α-Galactosidase from *Streptomyces griseoloalbus*: an enzyme with versatile applications

Thesis submitted under the Faculty of Sciences
Cochin University of Science and Technology
for the award of the degree of

Doctor of Philosophy

in

Biotechnology

ANISHA G.S.

Reg. No. 2784

Biotechnology Division,
National Institute for Interdisciplinary Science and Technology
Council of Scientific and Industrial Research
Thiruvananthapuram- 695019, INDIA

MAY 2008

DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr. P. Prema, Scientist F, in the Biotechnology Division of National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, and no part of this work has been included in any other thesis submitted previously for the award of any degree.

Thiruvananthapuram *ANISHA G.S.*

May 2008

राष्ट्रीय अंतर्विषयी विज्ञान तथा प्रौद्योगिकी संस्थान

(वैज्ञानिक एंव प्रौद्योगिकी अनुसंधान परिषद्)

(पहले क्षेत्रीय अनुसंधान प्रयोगशाला)

NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE AND TECHNOLOGY

(Council of Scientific & Industrial Research)

(formerly Regional Research laboratory)

इन्डस्ट्रियल इस्टेट डाक घर, तिरुवनन्तपुरम 695 019, भारत

Industrial Estate P.O., Thiruvananthapuram 695 019, India

05/05/2008

CERTIFICATE

This is to certify that the work presented in the thesis entitled "**α-Galactosidase from *Streptomyces griseoloalbus*: an enzyme with versatile applications**" is based on the original research done by **Mrs. Anisha G.S.** under my guidance and supervision at the Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum 695 019, India, and no part of this work has been included in any other thesis for any award of any degree.

Dr. P. Prema

Scientist F

Biotechnology Division

तार Telegram कानमग 'Consearch', फक्स Fax : +91-471-2491895, 2491712, 24910186, 2491842, 2490389

फान Phone [Epabx] (0471) 2490674, 2490224, 2490811, 2490851, 2490852

इमेल E-mail SPO@nistres.in, contact@nistres.in, rrltvm@md2.vsnl.net.in वेबसाइट Website http://www.nistres.in

ACKNOWLEDGEMENTS

This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that helped to get this done, but it will be tough to thank them enough. I will nonetheless try...

First and foremost I would like to thank my Ph.D. supervisor Dr. P. Prema for many insightful conversations during the development of the ideas in this thesis. I wish to express my wholehearted thanks to her for allowing me to choose this wonderful and interesting topic for my research and for being very supportive and confident in my abilities as a researcher. She had the faith to allow me to develop and pursue my own research interests.

A special gratitude is due to Prof. Ashok Pandey for leading the team BIOTECH so far and being insatiable in the quest for improvement and knowledge. He was also a particular leader and motivator, enabling all the team to face the hard work with a smile on the lips. His timely help, encouragement and valuable suggestions increased my interest in research.

It is my privilege to place on record my gratitude to Prof. T.K. Chandrashekar, Director, National Institute for Interdisciplinary Science and Technology, Trivandrum, for providing the necessary infrastructural facilities to carry out this work.

I am grateful to the Council of Scientific and Industrial Research, New Delhi, for the award of Junior and Senior Research Fellowships for my doctoral studies.

I am deeply indebted to Dr. Madhavan Nampoothiri, Dr. Rajeev Sukumaran and Dr. Sudheer Singh, scientists of team BIOTECH, whose thoughtful advice and stimulating criticisms often served to give me a sense of direction during my PhD studies. I am

also thankful to Dr. V.B. Manilal, Mr. Ajith Haridas and Mrs. Vijaya Prasad, Environmental Technology Division, NIIST, for their help and support.

I thank Dr. Sabu Thomas, Dr. Sreeja and Mrs. Indu Ramachandran, Rajiv Gandhi Centre for Biotechnology, Trivandrum, for making necessary arrangements for *N*-terminal sequencing. I am also grateful to Mr. M.K. Chandran for his help in scanning electron microscopy and to Mrs. Soumini, Ms. V.B. Gangadevi and Ms. Sajisha Sanjayan, Chemical Sciences Division, NIIST, for their excellent technical assistance in NMR spectra measurement. I also thank all the technical and non-technical staff of NIIST.

I am indebted to my many student colleagues for providing a stimulating and fun environment in which to learn and grow. The discussions, encouragement and critiques made by them were of essence to the progress of this work. I remember the joyful moments shared with my dear friends Asha Poorna, Nicemol Jacob, Sheejadevi, Sumitra, Sumantha, Babitha, Binod, Dhanya, Swetha, Syed, Aswathy, Sai, Shyam, Bindhumol, Reeta, Raji, Nisha, Gincy, Padma, Priya and Nimi. I am fortunate to have a friend like Nila who deserves special mention. She has been the best friend of mine, who stood by my side helping and supporting me in every way she could.

It is difficult to overstate my appreciation to Dr. Usha Balaraman, my teacher in All Saints' College, Trivandrum, who first kindled the research attitude in my mind. Not only a great mentor, she has also been a cornerstone in my professional development.

I cannot end without thanking my family, on whose constant encouragement and love I have relied throughout my period of research. Particular thanks, of course, to Asha, my sister and Rosamma John, my mother-in-law for their love and prayers. No words can suffice the encouragement and moral support rendered by my dear

husband Rojan P John. Without his support and striving development this work would never have reached the great result it did.

I express my heart-felt love and gratitude to my dear parents, Sathyanesan Daniel and Grace Sathyanesan. They have always supported my dreams and aspirations and have given me the freedom to choose my own career. They have always encouraged me to do my best in all my endeavors. They bore me, raised me, taught me, and loved me. To them I dedicate this thesis. Last but not the least, I acknowledge my little son, Joel John Rojan, whose innocent smile makes my days more enjoyable and worth living.

Nothing would have been possible without the blessings of our Lord, for "The Lord is good; His mercy is everlasting and his truth endures for all generations" - Psalms 100:5.

ANISHA G.S.

PREFACE

The present study is focused on the production, purification and characterization of multiple thermostable α-galactosidases from a novel actinomycete strain *Streptomyces griseoloalbus*. The Chapter I of the thesis covers the wide literature regarding α-galactosidases from various sources and their potential applications. The Chapter II deals with the isolation of α-galactosidase-producing actinomycetes and selection of the best strain. The Chapters III and IV describe the optimization of α-galactosidase production under submerged fermentation and solid-state fermentation respectively. The Chapter V describes the purification and characterization of multiple α-galactosidases and also the obvious existence of a novel galactose-tolerant enzyme. The Chapter VI illustrates the potential applications of α-galactosidases from *S. griseoloalbus* followed by the Chapter VII summarizing and concluding the results of the present investigation.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

%	Percentage
°C	Degree Celsius
δ	Chemical shift
µL	Micro litre
µM	Micro molar
µm	Micro meter
µmol	Micro mol
^2H_2O	Deuterized water
α-Gal	α-Galactosidase
Abs	Absorbance
ANOVA	Analysis of variance
BCP	Bromocresol purple
C_1	α-Galactosidase yield in the 1st fermentation cycle
CAZy	Carbohydrate active enzymes
CCD	Central composite design
CDW	Cell dry weight
C_{free}	α-Galactosidase yield of free cells
CFU	Colony forming units
C_{imm}	α-Galactosidase yield of immobilized cells
cm	Centimeter
CO_2	Carbon dioxide
COC	Coconut oil cake
CPF	Chick pea flour
C_x	α-Galactosidase yield in the xth fermentation cycle
Da	Dalton
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
Fru	Fructose
g	gram
V_{max}	Maximum reaction velocity
g/L	Gram per litre

G_1	Galactose liberated in the 1st catalytic cycle
Gal	Galactose
gds	Gram dry fermented substrate
GF	Guar flour
G_{free}	Galactose liberated by free enzyme
GH	Glycosyl hydrolase
G_{imm}	Galactose liberated by immobilized enzyme
Glu	Glucose
GOC	Groundnut oil cake
G_x	Galactose liberated in the xth catalytic cycle
h	Hour
H_2O	Water
kDa	kilo Dalton
K_m	Michaelis constant
kV	kilo Volt
L	Litre
LBG	Locust bean gum
M	Molar
m	*meta*
MF	Maize flour
mg	milligram
mg	milligram
mg/L	milligram/Litre
mg/mL	milligram/millilitre
MHz	mega Hertz
mL	Millilitre
mM	Milli molar
mm	millimeter
M_r	Relative molecular mass
MTCC	Microbial Type Culture Collection
MU-α-gal	Methyl umbelliferyl-α-D-galactopyranoside
NDO	Non-digestible oligosaccharides
NMR	Nuclear magnetic resonance
o	*ortho*
O_2	Oxygen

OD	Optical density
p	*para*
PAGE	Polyacrylamide gel electrophoresis
pH	Hydrogen ion concentration
pI	Isoelectric point
*p*NP	*p*-Nitrophenol
*p*NPG	*p*-Nitrophenyl-α-D-galactopyranoside
ppm	parts per million
RB	Rice bran
RFO	Raffinose-family oligosaccharides
rpm	Rotations per minute
RSM	Response surface methodology
SBF	Soybean flour
SCA	Starch casein agar
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SF	Sorghum flour
SM	Soymeal
SmF	Submerged fermentation
SSF	Solid-state fermentation
TLC	Thin layer chromatography
T_{max}	Maximum bearable temperature
T_{opt}	Optimum temperature
U	Enzyme unit
U/gds	Units per gram dry fermented substrate
U/mL	Units per millilitre
UV	Ultra violet light
v/v	Volume by volume
vvm	Vessel volume/min
w/v	Weight/volume
w/w	Weight/weight
WB	Wheat bran

Chapter I

INTRODUCTION AND REVIEW OF LITERATURE

1.1. INTRODUCTION

Enzymes were discovered in the second half of the nineteenth century by Eduard Buchner, and since then they have been extensively used in several industrial processes. Enzymes are extremely efficient and highly specific biocatalysts. Most of the machinery of living cells is made of enzymes. Since the beginning of biochemical investigation enzymes have held a special fascination for chemists and biologists. Thousands of them have been extracted from cells and have been purified and crystallized. Many others are recognized only by their catalytic action and have not yet been isolated in pure form. With the advancement in biotechnology over last three decades, especially in the area of genetics and protein engineering, enzymes have found their way into many new industrial processes. Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. Environmental pollution is no longer accepted as inevitable in technological societies. Over the past century, there has been a tremendous increase in awareness of the effects of pollution, and public pressure has influenced both industry and government. There is increasing demand to replace traditional chemical processes with biotechnological processes involving micro-organisms and enzymes such as pectinases (Bajpai 1999; Bruhlmann et al. 2000), xylanases (Beg et al. 2000), cellulases (Bajpai 1999), mannanase (Montiel et al. 2002), α-galactosidase (Clarke et al. 2000), and laccases and ligninases (Bajpai 1999; Onysko 1993), which not only provide an economically viable alternative but are also more environmentally friendly (Viikari et al. 2001).

