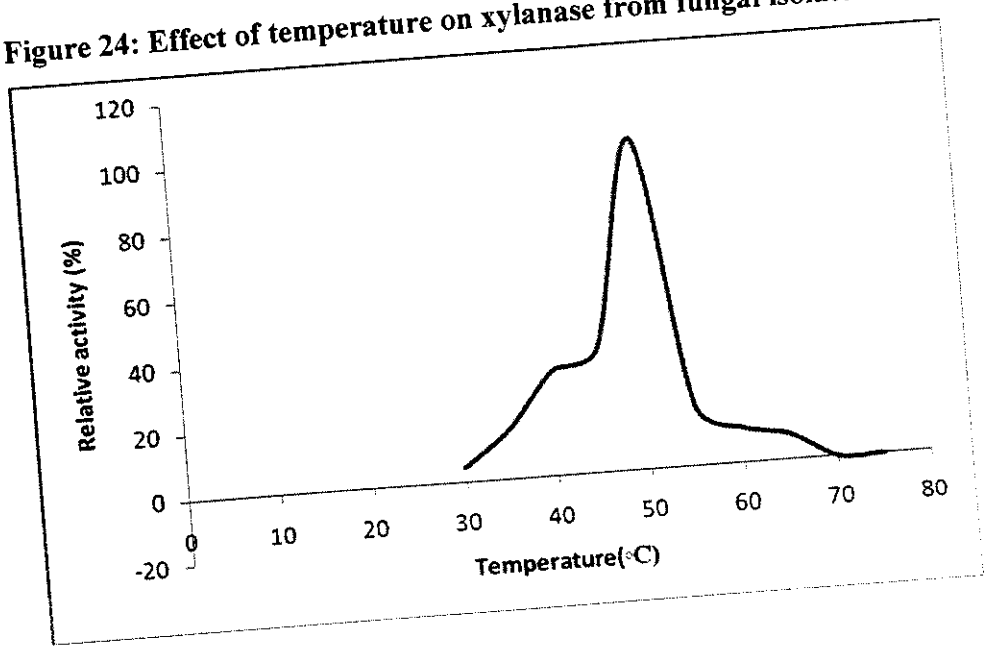


Figure 25: Effect of temperature on xylanase from fungal isolate 3

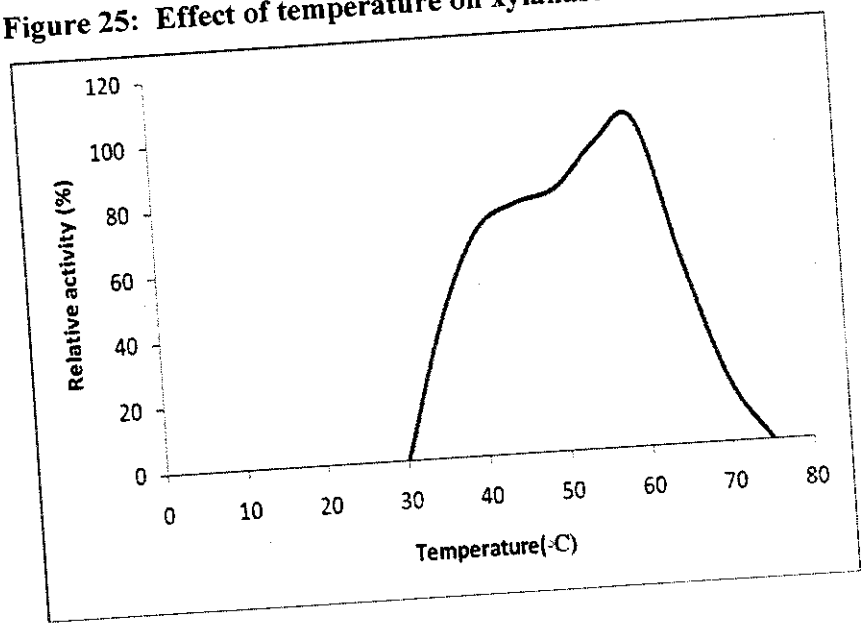


Table 20: Effect of metal ions on xylanase from fungal isolate 3

Metal ions	Conc.(mM)	Residual activity (%)
Control	Nil	100
$FeSo_4$	1	0
$AgNo_3$	1	0
$CaCl_2$	1	0
$HgCl_2$	1	0
$MgSo_4$	1	12.96
$FeCl_3$	1	21.76
KCl	1	30.32
$CuSo_4$	1	39.12