Thermostable enzymes are gaining wide and excellent applications in industrial sectors that take advantage of their function at high temperatures. Thermostable enzymes can either replace those enzymes currently used at less than optimal processing temperatures or be used in new biocatalytic applications. Generally enzymes that function optimally between 60 and 125 °C are regarded as thermostable. They have attracted increasing attention in recent years owing to their biotechnological

potential. They are already used in molecular biology (*Taq* polymerase), detergent industry (proteases) and starch processing (α-amylase) and are excellent catalytic candidates for numerous additional applications that require high temperature stability. The fact that extremozymes have significant financial impact for the companies that exploit them is evident from the example of *Taq* polymerase, which has sales of $ 80 million per annum (Gupta 1993). In general, parameters like temperature, pH and chemical as well as enzymatic stability are important for the industrial applicability of enzymes. The use of thermophilic enzymes reduces stability problems and in doing so, alleviates some of the expense of production and replacement in a reactor. Besides higher thermostability other expected advantages of thermophilic enzymes are increased chemoresistance, a longer useful shelf life and less contamination problems (Sonnleitner & Fiechter 1983).

Generally thermophilic organisms are considered as sources of thermostable enzymes. However, the temperature for an individual enzyme from an organism may have an optimum temperature quite different from that of the organism. If the temperature optimum of the enzyme is above that of the optimum temperature (T_{opt}) or the maximum bearable temperature (T_{max}) for the producing organism, it may be regarded as thermostable. It should therefore, not be surprising that even enzymes from psychrophilic or mesophilic organisms may be found to be stable and active at high temperatures (Gupta 1993).

The glycosidic bond is one of the most stable bonds in nature. With an estimated half life of around 5 million years (Wolfenden et al. 1998), the enzymes which catalyze the hydrolysis of this bond may be considered among the most powerful enzyme catalysts known. This, combined with the diversity of glycosidic bonds which may form between different sugar moieties in the order of 10^{12} possible hexasaccharide isomers (Laine 1994), creates a large and structurally diverse group of enzymes. These enzymes play central roles in diseases such as diabetes, and industrial processes like food technology. This ensures that the glycosyl hydrolases are a medically relevant, industrially important and scientifically interesting group of enzymes.

1.2. Glycosyl hydrolases

As a rule, about 1% of genes in a given genome encode glycosyl hydrolases and their homologues (Naumoff 2005). Glycoside hydrolases or glycosidases (EC 3.2.1.-) are a widespread group of enzymes that hydrolyze the glycosidic bonds between two carbohydrates or between a carbohydrate and an aglycone moiety. A large multiplicity of these enzymes is a consequence of the extensive variety of their natural substrates: di-, oligo-, and polysaccharides. Enzymes, which catalyze the hydrolysis of glycosidic linkages are widely distributed in nature and include α-galactosidase, β-galactosidase, invertase, maltase, β-glucosidase, amylase etc. The existence of glycosidases, also known as carbohydrolases, has been known for more than 100 years and they are the very first biological catalysts investigated. Glycosidases are truly remarkable enzymes. They are able to catalyze glycoside hydrolysis at rates of up to 1000 s^{-1}, offering rate enhancements of 10^{17} fold (Wolfenden et al. 1998; Yip & Withers 2006).

1.2.1. Classification of glycosyl hydrolases

The traditional nomenclature of glycosidases, the International Union of Biochemistry Enzyme Classification (E.C.) nomenclature system, is based on their substrate specificity and occasionally on the molecular mechanism of their action; such a classification, however, does not reflect the structural features and evolutionary relationships of these enzymes, and it is not appropriate for enzymes that act on several substrates (Henrissat 1991; Davies & Henrissat 1995; Henrissat & Davies 1997; Coutinho & Henrissat 1999a, b). A solution to this dilemma was proposed by Henrissat in 1991 by the development of a classification of glycosyl hydrolases based on their amino acid sequence similarities. Since there is a direct relationship between sequence and folding similarities, such a classification: (i) reflects the structural features of these enzymes better than their sole substrate specificity, (ii) helps to reveal the evolutionary relationships between these enzymes, and (iii) provides a convenient tool to derive mechanistic information (Henrissat 1991; Henrissat & Bairoch 1993). The

potential active site residues, which are strongly conserved among the similar family enzymes, can also be located by a detailed comparative analysis of the primary structures of glycosyl hydrolases. According to this new system which is complementary to the International Union of Biochemistry enzyme nomenclature, the glycosyl hydrolases from various sources including bacteria, fungi, plants and animals are classified into 112 families (as on May 2008) (http://afmb.cnrs-mrs.fr/CAZY/) and the number is still growing. Glycosyl hydrolases with a high degree of sequence homology are assigned to the same glycosyl hydrolase family (GH family). Enzymes in one GH family can have different substrate specificities and a different mode of action (endo or exo). However, it appears that within a GH family the catalytic mechanism, inverting or retaining, is conserved (Gebler et al. 1992). The catalytic residues are also conserved within a GH family (Henrissat & Bairoch 1996) as well as the protein fold (Davies & Henrissat 1995; Henrissat & Davies 1997).

As the fold of proteins is better conserved than their sequences, some of the families can be grouped in 'clans' (Henrissat & Bairoch 1996; Henrissat & Davies 1997), (i) when new sequences are found to be related to more than one family, (ii) when the sensitivity of sequence comparison methods is increased, or (iii) when structural determinations demonstrate the resemblance between members of different families. According to its definition, a clan is a group of families that are thought to have a common ancestry and are recognized by significant similarities in tertiary structure together with conservation of the catalytic residues and catalytic mechanism (Henrissat & Bairoch 1996). Currently 14 clans (GH-A to GH-N) are described, and in total they contain 46 GH families. The Carbohydrate Active Enzymes database (CAZy) (http://www.afmb.cnrs-mrs.fr/CAZY/) provides an up-to-date inventory of GH families.

1.2.2. Mechanisms of action of glycosyl hydrolases

Based on the nature of hydrolysis glycosyl hydrolases are of two types- exo-glycosidases and endo-glycosidases. The exo-glycosidases (e.g. galactosidases, glucosidases) act on the glycosidic bond present at the non-reducing end of the

saccharide chain whereas endo-glycosidases (e.g. amylases) act on the glycosidic bond within the saccharide chain.

The mechanism of action of glycosyl hydrolases involves the hydrolysis of glycosidic bonds in oligo- and polysaccharides by two carboxylic acid catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base (Sinnott 1990; McCarter & Withers 1994; Wang et al. 1994; Ly & Withers 1999; Rye & Withers 2000; Zechel & Withers 2000; Zechel & Withers 2001; Vasella et al. 2002). Depending on the spatial position of these catalytic residues, hydrolysis proceeds with one of the two stereochemical outcomes: net inversion or net retention of the anomeric configuration (Fig. 1.1).

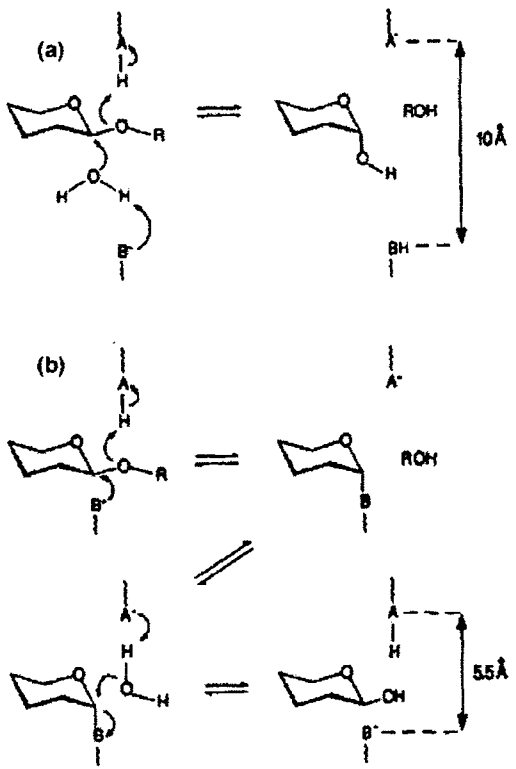


Fig. 1.1. Mechanisms of action of glycosyl hydrolases. (a) The inverting mechanism, in which protonation of the glycosidic oxygen and aglycon departure are accompanied by a concomitant attack of a water molecule that is activated by the base residue (B-). This single nucleophilic substitution yields a product with opposite stereochemistry to the substrate. (b) The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and nucleophilic assistance to aglycon departure is provided by the base B-. The resulting glycosyl enzyme is hydrolyzed by a water molecule and this second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate. (Davies & Henrissat 1995).

Inverting enzymes have a single displacement mechanism (Koshland 1953) in which bond breaking and bond making proceed in a single concerted step (Fig. 1.1a). In this case the glycosidic oxygen is initially protonated by the general acid catalyst forming an oxocarbenium ion (Zechel & Withers 2000), accompanied by a concomitant attack at the anomeric center of the sugar molecule by a water molecule, which is activated by a carboxylic base catalyst. The reaction product therefore has an anomeric configuration that is opposite to that of the substrate. As a consequence of the inclusion of an activated water molecule between the general base and the anomeric C1 atom, the catalytic carboxyl groups in inverting enzymes are generally about 6.5-10 Å apart (McCarter & Withers 1994; Davies & Henrissat 1995).