Table 21: Effect of metal ions on xylanase from fungal isolate 3

Metal ions	Conc.(mM)	Residual activity (%)
Control	Nil	100
$CaCl_2$	5	0
$AgNo_3$	5	0
$MgSo_4$	5	23.16
KCl	5	46.10
$FeCl_3$	5	53.90
$CuSo_4$	5	53.90
$FeSo_4$	5	57.58
$HgCl_2$	5	492.21

Table 22: Effect of sugars on xylanase from fungal isolate 3

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
Arabinose	5	0
Fructose	5	0
Xylose	5	0
Glucose	5	0
Sucrose	5	10.29
Raffinose	5	17.28
Maltose	5	31.07
Galactose	5	34.56
Melibiose	5	68.93
Lactose	5	79.42

Table23: Effect of sugars on xylanase from fungal isolate 3

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
Arabinose	10	0
Fructose	10	0
Xylose	10	0
Glucose	10	0
Sucrose	10	7.79
Raffinose	10	7.79
Maltose	10	19.26
Galactose	10	23.16
Melibiose	10	38.53
Lactose	10	65.37

Table 24: Effect of Reagents on Xylanase from fungal isolate 3

Sugars	Conc.(mM)	Residual activity (%)
Control	Nil	100
1,10-Phenanthroline	5	0
EDTA	5	23.26
Iodoacetate	5	46.72
Iodoacetamide	5	56.66
N-Bromosuccinimide	5	641.84

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The α-galactosidase from fungal isolate 3 was partially purified by acetone precipitation. The optimum pH, temperature, effect of metal ions, sugars and some reagentswere of partially purified enzyme were carried out. The results indicate that the enzyme has potential applications in food processing industry for the production of flatulence-free soymilk and legume products, etc.

The xylanase from fungal isolate 3 was partially purified by ammonium sulphate precipitation followed by dialysis. The optimum pH, temperature, effect of metal ions, sugars and some reagentswere of partially purified enzyme were carried out. The results indicate that the enzyme has potential applications in various field like paper and pulp industry, baking industry, etc .

The β-glucosidase from fungal isolate 2 was also partially purified by acetone precipitation. The optimum pH, temperature, effect of metal ions, sugars and some reagents on partially purified enzyme were carried out. The results indicate that the enzyme has potential applications in various fields like ethanol production, hydrolysis of lactose, etc.

REFERENCES

REFERENCES

1. Abiodun, P. (1991) 'Use of soya-beans for the dietary prevention and management of malnutrition in Nigeria', Acta paediatrica scandinavica (suppl.), vol. 374, pp. 175–182.
2. Chinen, I., Nakamura, T., and Fukuda, N. (1981) 'Purification and properties of α-galactosidase from immature stalks of *Saccharum officinarum* (sugar cane)', Journal of Biochemistry, vol. 90, pp.1453-1461.
3. Bruneton, J. (1995). Pharmacology, Phytochemistry, Medicinal plants, Lavoisier, Paris.
   Beebe, U.U., and Turgeon.R (1992). Planta, vol. 188, pp. 354.
4. Bourquelot, E. and Bridel, M. (1910), CR, Head. Scances Acad. Sci., vol.151, pp.760.
5. Choung, M. G. (2005) 'Determination of soluble carbohydrates in soybean seeds KoreanJournal of Crop Science', vol. 50, pp. 319-324.
6. Dey, P. M. and Pridham, J. B. (1972). Biochemistry of galactosidase. Advance Enzymology, vol. 36, pp. 91-13.
7. Dey, P.M., and Malhotra, O.P. (1969), Journal of Biochem and Biophys.vol. 185, pp.402 408
8. Dey, P.M., and Pridham, J.B. (1969a), Biochem. J, vol 115, pp.47-48.
9. Eneyskaya, E.V., Golubev, A.M., Kachurin, A.M., Savels, E.V., and Neustroev, K.N. (1998), Carbohydr. Res, vol.305,pp. 83
10. Fujimoto, Z., Kaneko, S., Momma, M., Kobayashi, H., and Mizuno, H. (2003) 'Crystal structure of rice α-galactosidase complexed with D-Galactose', Journal of Biological Chemistry, vol. 278, pp. 30-36.
11. Feldt-Rasmussen, U., Rasmussen, A. K., Mersebach, H., Rosenberg, K. M., Hasholt, L., Sorensen, S. A. (2002) 'Fabry disease a metabolic disorder with a challenge for endocrinologist. Hormone Research', vol.58, pp. 259-65.

12. Ferro, V.A., Bradbury, F., Cameron, P., Shakir. E., Rahma, S., Stimson WH (2003) 'In vitro susceptibilities of *Shigella flexneri* and *Streptococcus pyogenes* to inner gel of *Aloe barbadensis* Miller', *Antimicrobial agents and chemotherapy*, vol 47 pp. 1137-1139.

13. Hu, Y., Xu, J., Hu,Q.(2003) ' Evaluation of antioxidant potential of Aloe vera extracts', J.Agri. Food Chem, vol.51, pp.7788-7791.

14. Hara, K., Fujita, K., Kuwahara, N., Tanimoto, T., Hashomoto, H., Koizumi, K., and Holl, F.B and Vose, J.R (1980), Can. J. Plant Sci, vol.60,pp.1109.

15. Hansel,R ., Keller, K.,Rimpler, H., and Schneider,G. (1992) Hagers Handbuch der Pharmazeutischen Praxis New York, vol.5, pp.213-223.

16. Johnston, J.F.W. (1943) Phil. Mag. Vol. 23, pp. 14.

17. Kitahata, S., (1994).Biosci. Biotech. Biochem, vol. 58, pp. 652.

18. Keller, F. (1992), Plant Physiol., Vol. 99, pp. 1251.

19. Kotwal,S.M., Gote,M.M., Sainkar,S.R., Khan,M.J., and Khire,J.M. (1997) 'Production of alpha galactosidase by thermophilic fungus *Humicola* sp in solid-state fermentation in soymilk hydrolysis', Process biochemistry', vol.33, pp.337-343.

20. Lowry, O.H., Rosebrough,N.J., Farr,A.L., and Randall,R.L.(1951) 'Protein measurement with the folin phenol reagent', Journal of Biol Chem, vol.193,pp.265-275.

21. Malhotra, O. P., Dey, P. M. (1967) 'Purification and physical properties of sweet almond α- galactosidase. Biochemical Journal', vol. 103, pp. 508-513.

22. Mathew, C.D., and Balasubramaniyam, K. (1987), Indian J. Biochem. Biophys. Vol.24, pp.29-30.

23. Mulimani, V. H. and Ramalingam. (1995) 'Enzymic hydrolysis of raffinose and stachyose present in soymilk by soluble alpha galactosidase from *Gibberella fujikuroi*', Biochemistry and Molecular Biology International, vol. 36, pp.897–905.

24. Naumoff, D. G. (2004). Phylogenetic analysis of α-galactosidases of the GH27 Family. Molecular Biology, vol. 38, pp. 388–399.

25. Naim, M., Gestetner, B., Bondi, A., Birk, Y. (1976) 'Antioxidative and antihemolytic activities of soybean isoflavones', Journal of Agricultural and Food Chemistry, vol. 24, pp. 1174–1177.

26. PuchartV, Vrsanska M, Bhat, M.K and Biely P. (2000). Biochim. Biophys. Acta. 1524, 27.

27. Porter, J. E., Sarikaya, A., Herrmann, K. M., Ladisch, M. R. (1992) 'Effect of pH on subunit association and heat protection of soybean alpha–galactosidase', Enzyme and Microbial Technology, vol. 14, pp. 609-613.

28. Tanaka, M., Thananunkul, D., Chichester, C.O., Lee, T. C. (1976) 'Degradation of raffinose and stachyose in soybean milk by α-galactosidase from Mortierella vinacea', Entrapment of !-galactosidase with in polyacrylamide gel', Journal Food Science, vol. 41, pp. 173–175.

29. Van Laera, Billy, N., and Joseph, J.(1999) 'Alpha Galactosidase activity in high substrate concentration',Braz.J.Chem engg, vol.20,pp.26-31.

30. Ronald de vries, P.,Hetty van den broeck,C.,Esterdekkrs,Paloma manzanares,Leode graff and Jaap visser.(1999) 'Differential expression of three alpha galactosidase genes and a single beta galactosidase gene from Aspergillus niger',Applied and Environmental Microbiology, vol.65,pp.2453-2460.

31. Subba Rao K. and Pieringer R. A. (1970) 'Metabolism of glyceride glycolipids-IV: Enzymatic hydrolysis of monogalactosyl and digalactosyl diglycerides in rat brain', Journal of Neurochemistry, vol. 245, no.10, pp. 2520-2524.

32. Sumbul Shamim, S., Waseemuddin Ahmed., and Iqbal Azhar (2004) 'Antifungal activity of *Allium*, *Aloe*, and *Solanum* species',*Pharmaceutical Biology* , vol.46, pp 491–498.