The retaining reactions proceed *via* a double displacement mechanism involving the formation of a glycosyl-enzyme intermediate (Vernon 1967). In the first step the carboxylic acid acts first as an acid catalyst and protonates the glycosidic oxygen, while the other carboxylic acid acts as nucleophile and assists departure of the leaving group (Fig. 1.1b). This is the glycosylation step and a glycosyl-enzyme intermediate is formed. Subsequently, the first carboxylic acid will behave as a base catalyst and activate the incoming nucleophile (water), resulting in the hydrolysis of the glycosyl-enzyme intermediate. This is the de-glycosylation step. Each step proceeds *via* oxocarbenium ion-like transition states and inverts the configuration of the anomeric carbon. The two displacements thus result in overall retention of the anomeric configuration and the product formed has the same stereochemistry as the substrate. This mechanism requires a closer proximity of the two active carboxyl groups, generally around 5.5 Å (Davies & Henrissat 1995; McCarter & Withers 1994; Withers 2001).

For retaining enzymes, the incoming nucleophile can be a sugar molecule instead of water. This can lead to the formation of oligosaccharides with a higher degree of polymerization or containing a new linkage type. Such a reaction is called transglycosylation and the enzymes having this mechanism of action are called transglycosylases or glycosyl transferases (Sinnott 1990).

The single and double displacement mechanisms were first introduced in 1953 by Koshland, and have copious support from structural and mechanistic analyses of

enzymes across the numerous glycosidase families. These studies were also able to reveal that there is a direct relationship between primary sequence, and enzyme structure and mechanism, which authenticates the value of the classification system developed by Henrissat (Henrissat et al. 1995; Henrissat & Bairoch 1996; Henrissat & Davies 1997; Coutinho & Henrissat 1999a).

1.3. α-Galactosidases

α-Galactosidases or α-D-galactoside galactohydrolases (E.C. 3.2.1.22) are exo-glycosidases that cleave, with the optical configuration retained, the terminal non-reducing α-1-6-linked galactose residues from α-D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids. The presence of α-galactosidase was discovered for the first time in bottom yeast almost simultaneously by Bau and by Fischer & Lindner in 1895.

α-Galactosidases have been classified based on substrate specificity (Dey et al. 1993) and sequence similarity using hydrophobic cluster analysis (Henrissat & Bairoch 1996). With respect to substrate specificity, group I α-galactosidases hydrolyze oligosaccharides such as melibiose, raffinose, stachyose and verbascose; group II α-galactosidases are active on polysaccharide substrates, such as galactomannan and galactoglucomannan. By amino acid sequence homology, α-galactosidases are classified into four GH families: GH4, GH27, GH36 and GH57. Families GH4 and GH57 encompass several types of glycosyl hydrolases. α-Galactosidase activity has been demonstrated only for six enzymes of GH4 family and two enzymes of GH57 family (http://www.cazy.org/fam/acc_GH.html). The GH4 α-galactosidases include enzymes from *Alicyclobacillus acidocaldarius*, *Bacillus subtilis subtilis* str. 168, *Citrobacter freundii* M4, *Escherichia coli*, *Salmonella typhimurium* LT2 and *Sinorhizobium meliloti* 1021 and GH57 α-galactosidases are from *Pyrococcus furiosus* and *Thermococcus alcaliphilus*. The majority of the known α-galactosidases belong to GH27 and GH36 families. Most α-galactosidases of eukaryotic origin including *Aspergillus niger* AglA (den Herder et al. 1992) and AglB (de Vries et al. 1999) belong to family 27. Family 36 contains primarily bacterial α-galactosidases including α-galactosidase from *Bacillus*

stearothermophilus NUB 3621 (Fridjonsson et al. 1999a), *Thermus thermophilus* (Fridjonsson & Mattes 2001) and *Thermus* sp. Strain T_2 (Ishiguro et al. 2001). However some α-galactosidases of prokaryotic origin are also included in GH27 family. Similarly some eukaryotic α-galactosidases are also included in GH36 family. For example, α-galactosidase from *Streptomyces coelicolor* A3(2) (accession number CAB54169) is a member of GH27 family, where as that from *Trichoderma reesei* (accession number Z69254) is a member of GH36 family. There are also reports documenting the presence of α-galactosidases belonging to both families being produced by the same organism. For e.g. *Aspergillus niger* ATCC 46890 produces four major α-galactosidase forms (α-gal I-IV) of which α-gal I belongs to family 36 and α-gal II, III and IV, which appears to be isoforms of the same enzyme, show close similarity to family 27 α-galactosidases (Ademark et al. 2001).

The GH27 and GH36 families are thought to share a common ancestral gene forming the glycosyl hydrolase clan GH-D (http://afmb.cnrs-mrs.fr/CAZY/) with a common highly conserved $(\beta/\alpha)_8$ barrel or triosephosphate isomerase (TIM) barrel structure. Amino acid sequence analysis has revealed homology between the $(\beta/\alpha)_8$ domain of GH27 glycosidases and enzymes of GH31 and GH36 families (Naumoff 2001; 2002), which has made it possible to unify the three families into one α-galactosidase superfamily (Naumoff 2002). The hydrolytic reaction of glycoside hydrolase clan D is known to take place by retaining or double-displacement mechanism and the experimentally determined nucleophile of the catalytic residue is an aspartic acid which is highly conserved (Hart et al. 2000; Ly et al. 2000). Recently Comfort et al. (2007) has elucidated the mechanistic commonality of clan GH-D glycoside hydrolases based on the biochemical analysis of *Thermotoga maritima* GH36 α-galactosidase (*Tm*GalA).

1.3.1. α-Galactosides

α-Galactosides are glycosides containing terminal non-reducing α-D-galactosyl residue whose first carbon atom or the anomeric carbon atom is attached to a carbohydrate or non-carbohydrate moiety by an acetal linkage. The α-D-galactosyl

groups are ubiquitous in higher plants and are found in a variety of oligo-saccharides, polysaccharides, and few non-sugars such as glycerol, inositol, and certain lipids. Such α-galactosides are mainly the substrates for the enzyme α-galactosidase.

The most commonly used substrate for the routine assay of α-galactosidase is the chromogenic synthetic substrate *p*-nitrophenyl-α-D-galactopyranoside (Dey & Pridham 1969a; Kotwal et al. 1995; Rezessy-Szabó et al. 2003; Gote et al. 2004; Liu et al. 2007a, b, c), the hydrolysis of which liberates *p*-nitrophenol which is then estimated spectrophotometrically. The natural substrates of α-galactosidase include the oligosaccharides melibiose (α-D-Gal*p*(1→6)D-Glu), raffinose (α-D-Gal*p*(1→6) α-D-Glu*p*(1→2)β-D-Fru), stachyose (α-D-Gal*p*(1→6)α-D-Gal*p*(1→6)-α-D-Glu*p*(1→2)β-D-Fru) and verbascose (α-D-Gal*p*(1→6)α-D-Gal*p*(1→6)α-D-Gal*p*(1→6)-α-D-Glu*p*(1→2)β-D-Fru) and the polysaccharides like galactomannans and galacto(gluco)mannans (Naumoff 2004). Because of its action on melibiose (Fig. 1.2), a disaccharide of glucose and galactose, α-galactosidase is also known as melibiase.

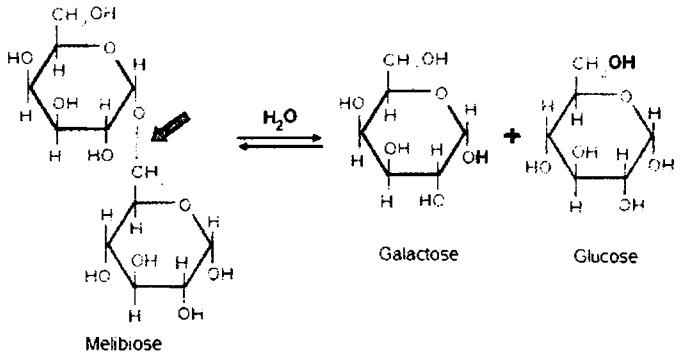


Fig. 1.2. Hydrolysis of melibiose by α-galactosidase. The block arrow points towards the α-1,6 linkage which is the site for action of α-galactosidase.

Raffinose, stachyose and verbascose, commonly called raffinose family oligosaccharides (RFO), are abundantly found in the seeds, roots, stem and leaves of the members of the family *Leguminoseae*, where they serve the purpose of reserve carbohydrate and also protection against frost and drought (Dey 1980). The structures

of the RFO, raffinose and stachyose, and the sites of cleavage by α-galactosidase are shown in Fig. 1.3.

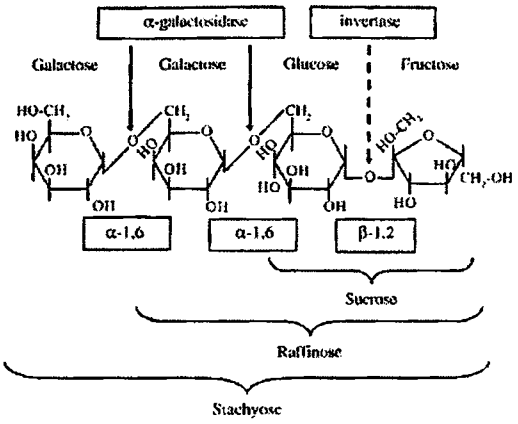


Fig. 1.3. Structures of raffinose and stachyose and the enzymes that catalyze their hydrolysis. The arrows point towards the sites of cleavage by the enzymes. (LeBlanc et al. 2004)

The polymeric substrates for α-galactosidase include the galactomannans and galacto(gluco)mannans which form the major cell wall components of higher plants (Aspinall 1980). The richest sources of galactomannans are the members of the family *Leguminoseae* (1-38% of seed dry weight) (Moreira & Filho 2008). In the family *Leguminoseae*, galactomannans are located in the seed endosperm, except for *Gymnocladus dioica* (Kentacky coffee bean), in which the polysaccharide lies in the inner side of the seed coat, and *Glycine max*, in which it occurs in the hulls. Galactomannans have also been found in species of *Annonaceae* (custard apple family), *Convolvulaceae* (morning glory family), *Ebenaceae* (ebony family), *Loganiaceae* (Buddleia family) and *Palmae* (Palm family) (Dea & Morrison 1975; Dey 1978; Moreira & Filho 2008). *Cyamopsis tetragonoloba* and the carob tree *Ceretonia siliqua* which are members of the family *Leguminosae*, are rich sources of galactomannans. *C. tetragonoloba* is an annual plant, grown in arid regions of India as a food crop for animals and its seed endosperm contains the galactomannan called

guar gum. Locust bean gum is another galactomannan extracted from the endosperm of the seeds of the carob tree *Ceretonia siliqua*, which grows in Mediterranean countries.