33. Shabalin, K.A., Kulminskaya, A.A., Savel'eV, A.N., Shishlyanikov and Neustroev, K.N. (2002), Enzyme Microbiol. Technol,vol 30,pp. 231-238.

34. Smith, A. K., Circle, S. J. 1972 Chemical composition of the seed. In: Soybeans: Chemistry and Technology, Vol. 1, pp. 61-92.

35. Shallenberger, R.S and Moyer, J.C.(1961), J. Agric. Food Chem., vol.9, pp.137.

36. S. Satish, K. A., Raveesha, G. R., Janardhana. (1999) 'Antibacterial activity of plant extracts on phytopathogenic Xanthomonas campestris', J.*Applied Microbiology* vol. 28, pp .145–147 .

37. Sanni, I.A., Ahrne,S., and Onilude,A.A.(1995) 'Production of alpha galactosidase by *Lactobacillus plantarum* isolated from diverse sources.Journal of basic microbiology',vol.35,pp.427-432.

38. Hin-chung wong, Chien-an hu, His-lien yeh, Wanchuang su, Hsuchchih and Chinfulin. (1986) 'Production, purification and characterization of alpha galactosidase from *Monascus pilosus'*, Applied and Environmental Microbiology, vol. 52, pp.1147-1152.

39. Harpez, N., Flowers, H.M., and Sharon, N. (1975).Arch. Biochem. Biophys, vol. 170, pp 676-680.

40. McCutchen, C.M., Duffaud, G.D., Leduc. P. Peterson., A.R.H., Khan., S.A., and Kelley.(1996), Biotech. Bioeng.m vol.52, pp. 332-334

41. Okthakara, A., Mitsutomi, M., and Uchida , Y. (1984) . Agr. Biol. Chm., vol 48, pp .1319.

42. Varbarnels, Mohly & Friere. (2001) 'Purification and characterization of Alpha galactosidase from *Lactobacillus fermentii'*, Physical chemistry,vol.57,pp.17-19.

43. Yagi, A., and Tankeo.S. (2003) 'Anti-inflammatory constituents,aloesin and aloemannan in *Aloe vera* species and effects of tanshinon', Mol.Biol, vol. 123, pp.517-521.

44. YananCao, Yaru Wang, Kun Meng, Yingguo Bai, Pengjun Shi ,Huiying Luo, Peilong Yang, ZhigangZhou, Zhifang Zang, and Bin Yao.(2009) 'A novel protease resistant alpha

galactosidase with high hydrolytic activity from *Giberella* sp.F75:gene cloning ,expression,and enzymatic characterization',Appl Microbiol Biotechnol,vol.83,pp.875-884.

45. Zhifang, G., Arthur, A. S.(1999) 'A novel alkaline alpha galactosidase from melon fruit with a substrate preference for raffinose ', Plant Physiology, vol.119, pp.979–98.

46. Zeilinger, S., Kristufek, D., Arisan-Atac,I., Hodits,R., and Ku-bicek,C.(1993) 'Conditions of formation, purification and characterization of an alpha galactosidase of *Trichoderma reesei* RTU', Applied and Environmental Microbiology,vol.30,pp.1347-1353.

47. Subramaniyan, S. and Prema, P. (2002) 'Biotechnology of Microbial Xylanases: Enzymology, Molecular biology, and Application', Critical Reviews in Biotechnology, Vol. 22, No. (1), pp. 33-64.

48. Collins, T., C. Gerday, and G. Feller, (2005) 'Xylanases, xylanase families and extremophilic xylanases', FEMS Microbiology reviews, Vol. 29, pp.3-23.

49. Bradford, M.M., (1976) 'A Rapid and Sensitive Method for The Quantitation of Microgram Quantities of Protein Utilizing The Principles of Protein Dye Binding', Analytical Biochemistry, Vol. 72, pp. 248-254.

50. Laemmli, U.K. (1970) 'Cleavage of structural proteins during the assembly of the head of bacteriophage T4', Nature, Vol. 27, pp. 680-685.

51. Bertrand, J.L., R. Morosoli, F. Shareck, and D. Kluepfel, (1989) 'Expression of the xylanase gene of Streptomyces lividans and production of the enzyme on natural substrates', Biotechnology and Bioengineering, Vol. 33, pp. 791–794.

52. Liu, W., W. Zhu, Y. Lu, J. Kong, and G. Ma, (1998) 'Production, partial purifcation and characterization of xylanase from Trichosporon cutaneum SL409', Process Biochemistry, Vol. 33, pp. 331–336.