The amount and distribution of α-1,6-D-galactosyl side groups along the β-1,4-D-mannopyranose backbone in galactomannans depends on the species. For example, guar galactomannan (guar gum) contains 38-40% galactose whereas the amount of galactose in locust bean galactomannan (locust bean gum) is 22-24% (Barry et al. 1981). Water-soluble galactoglucomannan contains β-1,4-linked D-mannopyranose and D-glucopyranose in the backbone and α-1,6-linked D-galactopyranose as side groups in a ratio of 3:1:1, respectively. In the less soluble polysaccharide which is often referred to as glucomannan, the ratio is 3:1:0.1. In addition galactose is known to be a component in complex slime and gum substances. The complete hydrolysis of polymeric galactoglucomannans require the concerted action of three different enzymes- α-galactosidase, β-mannosidase and endo-β-D-mannanase (Zeilinger et al 1993). The structures of locust bean gum and guar gum and the sites of enzymatic hydrolysis are shown respectively in Figs. 1.4 and 1.5.

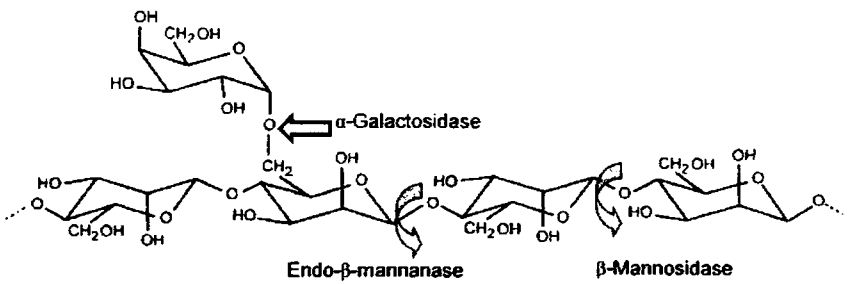


Fig. 1.4. Structure of locust bean gum and the enzymes that catalyze its hydrolysis. Locust bean gum is a linear polymer of β-(1,4)-D-mannose units. Approximately every fourth mannose unit is substituted with a small side chain of 1,6 linked α-galactose sugar (Sittikijyothin et al. 2005).

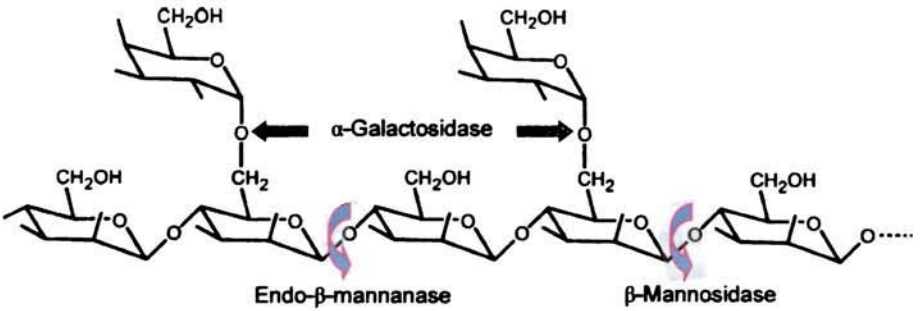


Fig. 1.5. Structure of guar gum and the enzymes that catalyze its hydrolysis. The linear β-1,4-D-mannopyranose backbone is substituted every two residues by a α-D-galactose residue at C-6 of mannose with 1→6 glycosidic bonds (Duffaud et al. 1997).

1.3.2. Sources of α-galactosidases

α-Galactosidases are widely distributed in nature, being found in a variety of plants, animals including both vertebrates and invertebrates and extensively in micro-organisms including bacteria, fungi and yeasts.

1.3.2.1. Plant sources of α-galactosidases

α-Galactosidase is widely distributed in the plant kingdom and is most widely studied in plant seeds. Among plants the presence of α-galactosidase was reported for the first time in sweet almond emulsion (Helferich & Appel 1932). The enzyme in plants has been greatly investigated from seeds in relation to germination (Dey & Pridham 1969a, b; Barham et al. 1971; Mc Cleary & Matheson 1974; McCleary 1983; Guimarães et al. 2001; Kim et al. 2002; Ren et al. 2007; Shen et al. 2008). Raffinose family oligosaccharides (RFOs) make up a substantial part (40%) of the soluble sugars found in soybean seeds. Verbascose, stachyose and raffinose (RFO, α-galactosyl derivates of sucrose) are associated with desiccation tolerance and storability of seeds (Obendorf 1997). RFOs are accumulated in soybean seeds during maturation, when all enzymes involved in the RFOs synthesis are active. RFOs are found in the cotyledons

of mature soybeans, located in the cytosol, while hydrolytic enzymes such as α-galactosidase are located in the protein bodies. The hydration of seeds during imbibition possibly induces α-galactosidase activity, leading to the breakdown of RFOs, which supplies the germinative energy (Herman & Shannon 1985). During soybean seed germination, the content of RFOs decrease substantially, while the α-galactosidase activity increases. RFO degradation during germination by endogenously synthesized α-galactosidase is also well-documented (Porter & Ladisch 1991; Modi et al. 2000; Guimarães et al. 2001; Machaiah & Pednekar 2002).

The enzyme α-galactosidase is also reported to be present in cucurbits (Thomas & Webb 1977; Gaudreault & Webb 1986; Irving et al. 1997; Gao & Schaffer 1999). The galactosyl-Suc sugars stachyose and raffinose, together with sucrose, are the primary translocated sugars in the phloem of cucurbits (Schaffer et al. 1996), including melon (*Cucumis melo*) (Chrost & Schmitz 1997). The very low concentrations of raffinose and stachyose in fruit tissues of *C. melo* (Chrost & Schmitz 1997) suggest that galactosyl-Suc unloaded from phloem is rapidly metabolized, with the initial hydrolysis by α-galactosidase. It is likely that α-galactosidase, as the initial enzyme in the metabolic pathway of stachyose and raffinose catabolism (Keller & Pharr 1996), plays an important role in the carbohydrate partitioning in the cucurbits (Gao & Schaffer 1999). This enzyme is also found in stalks of the *Saccharum officinarum* (Chinen et al. 1981), endosperm of coconut (Balasubramaniam & Mathew 1986) etc. Diverse forms of α-galactosidase occur occasionally in many plants (Dey & Pridham 1969a, 1969b, 1972; Thomas & Webb 1977; Haibath et al. 1991). Despite its wide distribution and diversity, α-galactosidases seem to be less abundant in fruits compared with other plant organelles because biochemical/physiological aspects in fruits differ from those of other tissues or organelles in plants (Dey & Pridham 1969a, 1969b, 1972; Thomas & Webb 1977; Haibath et al. 1991). Recently it has been isolated form tomato fruit (Feurtado et al. 2001), grape flesh (Kang & Lee 2001), cultured rice (Kim et al. 2002). Very recently it has been isolated from sunflower seeds (Kim et al. 2003). α-Galactosidases are generally involved in metabolic utilization of RFOs and galactomannan, which are even more widely distributed in storage organs such as seeds, roots and tubers (Mc Cleary &

Matheson 1974; Kandler & Hopf 1980; Porter & Ladisch 1991; Modi et al. 2000; Guimarães et al. 2001; Machaiah & Pednekar 2002).

Plant α-galactosidases from numerous sources have been studied, and multiple forms of the enzyme have been described (Keller & Pharr 1996). These can be divided into two groups, acid and alkaline, based on their activity response to pH. Most studies have dealt with the acid forms of the enzyme, which play important roles in seed development and germination (Keller & Pharr 1996). While the acid α-Gal type is most likely active in the acidic environment of the vacuole and the apoplasm, the alkaline α-Gal type probably catalyzes galactose release in the more neutral or alkaline cytoplasm (Gao & Schaffer 1999; Carmi et al. 2003; Lee et al. 2004). In the cucurbits Gaudreault & Webb (1986) have described an alkaline α-galactosidase from young leaves of *Cucurbita pepo*, in addition to multiple acid forms of the enzyme. The alkaline form is unique in that it shows a high affinity for stachyose and little activity towards raffinose compared with the acid forms, for which raffinose is found to be preferred substrate. However, Gao & Schaffer (1999) have identified a novel alkaline form of α-galactosidase with activity toward a broader spectrum of galactosyl saccharides, particularly, raffinose.

1.3.2.2. Animal sources of α-galactosidases

There are only few reports available in the literature documenting α-galactosidases from animals the first being from *Helix promatia* (Bierry 1913). Among animals α-galactosidase is most studied in insects. In insects α-galactosidase is present in the midgut. Terra & Ferreira (1994) have reported that the insect midgut α-galactosidases are soluble and have acidic pH optima. However, *Stomoxys calcitrans* α-galactosidase activity is equally distributed between soluble and particulate fractions (Deloach & Spates 1984). In the maize grain-feeding weevil, *Sitophilus zeamais*, a weak α-galactosidase is detected (Baker 1991). In *Rhynchosciara americana*, α-galactosidase is restricted to midgut cells (Terra et al. 1979), whereas in heteropteran bugs α-galactosidase occurs as a soluble enzyme in luminal contents (*Rhodnius prolixus*) (Terra et al. 1988) or in the space between microvillar and perimicrovillar

membranes (*Dysdercus peruvianus*) (Silva & Terra 1997). The only attempts to resolve insect midgut α-galactosidases have been performed in *D. peruvianus* (Silva & Terra 1997), *Psacothea hilaris* (Scrivener et al. 1997), *Abracris flavolineata* (Ferreira et al. 1999), *Tenebrio molitor* and *Spodoptera frugiperda* (Grossman & Terra 2001). There are three midgut α-galactosidases in the larvae of *Tenebrio molitor* and *Spodoptera frugiperda*. Most galactoside digestion occur in the lumen of the first two third of the larval midguts, since α-galactosidase activity predominates there.

High titres of α-galactosidase have been reported in rats especially in the cytoplasm of epithelial cells of Brunner's glands in the intestine. Cechowska-Pasko et al. (2002) reported α-galactosidase activity in the skin of fasted rats. Blood cells and bone marrow of some animals are found to contain α-galactosidase (Szmigielski 1966; Monis & Wasserkrug 1967).

The presence of α-galactosidase has also been reported in humans, especially in spleen, liver, placenta, saliva etc (Dean & Sweeley 1979; Bishop & Desnick 1981; Oh et al. 2008). Human α-galactosidase is a lysosomal hydrolase that catalyzes the cleavage of the terminal α-galactose of globotriaosylceramide (GL-3) and ceramidetrihexoside (Desnick 2001). Further metabolism of globotriaosylceramide and ceramidetrihexoside results in the formation of ceramide, an indispensable precursor for glycosphingolipids. Glycosphingolipids are present in all cell membranes where they act as structural components of cell membranes, and participate in a variety of immune recognition processes and in signalling mechanisms (Gillard et al. 1993; Hakomori 2003). The lysosomal α-galactosidase is synthesized as a precursor that is processed to a mature form by specific *N*-glycosylation and proteolytic cleavage of a signal peptide (LeDonne et al. 1983). Mutations in the α-galactosidase A gene result in the defective activity of this enzyme in Fabry disease patients.

1.3.2.3. Microbial sources of α-galactosidases

Among micro-organisms α-galactosidase was detected for the first time in brewer's yeast (Bau 1895; Fischer & Lindner 1895). Later its presence was detected in *Saccharomyces carlsbergensis* (Lazo et al. 1978), *Pichia guilliermondii* (Church et al.

1980), *Candida javanica* (Cavazzoni et al. 1987), *Aureobasidium pullulans* (Kremnicky & Biely 1997) etc. Many bacteria, including the filamentous actinomycetes, have also been reported to contain α-galactosidase activity. The bacterial sources of α-galactosidase include *Bacillus stearothermophilus* (Talbot & Sygusch 1990), *Lactobacillus fermentum* (Garro et al. 1996), *Bifidobacterium adolescentis* (Leder et al. 1999), *Bifidobacterium breve* (Xiao et al. 2000) etc. The filamentous actinomycetes including *Streptomyces erythrus* (Elshafei et al. 2001) *S. coelicolor* A3(2) (Kondoh et al. 2005) and *Saccharopolyspora erythraea* (Post & Luebke 2005) are sources of α-galactosidase. Recently its presence is reported in extreme thermophilic eubacterium *Rhodothermus marinus* (Gomes et al. 2000), marine bacterium *Pseudoalteromonas sp.* (Bakunina et al. 1998) and lactic acid bacterium *Carnobacterium piscicola* (Coombs & Brenchley 2001). In fungi it is found in *Corticium* (Kaji & Yoshihara 1972), *Cephalosporium* (Zaprometova & Ulezlo 1981), *Pycnoporous* (Ohtakara et al. 1984), *Monascus* (Wong et al. 1986), *Scopulariopsis* (McKay 1991), *Trichoderma* (Zeilinger et al. 1993), *Aspergillus* (McKay 1991; Somiari & Balogh 1995; Ademark et al. 2001; Shankar & Mulimani 2007), *Gibberella,* (Mulimani & Ramalingam 1995), *Mortierella* (Galas & Miszkiewicz 1996), *Penicillium* (Luonteri et al. 1998a, b), *Humicola* (Kotwal et al. 1999), *Thermomyces* (Puchart et al. 2000), *Rhizopus* (Bei-Zhong et al. 2003) and many other fungi imperfecti (Zaprometova & Ulezlo 1988). Presence of α-galactosidase in higher fungi has also been reported (Li & Shetlar 1964). Very recently an unusual intracellular α-galactosidase has been isolated from the hyperthermophilic crenarchaeon *Sulfolobus solfataricus* P2 (Brouns et al. 2006), an aerobic micro-organism that lives in terrestrial volcanic pools of high acidity. At present, this *Sulfolobus* enzyme not only constitutes a distinct type of thermostable α-galactosidase within glycoside hydrolase clan D but also represents the first member from the *Archaea.*

α-Galactosidases are located intracellularly in most cases when grown in submerged cultivation. Kotwal et al. (1999) reported the presence of a constitutive intracellular α-galactosidase in the thermophilic fungus *Humicola* sp. α-Galactosidases from *Bifidobacterium adolescentis* DSM 20083 (Leder et al. 1999) and

Table 1.1. Microbial sources of α-galactosidase

Microbial source	Localization	Reference
Aspergillus foetidus	Extracellular	Liu et al. 2007a, b, c
Aspergillus oryzae	Extracellular	Shankar & Mulimani 2007
Aspergillus fumigatus	Extracellular	de Rezende et al. 2005
Aspergillus niger NCIM 839	Extracellular	Srinivas et al. 1993
Aspergillus tamarii	Intra/Extracellular	Civas et al. 1984a, b
Humicola sp.	Intracellular	Kotwal et al. 1998, 1999
Thermomyces lanuginosus	Intracellular	Rezessy- Szabó et al. 2003
Morteirella vinacea	Cell-bound	Shibuya et al. 1997
Monascus pilosus	Intracellular	Wong et al. 1986
Penicillium simplicissimum	Extracellular	Luonteri et al. 1998a, b
Penicillium purpurogenum	Extracellular	Shibuya et al. 1995
Penicillium ochrochloron	Extracellular	Dey et al. 1993
Phanerochaete chrysosporium	Intracellular	Brumer et al. 1999
Trichoderma reesei	Extracellular	Golubev & Neustroev 1993
Trichoderma reesei RUT C-30	Extracellular	Zeilinger et al. 1993
Gibberella fujikuroi	Intracellular	Thippeswamy & Mulimani 2002
Ganoderma lucidum	Intracellular	Sripuan et al. 2003
Cladosporium cladosporides	Extracellular	Mansour & Khalil 1998
Candida guilliermondii H-404	Intra/extracellular	Hashimoto et al. 1993
Candida javanica	Cell-bound	Cavazzoni et al. 1987
Debaryomyces hansenii UFV-1	Intra/extracellular	Viana et al. 2007
Absidia sp. WL511	Extracellular	Li et al. 2006
Bifidobacterium adolescentis	Intracellular	Leder et al. 1999
Bifidobacterium breve	Intracellular	Xiao et al. 2000
Bacteroides fragilis	Extracellular	Berg et al. 1980
Bacillus stearothermophilus	Extracellular	Gote et al. 2004
Lactobacillus fermentum	Intracellular	Garro et al. 1996
Thermotoga neopolitana	Intracellular	Duffaud et al. 1997
Bacillus sp. JF_2	Extracellular/Cell-bound	Li et al. 1997
Bacillus sp.	Intracellular	Jin et al. 2001
Saccharopolyspora erythraea	Extracellular	Post & Luebke 2005
Streptomyces erythrus	Extracellular	Elshafei et al. 2001
Streptomyces coelicolor A3(2)	Extracellular	Kondoh et al. 2005
Streptomyces olivaceus	Extracellular	Suzuki et al. 1966

Thermomyces lanuginosus (Rezessy-Szabó et al. 2003) are also intracellular. However, Flórez et al. (1981) reported that most of the enzyme is externally bound to the cells under their growth conditions for *Saccharomyces carlsbergensis*. In solid-substrate fermentation with *Aspergillus awamori* (Smiley et al. 1976) and *Morteirella vinacea* (Suzuki et al. 1969) the enzyme is readily extracted from the wheat bran by simple washing procedures suggesting an extracellular location. α-Galactosidase has been found to occur in commercial preparations of α-amylase from *Aspergillus niger* which suggests extracellular secretion (Bahl & Agarwal 1969). *Aspergillus foetidus* produces a scretory α-galactosidase under submerged as well as soli-state cultivation (Liu et al. 2007a, b, c). Table 1.1 lists the various microbial sources of α-galactosidase and its location in the organism.

1.3.3. Microbial production of α-galactosidases

Microbial enzymes have the advantage of high production yields and are most suitable for technological applications mainly due to their extracellular localization and broad stability profiles compared to corresponding enzymes from plants or animals. Furthermore, microbial enzymes provide a greater diversity of catalytic activities and can be produced more economically. The majority of enzymes, including α-galactosidase, currently used in industry are of microbial origin.

The submerged fermentation for aerobic micro-organisms is now well known and widely used method for the production of α-galactosidase (Suzuki et al. 1974; Delente et al. 1974; Olivieri et al. 1984; Li et al. 1997; Kotwal et al. 1999; Jin et al. 2001; Elshafei et al. 2001; Rezessy-Szabó et al. 2003; Gote et al. 2004; Liu et al. 2007a) (Table 1.2). Although several α-galactosidase preparations are produced by solid-state fermentation method (Annunziato et al. 1986; Kotwal et al. 1998; Shankar et al. 2006; Liu et al. 2007b, 2007c), this fermentation technique has not yet been exploited industrially for α-galactosidase production. Both the methods of fermentation have advantages and disadvantages. However the relative yield and ease of convenience are deciding factors to choose the fermentation method.

The carbon source used for the induction of α-galactosidases has been found to have a marked effect on the properties of the α-galactosidase produced. *Bacteroides ovatus* has been reported to produce two inducible α-galactosidases: α-galactosidase I, which is able to hydrolyze galactomannan, is induced by guar gum but not by other galactosides, whereas synthesis of α-galactosidase II, incapable of acting on guar gum, is induced by galactose, melibiose, raffinose and stachyose (Gherardini et al. 1985). Similarly *Aspergillus tamarii* produces two mycelial α-galactosidases when raffinose is used as the carbon source and one secretory α-galactosidase when cultivated in presence of galactomannan. (Civas et al. 1984a). The production of α-galactosidase I appeared to be regulated co-ordinately with the mannanse acivity, which degrades the backbone of the substrate. Another galactomannan, locust bean gum, has been reported to induce α-galactosidase production by *Trichoderma reesei* Rut C-30 (ATCC 56765) (Zeilinger et al. 1993) and *Bacillus stearothermophilus* (Talbot & Sygush 1990). As in the case of *B. ovatus* α-galactosidase I (Gherardini et al. 1985), in *B. stearothermophilus* the maximum α-galactosidase activity did not occur until 5 days after inoculation, suggesting that the mannanase was required to depolymerise the galactomannan to oligosaccharides before α-galactosidase was significantly expressed (Talbot & Sygush 1990). In *T. reesei* a low constitutive amount of α-galactosidase is present and it has been suggested that this enzyme releases galactose from locust bean gum and thereby triggers the production of inducible α-galactosidase (Zeilinger et al. 1993). α-Galactosidase has also been reported to be produced constitutively by the thermophilic fungus *Humicola* sp. (Kotwal et al. 1995), *Streptomyces mutans* (Aduse-Opoku et al. 1991) and *Bacillus* sp. (Akiba & Horikoshi 1976), although notable increases in activity have been observed after melibiose, raffinose or lactose supplementation. Wong-Leung et al. (1993) used extracts of sugarcane and soybean wastes as carbon sources for *Monascus anka* M9 IAM. Both supported the growth of the fungus, but sugarcane waste was superior for the production of α-galactosidase.