53. Ding, C.H., Z.Q. Jiang, X.T. Li, L.T. Li, and I. Kusakabe, (2004) 'High activity xylanase production by Streptomyces olivaceoviridis E-86', World Journal of Microbiology & Biotechnology, Vol. 20, pp. 7–10.

54. Bajpai, P. (1999) 'Application of enzymes in the pulp and paper industry', Biotechnol. Progr, Vol. 15, pp. 147–157.

55. CAI, J.M., W.Ke, Y. Zhow, Z. Jie, J. Bang and R. Ruipen. (1997) 'Production of xylanase by *Penicillium* sp. P1 using solid state fermentation', ShipinYu Fajio Gongye, Vol. 23(4), pp. 30-33.

56. Camacho, N.A. and G.O. Aguilar. (2003) 'Production, purification and characterization of low molecular mass xylanase from *Aspergillus* sp. and its application in baking', Appl. Biochem. Biotechnol, Vol. 104, pp. 159–172.

57. Carmona, E.C., R.B.B. Marcia, A.P.K. Aline and A.J. Jao. (1998) 'Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor'*. FEMS. Microbiol. Letters, Vol. 166, pp. 311-315.

58. Cesar, T. and V. Mrsa. (1996). 'Purification and properties of xylanase produced by *Thermomyces lanuginosus'*, Enzyme Microb. Technol, Vol. 19, pp. 289–296.

59. Christov, L.P., G. Szakacs, and H. Balakrishnan. (1999) 'Production, partial characterization and use of fungal cellulase-free xylanases', Process Biochem, Vol. 34, pp. 511–517.

60. Coelho, G.D. and E.C. Carmona. (20030) 'Xylanolytic complex from *Aspergillus giganteus:* production and characterization', J. Basic Microbial, Vol. 43, No. 4, pp. 269-277.

61. Dervilly, G., C. Leclercq, D. Zimmermann, C.Roue, J.F. Thibault and L. Saulnier. (2002) 'Isolation and characterization of high molar mass watersoluble arabinoxylans from barley and barley malt', Carbohydr. Polym, Vol. 47, pp. 143–149.

62. Huang. (1991) 'Purification and characterization of an endoxylanase from *Trichoderma koningii* G-39', Biochem. J, Vol. 278, pp. 329-333.

63. Ilieva, S., A. Atanas, P. Adriana, M. Diliana, P. Rumiana and P. Nadejda. (1995) 'Xylanase production by *Aspergillus awamori* k-1', Sv. Kliment Okhridski Biol. Fak, Vol. 88, No. 4, pp. 63-68.

64. Latif, F., M. Asgher, R. Saleem, A. Akram and R. Legge. (2006). 'Purification and characterization of xylanase produced by *Chaetomium thermophile* NIBGE', World J. Microbiol. Biotechnol, Vol. 22, pp. 45–50.

65. Rashid, A.N. (1999) 'Characterization of xylanase produced by *Bacillus pumilus* strain PJ19', J. Microbial Biotechnol, Vol. 9, No. 2, pp. 157-162.

66. Almeida EM, Polizeli MLTM, Terenzi HF, Jorge JA. (1995) 'Purification and biochemical characterization of β-xylosidase from Humicola grisea var. thermoidea', FEMS Microbiol Lett, Vol. 130, pp. 171–176.

67. Camacho NA, Aguilar OG. (2003) 'Production, purification and characterization of a low molecular mass xylanase from Aspergillus sp. and its application in bakery', Appl Biochem Biotechnol, Vol. 104, pp. 159–172.

68. Carmona EC, Brochetto-Braga MR, Pizzirani-Kleiner AA, Jorge JA. (1998) 'Purification and biochemical characterization of an endoxylanase from Aspergillus versicolor', FEMS Microbiol Lett, Vol. 166, pp. 311–315.

69. Dervilly G, Leclercq C, Zimmerman D, Roue C, Thibault JF Sauliner L. (2002) 'Isolation and characterization of high molecular mass water-soluble arabinoxylans from barley malt', Carbohydr Polym, Vol. 47, pp. 143–149.

70. Fernández-Espinar M, Piñaga F, de Graaff L, Visser J, Ramón D, Vallés S. (1994) 'Purification, characterization and regulation of the synthesis of an Aspergillus nidulans acidic xylanase', Appl Microbiol Biotechnol, Vol. 42, pp.55–562.