Cheap agriculture residues like, wheat bran or wheat flour, rice bran, soy flour, soybean cake or soybean meal, sorghum, corn, millet etc, are also used for production of α-galactosidase (Suzuki et al. 1972; Kotwal et al. 1995; Coombs & Brenchley 2001;

Jin et al. 2001; Liu et al. 2007a). Galactose and several galactose containing oligosaccharides, such as melibiose, raffinose and stachyose have commonly been used for the induction of α-galactosidases, especially when agricultural residues are used as a carbon sources in the medium (Li et al. 1997; Jin et al. 2001; Gote et al. 2004). These low molecular weight compounds have been effective inducers for the production of intracellular enzymes by *Monascus* sp. (Imanaka et al. 1972), *Monascus pilosus* (Wong et al. 1986), *Corynebacterium murisepticum* ATCC 21474 (Nadkarni et al. 1992) and *Micrococcus* sp. (Akiba & Horikoshi 1976), and for both cell-associated and extracellular enzymes of the yeast *Torulaspora delbruecki* IFO 1255 (Oda & Tonomura 1996). In addition to galactose, L-arabinose and corresponding polyols induced α-galactosidase in *T. reesei* (Zeilinger et al. 1993).

Foda et al. (1995) screened 38 fungal strains for α-galactosidase production using Czapek-Dox agar medium supplemented with melibiose or galactose. Only five strains produced appreciable amounts of enzyme, *Penicillium janthinellum* being superior for the formation of both intra and extracellular α-galactosidase. In further studies carried out with this fungus, galactose, lupin seed powder and soybean were shown to the best carbon sources for α-galactosidase production.

In few cases, waste effluent and waste by-products have been utilized for cultivating the organism and producing α-galactosidase in the fermentation medium (Arnaud et al. 1976; Wong-Leung et al. 1993). Soy effluent stream, waste liquor generated during production of Dofu, a traditional Chinese soybean bread food, contains 0.69% of protein and 0.96% of total sugar which 0.3% is raffinose. It has been reported to be effective as an excellent source of carbon and nitrogen for α-galactosidase production by *Bacillus* sp. JF_2 (Li et al. 1997).

Recently an unusual α-galactosidase which was produced exclusively in the presence of a specific inducer 6-deoxy-D-glucose (quinovose) was reported from the filamentous fungus *Talaromyces flavus* CCF 2686 (Simerská et al. 2007).

Table 1.2. Microbial production of α-galactosidase by submerged fermentation

Microbial strain	Carbon source/Inducer	α-Galactosidase yield*	Reference
Aspergillus oryzae	Soybean carbohydrate	1.65 U/mL	Cruz & Park 1982
Aspergillus foetidus ZU-G1	Soybean meal + wheat bran	64.75 U/mL	Liu et al. 2007a
Aspergillus fumigatus	Galactose	35.68 U/mL	de Rezende et al. 2005
Humicola sp.	Wheat bran extract	5.54 U/mL	Kotwal et al. 1995
Penicillium ochrochloron	Guar gum	n.a.	Dey et al. 1993
Monascus pilosus	Galactose	13.9 U/mL	Wong et al. 1986
Thermomyces lanuginosus CBS 395.62/b	Sucrose	90 U/mL	Rezessy-Szabó et al. 2003
Trichoderma reesei RUT C-30	Locust bean gum + galactose	0.195 U/mL	Zeilinger et al. 1993
Candida javanica	Melibiose + raffinose	2 U/mg protein	Cavazzoni et al. 1987
Bacillus sp. JF_2 strain	Soy effluent stream	0.6 U/mL	Li et al. 1997
Bacillus sp. JF strain	Wheat flour + Soybean seed flour	27.4 U/mL	Jin et al. 2001
Bacillus stearothermophilus	Soybean meal	1.08 U/mL	Gote et al. 2004
Streptomyces erythrus	Galactose	9.94 U/mL	Elshafei et al. 2001
Absidia sp.	n.a.	3.8	Suzuki et al. 1974
Absidia griseola var. *Iguchii* ATCC 20431	Lactose	23 U/mL	Narita et al. 1976
Saccharomyces carlsbergensis	n.a.	1.0	Flórez et al. 1981

n.a.- Information not available.

* The values given are not necessarily optimal for the relevant enzymes in all cases.

There have been numerous reports on the production of α-galactosidase by solid-state fermentation process. Table 1.3. lists the various micro-organisms reported for producing α-galactosidase by solid-state fermentation method. The first report on α-galactosidase production from *Mortierella vinacea* by 'koji' method appeared in 1969 (Suzuki et al. 1969). The organisms reported to be producing α-galactosidase in wheat bran based solid-state fermentation are *Aspergillus awamori* (Silman et al. 1980), *Aspergillus oryzae* (McKay 1991) and *Aspergillus niger* (Somiari & Balogh 1992). Cruz & Park (1982) and Kotwal et al. (1998) reported production of α-galactosidase in SSF system by *Aspergillus oryzae* and *Humicola* sp., respectively. Cruz & Park (1982) reported the supplementation of wheat bran with soybean carbohydrate etract for the highest production of α-galactosidase by *A. oryzae*.

Soy flour and soy beans are considered as the most ideal substrates for α-galactosidase production in SSF (Annunziato et al. 1986; Kotwal et al. 1998; Shankar et al. 2006). Sonia et al. (2005) reported sorghum straw-based solid-state fermentation process for the production of cellulose-free xylanase and associated hemicellulases including α-galactosidase by the indigenous thermophilic *Thermomyces lanuginosus* (D_2W_3). Recently Shankar & Mulimani (2007) reported red gram plant waste mixed with wheat bran as the best solid substrate for α-galactosidase production by *Aspergillus oryzae*.

Srinivas et al. (1994) described the use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the α-galactosidase production by *Aspergillus niger* MRSS 234 in solid-state fermentation system. Liu et al. (2007c) reported the enhancement of α-galactosidase yield from *Aspergillus foetidus* grown under solid-state fermentation conditions on wheat bran supplemeted with soymeal.

Table 1.3. Microbial production of α-galactosidase by solid-state fermentation

Microbial strain	Solid-substrate/Inducer	α-Galactosidase yield[a]	Reference
Aspergillus niger	Wheat bran	0.025 U/gds	Somiari & Balogh 1995
Aspergillus niger	Rice bran	0.016 U/gds	Somiari & Balogh 1995
Aspergillus niger NCIM 839	Wheat bran + guar flour + lactose	87.0 U/gds	Srinivas et al. 1993
Aspergillus oryzae	Soyflour (defatted)	10.4 U/gds	Annunziato et al. 1986
Aspergillus oryzae	Wheat bran + soybean carbohydrate solution	2.58 U/mL of enzyme extract	Cruz & Park 1982
Aspergillus oryzae	Pigeon pea plant waste	5.12 U/gds	Shankar et al. 2006
Aspergillus oryzae	Red gram plant waste	3.4 U/gds	Shankar & Mulimani 2007
Aspergillus foetidus	Wheat bran + soybean meal	2207.19 U/gds	Liu et al. 2007c
Humicola sp.	Soy flour	44.6 U/gds	Kotwal et al. 1998
Penicillium sp.	Wheat bran + soymeal + beet pulp	185.2 U/gds	Wang et al. 2004
Thermomyces lanuginosus (D_2W_3)	Sorghum straw	13.4 U/gds	Sonia et al. 2005
Absidia sp. WL511	Soybean meal	117.8 U/gds	Li et al. 2006

[a] The values given are not necessarily optimal for the relevant enzymes in all cases.

1.3.4. Properties of microbial α-galactosidases

1.3.4.1. Molecular mass and isoelectric point

α-Galactosidases have been purified from several strains of fungi, bacteria and yeast. Most of the α-galactosidases produced by fungi have been isolated from different strains of *Aspergillus* and *Penicillium* (Table 1.4). *Aspergillus* α-galactosidases form a heterogeneous group with highly variable molecular properties. Most of the fungal α-galactosidases are monomeric proteins, with an average molecular size of 50 kDa (Puchart et al. 2000; Shibuya et al. 1995, 1997); nevertheless few multimeric forms are also reported from fungi and yeast (Ohtakara & Mitsutomi 1984; Rios et al. 1993;

Sumner-Smith et al. 1985). Despite the heterogeneity in the molecular masses, the isoelectric points determined for *Aspergillus* α-galactosidases have been rather similar ranging from 4.2 to 4.8 (Manzanares et al. 1998; Ademark et al. 2001). *Penicillium* α-galactosidases form a more homogeneous group with molecular masses between 55 and 67 kDa as determined by SDS-PAGE, but the isoelectric points vary between 4.0 and 7.0 (Shibuya et al. 1995; Luonteri et al. 1998a). *Cephalosporium acremonium* 237 (Zaprometova & Ulezlo 1988) and *Monasucs pilosus* (Wong et al. 1986) also produce large α-galactosidases with molecular masses of 240 and 150 kDa respectively, as determined by gel filtration.

Bacterial α-galactosidases are also complex heterogeneous group of enzymes with a more complex structure and their molecular masses vary considerably between 45 kDa to 400 kDa (Halstead et al. 2000; Ishiguro et al. 2001). α-Galactosidase from *Corynebacterium murisepticum* is a homotetramer of 320 kDa protein (Nadkarni et al. 1992). Most complex structure of α-galactosidase has been observed in *Thermus* sp. Strain T_2, having molecular mass of 400 kDa and existing in solution as an octameric form (Ishiguro et al. 2001). However, the α-galactosidase from *Thermotoga neapolitana* is active as a monomer of 61 kDa protein (King et al. 1998). The α-galactosidase from *Bacillus stearothermophilus* NCIM 5146 is a dimeric protein with a molecular mass of 165.9 kDa and pI 4.9 (Gote et al. 2006). *Streptomyces coelicolor* A3(2) family 36 α-galactosidase is a monomeric protein with a molecular mass of 58 kDa (Kondoh et al. 2005).