71. Fujimoto H, Ooi T, Wang S-L, Takizawa T, Hidaka H, Murao S, Arai M. (1995) 'Purification and properties of three xylanases from Aspergillus aculeatus', Biosci Biotechnol Biochem, Vol. 59, pp. 538–540.

72. Georis J, Giannotta F, de Buyl E, Granier B, Frère J-M. (2000) 'Purification and properties of three endo-β-1,4-xylanases produced by Streptomyces sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulp', Enzyme Microb Technol, Vol. 26, pp. 178–186.

73. Ghanen NB, Yusef HH, Mahrouse HK. (2000) 'Production of Aspergillus terreus xylanase in solid-state cultures: application of the Plackett–Burman experimental design to evaluate nutritional requirements', Bioresour Technol Vol. 73, pp. 113–121.

74. Ghareib M, El Dein MMN. (1992) 'Purification and general properties of xylanase from Aspergillus terreus', Zentralbl Mikrobiol, Vol. 147, pp.569–576.

75. Akiba, S., Y. Kimura, K. Yamamoto and H. Kumagai. (1995). 'Purification and characterization of a protease-resistant cellulase from *Aspergillus niger'*, J. Ferment. Bioeng, Vol. 79, pp. 125-130.

76. Chen, G., J. Du, L. Zhuang and P. GAO (2001) 'Purification and properties of endoglucanases from *Aspergillus aculeatus* SM-L22', Wei Sheng Wu Xue Bao, Vol. 41, No. 4, pp. 469-474.

77. GAO, J., H. Weng, Y. Xi, D. Zhu and S. Han. (2008) 'Purification and characterization of a novel endo-beta-1, 4-glucanase from the thermoacidophilic *Aspergillus terreus*', Biotechnol. Lett, Vol. 30, No. 2, pp. 323-327.

78. Hurst, P.L., J. Nelson, P.A. Sullivan and M.G. Shepherd, (1977) 'Purification and properties of a cellulose from *Aspergillus niger'*, Biochem. J., Vol. 165, pp. 33-41.

79. Lee, D. W. *et al.*, (1999) 'Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1', *FEMS Microbiol. Lett*, Vol. 170, pp. 393–400.

80. Tsujibo, H., Nishino, S. N. and Inamori, Y., (1990) 'Purification and properties of three types of xylanases produced by an alkaliphilic actinomycete', *J. Appl. Bacteriol.*, Vol. 69, pp. 398–405.

81. Beg, Q. K., Bhushan, B., Kapoor, M. and Hoondal, G. S., (2000) 'Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3', *J. Indian Microbiol. Biotechnol*, Vol. 24, pp. 396–402.

82. Gilbert, M., Breu, C., Aguchi, M. and Saddler, J. N., (1992) 'Purification and characterization of a xylanase from the thermophilic Ascomycetes *Thielavia terrestris* 2558', Appl. *Biochem. Biotechno*, Vol. 34, pp. 247–259.

83. Lee, D. W. *et al.*, (1999) 'Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1', *FEMS Microbiol. Lett*, Vol. 179, pp. 393–400.

84. Chaudhary, P. and Deobagkar, D.N. (1997) 'Purification and Characterization of Xylanases from *Cellulomonas* sp. N.C.I.M.2353', *Biotechnology and Applied Biochemistry,* Vol. 25, pp.127-133.

85. Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B., and Claeyssens, M. (1996) 'Purification and Characterization of Two Low Molecular Mass Alkaline Xylanasesfrom *Fusarium oxysporum* F3', *Journal of Biotechnology*, Vol. 51, pp. 181-189.

86. Elegir, G., Szakacs, G., and Jeffries, T.W. (1994) 'Purification, Characterization, and Substrate Specificities of Multiple Xylanases from *Streptomyces* sp. Strain B-12-2', *Applied and Environmental Microbiology,* Vol. 60, No. 7, pp. 2609-2615.

87. Tsujibo, H., Sakamoto, T., Nishino, N., Hasegawa, T., and Inamori, Y. (1990) 'Purification and Properties of Three Types of Xylanases Produced by An Alkalophilic Actinomycete', *Journal of Applied Bacteriology'* Vol. 69, pp. 398-405.

88. Matte, A. and Forberg, C.W. (1992) 'Purification, Characterization and Mode of Action of Endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85', *Applied and Environmental Microbiology,* Vol. 58, No. 1, pp. 57-158.

89. Ruiz-Arribas, A., Fernandez-Abalos, J.M., Sanchez, P., Grada, A.N., and Santamaria, R.I. (1995) 'Overproduction, Purification, and Biochemical Characterization of a Xylanase (*xyl*1) from *Streptomyces halstedii* JM8', *Applied and Environmental Microbiology,* Vol. 1, No. 6, pp. 2414-2419.

90. Grabski, A.C. and Jeffries, T.W. (1991) 'Production, Purification, and Characterization of β–(1, 4)-Endoxylanase from *Streptomyces roseiscleroticus*', *Applied and Environmental Microbiology*, Vol. **57**, No. 4, pp. 987- 992.

91. Sakka, K., Kojima, Y., Kondo, T., Karita, S., Shimada, K., and Ohmiya, K. (1994) 'Purification and Characterization of Xylanase A from *Clostridium stercorarium* F-9 and a Recombinant *Escherichia coli*', *Bioscience, Biotechnology and Biochemistry*, Vol. 58, No. 8, pp. 1496-1499.

92. Akiba, S., Y. Kimura, K. Yamamoto and H. Kumagai, (1995) 'Purification and characterization of a protease-resistant cellulase from *Aspergillus niger'*, J. Ferment. Bioeng, Vol. 7, pp. 125-130.

93. Anish, R., M.S. Rahman and M. Rao, (2006) 'Application of cellulases from an alkalothermophilic *Thermomonospora* sp. in biopolishing of denims', Biotechnol. Bioeng, Vol. 96, No. 1, pp. 48-56.

94. Bhat, M.K. and S. Bhat, (1997) 'Cellulose degrading enzymes and their potential industrial applications', Biotechnol. Adv., Vol. 1, pp. 583-620.

95. Bradford, M.M., (1976) 'A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding', Anal. Biochem, Vol. 72, pp. 248-254.

96. Chen, G., J. Du, L. Zhuang and P. Gao, (2001) 'Purification and properties of endoglucanases from *Aspergillus aculeatus* SM-L22', Wei Sheng Wu Xue Bao, Vol. 41, No. 4, pp. 469-474.

97. Elshafei, A.M. *et al.*, (2009) 'Purification and properties of an endoglucanase of *Aspergillus terreus* DSM 826', J. Basic Microbiol., Vol. 49, No. 5, pp. 426-432.

98. Gao, J., H. Weng, Y. Xi, D. Zhu and S. Han, (2008) 'Purification and characterization of a novel endo-beta- 1,4-glucanase from the thermoacidophilic *Aspergillus terreus*', Biotechnol. Lett, Vol. 30, No. 2, pp. 323-327.

99. Halliwell, G., (1965) "Hydrolysis of fibrous cotton and reprecipitated cellulose by cellulolytic enzymes from soil microorganisms', Biochem. J., Vol. 95, pp. 270-275.

100. Hong, J., H. Tamaki, S. Akiba, K. Yamamoto and H. Kumagai, (2001) 'Cloning of a gene encoding a highly stable endo-beta-1, 4-glucanase from *Aspergillus niger* and its expression in yeast', J Biosci. Bioeng, Vol. 92, No. 5, pp. 34-441.

101. Hurst, P.L., J. Nelson, P.A. Sullivan and M.G. Shepherd, (1977) 'Purification and properties of a cellulose from *Aspergillus niger*', Biochem. J., Vol. 165, pp.33-41.

102. Laemmli, U.K., (1970) 'Clevage of structure proteins during the assembly of the head of bacteriophage T4', Nature, Vol. 227, pp. 680-685.

103. Mandels, M., R. Andreotti and C. Roche, (1976) 'Measurment of saccharifying cellulose. Biotechnol. Bioeng', Symp, Vol. 6, pp.21-34.

104. Nazir, A., R. Soni, H.S. Saini, R.K. Manhas and B.S. Chadha, (2009) 'Purification and characterization of an endoglucanase from *Aspergillus terreus* highly active against barley â-glucan and xyloglucan', World J. Microbiol. Biotechnol, Vol. 25, pp. 1189-1197.

105. Ole, K., T.V. Borchert and C.C. Fuglsang, (2002) 'Industrial enzyme applications', Curr. Opin. Biotech, Vol. 13, pp.345-351.

106. Waksman, G., (1991) 'Purification and characterization of two endo-1-4- beta-D-glucanases from *Sclerotinia sclerotium*', Biochem. Biophys. Acta, Vol. 1073, pp. 49-55.

107. Asim Esen (1920) 'Purification and Partial Characterization of Maize (Zea mays L.) β-Glucosidase', - Plant Physiol, Vol.198, pp.174-182.