On the basis of subunit molecular mass, bacterial α-galactosidases have been classified into two groups (Ishiguro et al. 2001). The first group consists of α-galactosidases from *Streptococcus mutans* (Aduse-Opoke et al. 1991), *Bacillus stearothermophilus* (Fridjonsson et al. 1999a), *Escherichia coli* Raf A (Aslanidis et al. 1989), *Bifidobacterium breve* (Xiao et al. 2000) and *Pseudomonas fluorescens* (Halstead et al. 2000), which have molecular mass of more than 80 kDa. The α-galactosidases from *Thermus* sp. strain T_2, *Thermus brockianus* (Fridjonsson et al. 1999b), *Thermotoga maritima* (Liebl et al. 1998) and *Thermotoga neopolitana* (King et al. 1998) belong to the second group, possessing molecular mass ranging from 53-65

kDa. The isoelectric points of bacterial α-galactosidases range from 4.5 to 6.9 (Berg et al. 1980; Gote et al. 2006).

1.3.4.2. Multi-molecular forms of α-galactosidases

Although the phenomenon of multiple forms of one enzyme has been known for many years, it was not until the development of the zymogram technique in 1957, that the occurrence of isozymes was confirmed and that they were really investigated (Hunter & Markert 1957). Soon, multiple forms of enzymes were demonstrated in a variety of micro-organisms and tissues, both plant and animal, and their occurrence was revealed to be relatively common (Shaw 1969).

The existence of multi-molecular forms of α-galactosidases has been reported from a few micro-organisms (Table 1.4). The underlying biochemical cause of α-galactosidase multiple forms may be post translational modifications such as proteolytic cleavage or differential glycosylation of the proteins. *Thermomyces lanuginosus* α-galactosidase exhibits micro-heterogeneity due to differential glycosylation of the protein (Puchart et al. 2000). Some times isoenzymes are the products of two distinct genes. Two α-galactosidase isoenzymes, regulated by two different genes, *agaA* and *agaB* were detected in *Bacillus stearothermophilus* KVE39 (Ganter et al. 1988). The expression of isoenzymes can vary markedly as a function of carbon and nitrogen source available in the medium for growth of the micro-organism (Naessens & Vandamme 2003). Two forms of α-galactosidases, one specific for galactooligosaccharides and the other specific for galactomannans were purified from *Morteirella vinacea* (Suzuki et al. 1970), *Aspergillus tamarii* (Civas et al. 1984a) and *Bacteroides ovatus* (Gherardini et al. 1985). Multiple proteins exhibiting α-galactosidase activity, but differing in molecular masses and *pI*s have been reported from *A. niger* (Somiari & Balogh 1995; Manzanares et al. 1998; Ademark et al. 2001).

One of the earlier views on the occurrence of multiform enzymes was that it violated the principle of biological economy for an organism to produce different enzymes where one enzyme would do (Shaw 1969). Since then it has been repeatedly demonstrated that the apparently wasteful hyper-production of multiple forms of enzymes is usually counterbalanced by specific advantages for the producing strain.

Although multi-form enzymes catalyse the same reaction, they may have different kinetic parameters, different regulatory characteristics and different stabilities. They mostly provide added flexibility and adaptability, such that the organism can cope with a wide variety of changes in environment as well as metabolic need. Multiform enzymes with distinct selectivities and different physiological roles or regulation, allow fine tuning of cell metabolism in function of changing nutrient availability (Kalcheva et al. 1994; Beecher et al. 1996; LeClerc & Grahame 1996).

1.3.4.3. Glycoprotein nature

Most of the fungal α-galactosidases are glycosylated proteins (Adya & Elbein 1977; Manzanares et al. 1998; Kotwal et al. 1999; Ademark et al. 2001; Varbanets et al. 2001). However, the α-galactosidase purified from *Rhizopus* sp. is a non-glycosylated protein (Cao et al. 2007). Generally bacterial α-galactosidases are non-glycosylated (Gote et al. 2006). The carbohydrate content of α-galactosidases has been estimated only in a few cases and very few studies on its composition and structural analysis have been carried out. The α-galactosidase from *Mortierella vinacea* contains 10.8% neutral sugars and 2.7% D-glucosamine (Suzuki et al. 1970). The α-galactosidases from *Aspergillus tamarii* (Civas et al. 1984b) contain *N*-acetylglucosamine, mannose, glucose and galactose in the molar proportion of 1:6:1.5. However the enzyme from *Aspergillus niger* (Adya & Elbein 1977) contain only mannose and glucosamine in the molar proportion 3:1. The nature of glycopeptide determined in this enzyme, exhibited $(man)_n$-$(GlcNAc)_2$-Asn like structure. The carbohydrate moieties were also detected in a few yeast α-galactosidases (Lazo et al. 1977; Cavazzoni et al. 1987). The α-galactosidases from *Cephalosporium acremonium* (Zaprometova & Ulezlo 1990) contain about 27% of neutral sugars; its carbohydrate composition was *N*-acetylglucosamine, mannose, galactose, and sialic acid in the molar proportion of 2:7:3:11. The α-galactosidase from *Thermomyces lanuginosus* contained 5.3% carbohydrates which comprised of 56% D-mannose, 8% D-galactose, 36% D-glucosamine and <1% D-glucose (Rezessy-Szabó et al. 2007). The carbohydrates are believed to play an important role in stabilizing the enzyme structure,

activity and stability (Mikami et al. 1992). The resistance of glycoproteins to proteolytic attack suggests a possible protective effect of the carbohydrate moiety (Civas et al. 1984b).

1.3.4.4. Effect of pH and temperature

Generally, bacterial α-galactosidases have a pH optimum in the range of 6.0 to 7.5 (Gherardini et al. 1985), while the pH optimum of the fungal and yeast α-galactosidases is in the range of 3.5 to 5.0 (Ulezlo & Zaprometova 1982). The purified *Streptomyces coelicolor* family 36 α-galactosidase is most active at pH 7.0 and is stable between pH 7.0 and 9.5 over 1 h (Kondoh et al. 2005). Most acidic pH optimum for α-galactosidase has been observed in fungus *Corticium rolfsii* (Kaji & Yoshihara 1972) and *Aspergillus niger* (Ademark et al. 2001), active at pH 2.5-4.5. The optimal pH range for the action of the *Penicillium* α-galactosidases is pH 4.0-6.0 (Luonteri et al. 1998a) and that of *Thermomyces lanuginosus* is pH 5.0-5.5 (Rezessy-Szabó et al. 2007). A temperature-dependent shift in the pH optima has been observed in the case of α-galactosidase from *Lactobacillus fermentum* (Schuler 1985). Like wise α-galactosidases from *Aspergillus niger* (Lee & Wacek 1970) and *Penicillium duponti* (Arnaud et al. 1976) shows substrate dependent pH optima.

Depending upon the source of origin, α-galactosidases differ with respect to their temperature optima and thermal stability. Due to the elevated temperatures used during the sugar manufacturing process, as well as in other industrial applications, stability and activity at high temperatures are important properties of α-galactosidases (Fridjonsson & Mattes 2001). It is important that the mode of action and stability of α-galactosidases are met with industrial demands as these enzyme properties reduce expenses for cooling and re-heating. The α-galactosidase from *Diplococcus* (Li et al. 1963), *E. coli* (Schmitt & Rotman 1966) and the family 36 α-galactosidase from *Streptomyces coelicolor* A3(2) (Kondoh et al. 2005) are thermolabile. α-Galactosidase from *Penicillium purpurogenum* is heat stable only below 40 °C (Shibuya et al. 1995). *Morteirella vinacea* (Shibuya et al. 1997) and *Monascus pilosus* (Wong et al. 1986) α-galactosidases are stable below 55 °C. However thermostable α-galactosidases have

been reported from a wide variety of microbial sources. The *Thermotoga neapolitana* 5068 (TN5068) α-galactosidase is the most thermoactive α-galactosidase hitherto isolated, with a temperature optimum of 100-103 °C and a half life of 2 h at 90 °C and 3 min at 100 °C (Duffaud et al. 1997). Very recently a α-galactosidase has been isolated from a hyperthermophilic archaeon *Sulfolobus solfataricus* with a temperature optimum of 90 °C (Brouns et al. 2006). The α-galactosidase from *Thermoanaerobacterium polysaccharolyticum* (King et al. 2002) remains stable at 70 °C for 36 h. The trimeric and tetrameric forms of α-galactosidase have high thermal stability compared to monomeric and dimeric forms (Gote et al. 2006).

1.3.4.5. Effect of metal ions and sugars

A wide range of chemicals are known to influence the activity of α-galactosidases. The divalent metal cations like Hg^{2+}, Ag^{2+} and Cu^{2+} are found to have considerable inhibition effect on α-galactosidase (Fridjonsson et al. 1999b; Gote et al. 2006) which usually suggests reaction with thiol groups and/or carboxyl, amino and imidazolium group of histidine in the active site (Dey & Pridham 1972). However there are some reports suggesting a stabilizing/activating effect for Cu^{2+} (Sripuan et al. 2003), Mn^{2+}, Mg^{2+} and K^{+} (Burstein & Kepes 1971; Elshafei et al. 1993; Scigelova & Crout 2000; Rezessy-Szabó et al. 2007). Most other metal cations are found to have no or little effect on α-galactosidase activity (Sripuan et al. 2003; Kondoh et al. 2005; Gote et al. 2006; Rezessy-Szabó et al. 2007).

α-Galactosidase activity is also influenced by some sugars and sugar derivatives. Generally, galactose, melibiose, raffinose and stachyose are reported to have an inhibitory effect on α-galactosidase activity when assayed with *p*-nitrophenyl-α-D-galactopyranoside (Pederson & Goodman 1980; Rios et al. 1993; Scigelova & Crout 2000; Gote et al. 2006). This may be due to the fact that the galactosyl residues of these compounds are analogous to the galactosyl residue of *p*NPG with which they compete for the active site of the enzyme. But Suzuki et al. (1970) reported a mixed type of inhibition by D-galactose on α-galactosidase from *Mortierella vinacea* suggesting its competitive and non-competitive binding on the enzyme. α-Galactosidase from *M.*

vinacea (Suzuki et al. 1970) and *Aspergillus ficcum* (Zapater et al. 1990) were inhibited non-competitively by D-Glucose where as α-galactosidase from *A. ficcum* shows uncompetitve inhibition by mannose (Zapater et al. 1990). Luonteri et al. (1998a) reported three α-galactosidases from *Penicillium simplicissimum* of which AGL II showed more resistance to product inhibition by galactose than the other two enzymes, AGL I and AGL III.