108. Badal Saha, C. and Rodey, J. Bothast (1996) 'Production, Purification, and Characterization of a Highly GlucoseTolerant Novel f)-Glucosidase from *Candida peltata a* pplied and Environmental microbiology, Vol. 62, pp. 3165-3170.

109. Cesar .Vanderlei. Nascimento. Flávio Henrique. Moreira Souza. Douglas. Chodi. Masui.Francisco. Assis. Leone. Rosane. Marina. Peralta. João Atílio Jorge.Rosa Prazeres and Melo Furriel. (2010) 'Purification and Biochemical Properties of a Glucose-Stimulated α-D-Glucosidase Produced by *Humicola grisea* var. *Thermoidea* Grown on Sugarcane Bagasse',- Journal of Microbiology ,Vol. 48, No. 1, pp. 53-62.

110. Christine Riou. Jean-michel Salmon. Marie-jose Vallier. Ziya gu nata. and Pierre barrel.(1998) 'Purification, Characterization, and Substrate Specificity of a Novel Highly Glucose-Tolerant β-Glucosidase from *Aspergillus oryzae'*, Applied and Environmental microbiology, Vol. 64, No. 10, pp. 3607–3614.

111. George Tsao,T. Liming Xia.Ningjun Cao and Cheng Gong,S (2000) 'Solid-State Fermentation with Aspergillus niger for Cellobiase Production',- Applied Biochemistry and Biotechnology, Vol. 84–86.

112. Korish, M. & Salem, M (2007) 'A Broad -specificity β-Glucosidase from a wild type of yeast isolate and its potential use in food industry', - Food science and Technology, Vol.4 No.1,pp.63-72.

113. Korotkova.O.G. Semenova.M.V. Morozova.V.V. Zorov.I.N. Sokolova.L.M. Bubnova.T.M. Okunev.O.N. and Sinitsyn.A.P.(2009) 'Isolation and Properties of Fungal β_Glucosidases', Vol. 74, No. 5, pp. 569-57.

114. Kunio Ohmiya. Makoto Shirai. Yasumasa Kurachi and Shoichi Shimizu (1985), 'Isolation and Properties of β-Glucosidase from Ruminococcus albus',- Journal of Scientific and Industrial Research, Vol.64.

115. Ming Chena.Liming Xiaa.Peijian Xueb. (2007) 'Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate',- International Biodeterioration & Biodegradation , pp.85-89.pp

116. Mohamed, A. Abdel-Naby. Mona Osman, Y and Ahmed,F. Abdel-Fattah (1998) 'Purification and Properties of Three Cellobiases from Aspergillus niger A20',- Applied Biochemistry and Biotechnology, Vol. 76.

117. Pothiraj, C.Balaji and Eyini, M. (2006) 'Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste',- African Journal of Biotechnology ,Vol. 5 (20), pp. 1882-1885.

118. Rajeev Sukumaran, K. Reeta Rani Singhania and Ashok Pandey (2005) 'Microbial Cellulases-Production, Applications and Challenges',- Journal of Scientific and Industrial Research ,Vol.64,pp-832-844.

119. Tarek, A. Moussa.A and Nagwa, A. Tharwat. (2007) 'Optimization of cellulase and β-glucosidase induction by sugarbeet pathogen Sclerotium rolfsii'- African Journal of Biotechnology, Vol. 6 (8), pp. 1048-1054.

120. Xin-Liang Li. Lars Ljungdahl, G. Eduardo Ximenes, A. Huizhong Chen. Carlos Felix,R. Michael Cotta,A and Bruce Dien,S. (2004) 'Properties of a Recombinant β-Glucosidase from Polycentric Anaerobic Fungus Orpinomyces PC-2 and Its Application for Cellulose Hydrolysis',- Applied Biochemistry and Biotechnology, Vol 2,pp 113–116.

121. Yin kiong Hoh. Hock-Hin Yeoh and Teck Koon Tan (1992) 'Properties of β-Glucosidase purified from Aspergillus niger mutants USDB 0827 and USDB 0828',- Applied Microbiology Bio-Technology ,Vol.37, pp 590-593.

122. Zorov, I.N. Gusakov, A. V. Baraznenok, V.A. Bekkarevich, A.O. Okunev, O.N. Sinitsyn, A.P and Kondrateva, E.G *(2001)* 'Isolation and Properties of Cellobiase from *Penicillium verruculosum'*, - Applied Biochemistry and Microbiology, *Vol. 37, No. 6, pp. 587–592.*