1.3.4.6. Substrate specificity

α-Galactosidases are very specific with regard to anomer selectivity of the substrate though they show some flexibility in glycone and aglycone specificity. The anomeric configuration of the product liberated by the action of α-galactosidase is specifically α-, despite the type of α-galactosidic linkage in the substrate, e.g. α-1-2, α-1-3, α-1-4, α-1-6 etc. (Zaprometova & Ulezlo 1990; Puchart et al. 2000; Varbanetes et al. 2001; Comfort et al. 2007). However many enzymes do not show absolute specificity for glycone residues, and hydrolyze structural analogues β-L-arabinopyranosides and α-D-fucopyranosides (Berg et al. 1980). The aglycone group of the substrate may or may not have marked effect on hydrolysis; hence many α-galactosidases can hydrolyze for e.g. methyl-, ethyl-, *n*-propyl, α-naphthyl galactose, *p*-nitrophenyl-α-D-galactopyrnoside, melibiose and raffinose (Halstead et al. 2000; Shabalin et al. 2002). Most microbial α-galactosidases have in common the fact that they can hydrolyze the synthetic or aryl glycosides like *p*NPG and oNPG more extensively than the natural α-galactosides like melibiose, raffinose and stachyose (Varbanets et al. 2001), indicating that aryl glycosides are the better substrates than the alkyl derivatives. Moreover, α-galactosidase from *Aspergillus niger* hydrolyzed exclusively the synthetic substrate and failed to split off the terminal α-1,6-bound galactose in linear structures like the melibiose, raffinose and stachyose (Kaneko et al. 1991).

Table 1.4. Characteristics of α-galactosidases from different micro-organisms

Micro-organism	M_r (kDa)	pI	pH_{opt}	T_{opt} (°C)	Active against	K_m for *p*NPG (mM)	V_{max}	Reference
Rhizopus sp. F78 ACCC30795	210	n.a.	4.8	50	*p*NPG, Melibiose, Raffinose, Stachyose	2.9	246.1 μmol/min/mg	Cao et al.2007
Talaromyces flavus	63	n.a.	3.5-4.5	50	*p*NPG, Raffinose, Stachyose, Galactomannans	0.54	0.21 mM/min	Simerská et al. 2007
Thermomyces lanuginosus CBS 395.62/b	93	3.9	5-5.5	65	*p*NPG, Raffinose, Stachyose	1.13	2498 μmol/min/mg	Rezessy-Szabó et al. 2007
Thermomyces lanuginosus	57	5.2	4.5-5.0	65-70	*p*NPG, Melibiose, Raffinose	0.5	52.4 U/mg	Puchart et al. 2000
Trichoderma reesei RUT C-30	50	5.2	4	60	*p*NPG, Melibiose, Raffinose, Stachyose, Locust bean gum	1.2	30.1 U/mg	Zeilinger et al. 1993
Morteirella vinacea								Shibuya et al. 1997
α-Galactosidase I	240	5.4	3-4.0	60	Oligosaccharide chains	n.a.	n.a.	
α-Galactosidase II	60	8.5	3-4.0	60	Galactomanno-oligosaccharides, Galactomannans	n.a.	n.a.	
Aspergillus fumigatus	54.7		4.5	55	*p*NPG, Melibiose	0.38	0.16 μmol/min/mg	de Rezende et al. 2005
Aspergillus niger	45	n.a.	4-4.5	n.a.	*p*NPG, Raffinose, Stachyose, Melibitol, Galactomannans	0.18	1.5 μmol/min/mg	Adya & Elbein 1977
Aspergillus niger ATCC 46890								Ademark et al. 2001
α-Gal I	350	4.15	4.5	60	*p*NPG, Melibiose, Raffinose, Stachyose	1.4	18000 nkat/mg	
α-Gal II	117	4.5	4.5	60	*p*NPG, Melibiose, Raffinose, Stachyose, Galactomanno-oligosaccharides	0.22	3600 nkat/mg	
α-Gal III	117	4.7	4.5	60	*p*NPG, Melibiose, Raffinose, Stachyose	0.27	3000 nkat/mg	
α-Gal IV	117	4.8	4.5	60	*p*NPG, Melibiose, Raffinose, Stachyose, Galactomanno-oligosaccharides	0.24	3200 nkat/mg	
Morteirella vinacea	n.a.	n.a.	4-6	n.a.	*p*NPG, oNPG, Methyl-α-D-galactoside, Melibiose, Raffinose, Stachyose, 4-o-α-D-Galactopyranosyl-D-galactose, 6-o-α-D-Galactopyranosyl-*o*-β-D-galactopyranosyl-1-glycerol, Methyl- β-L-arabinoside	0.43	143.5 μmol/min/mg	Suzuki et al. 1970
Penicillium simplicissimum								Luonteri et al. 1998a
AGLI	61	5.2	3-4.5	40	*p*NPG, Raffinose family oligosaccharides, Polymeric galacto(gluco)mannans	n.a.	n.a.	

AGL II	84	4.4	4-5	60	*p*NPG,Raffinose family oligosaccharides	0.75	26600 nkat/mg	
AGL III	61	7.0	3-4.5	45	*p*NPG,Raffinose family oligosaccharides, Polymeric galacto(gluco)mannans			
Penicillium purpurogenum	67	4.1	4.5	55	*p*NPG,Galactomanno-oligosaccharides	n.a.	n.a.	Shibuya et al. 1995
Pycnoporus cinnabarinus	210	3.5	5	75	*p*NPG	0.31	630 µmol/min/mg	Ohtakara et al. 1984
Monascus pilosus	150	n.a.	4.5-5	55	*p*NPG,Melibiose, Raffinose, Stachyose	0.8	39 µmol/min/mg	Wong et al. 1986
Ganoderma lucidum	249	n.a.	6	70	Melibiose, Raffinose, Stachyose	0.4	n.a.	Sripuan et al. 2003
Candida guilliermondii H-404 α-Galactosidase I								Hashimoto et al. 1993
	270	6.16	4.5	75	*p*NPG, *o*NPG, *m*NPG, Melibiose, Raffinose, Stachyose	0.6	n.a.	
α-Galactosidase II	270	6.21	4.5	75	*p*NPG, *o*NPG, *m*NPG, Melibiose, Raffinose, Stachyose	0.61	n.a.	
Phanerochaete chrysosporium	250	n.a.	3.75	n.a.	*p*NPG	0.198	n.a.	Brumer et al. 1999
Bacillus stearothermophilus	247	n.a.	7-7.5	60	*p*NPG, Melibiose, Raffinose, Stachyose, Galactomannans	0.25	195 U/mg	Talbot & Sygusch 1990
Bacillus stearothermophilus NCIM 5146	165.9	4.9	6.5-7	65	*p*NPG, Melibiose, Raffinose, Stachyose, Galactomannans (limited activity)	0.5	833 U/mg	Gote et al. 2006
Lactobacillus plantarum	194.5	n.a.	5.8	45	*p*NPG	0.079	2838 µmol/min/mg	Garro et al. 1996
Thermotoga neopolitana 5068	61	n.a.	7.5	100-105	Polymeric galactomannans	n.a.	n.a.	Duffaud et al. 1997
Bifidobacterium adolescentis	344	n.a.	5.5	55	*p*NPG, Melibiose, Raffinose, Stachyose, α-1,3-D-Galactobiose, α-1,4-D-Galactobiose, Gal-α-1,3-gal-β-1,4-gal, Gal-α-1,3-gal-β-1,4-gal- α-1,3-gal	0.957	n.a.	Leder et al. 1999
Bifidobacterium breve	160	n.a.	5.5-6.5	37	Melibiose, Raffinose, Stachyose	n.a.	n.a.	Xiao et al. 2000
Bacteroids fragilis	125	6.2	5.5	n.a.	*p*NPG	n.a.	n.a.	Berg et al. 1980
Saccharopolyspora erythraea	45	n.a.	6.1	65	Melibiose, Raffinose, Stachyose	0.65	31 µmol/min/mg	Post & Luebke 2005
Streptmyces coelicolor A3(2)	58	n.a.	7	40	*p*NPG, Raffinose, Stachyose	n.a.	n.a.	Kondoh et al. 2005

n.a. information not available

As mentioned earlier α-galactosidases are of two types, one group active only on oligomeric substrates and the other group active on both oligomeric and polymeric substrates. Generally GH27 α-galactosidases are active on both polymeric and oligomeric substrates where as GH36 α-galactosidases are active only on oligomeric substrates. Many of the α-galactosidases in family 36 are large enzymes with a tetrameric structure (Aslanidis et al. 1989; Aduse-Opoku et al. 1991; Fridjonsson et al. 1999a, b; van den Broek et al. 1999), and hence their ability to release galactose from polymers is probably sterically restricted. The large trimeric or tetrameric α-galactosidases of *Aspergillus tamarii* (Civas et al. 1984a, b), *Penicillium simplicissimum* (Luonteri et al. 1998a, b), *Bacteroides ovatus* (Gherardini et al. 1985) and *Bacillus stearothermophilus* (Talbot & Sygusch 1990) have little or no activity toward polymeric substrates. The two fungal α-galactosidases in family 36, *Trichoderma reesei* AGL II (Margolles-Clark et al. 1996) and *Aspergillus niger* α-gal I (Ademark et al. 2001), are more specific toward oligosaccharides such as melibiose and raffinose and have little or no activity on polymeric substrates. The α-galactosidases in family 27 are generally smaller and at least some of them are monomers (Shibuya et al. 1997; Luonteri et al. 1998a). Many of the α-galactosidases in family 27, such as *Trichoderma reesei* AGLI (Margolles-Clark et al. 1996), *Mortierella vinacea* α-galactosidase II (Shibuya et al. 1997), *Penicillium simplicissimum* AGLI (Luonteri et al. 1998a, b) and *Aspergillus niger* AglB (Manzanares et al. 1998; de Vries et al. 1999), can release galactose from intact galactomannan polymers. Recently an α-galactosidase with negligible hydrolytic activity towards melibiose, but active on locust bean gum and guar gum, was purified from the filamentous fungus *Talaromyces flavus* CCF 2686 (Simerská et al. 2007).

1.3.4.7. Transglycosylation reaction

Many microbial α-galactosidases have been shown to possess transglycosylation activities in addition to hydrolytic activity (Li et al. 1964; Mitsutomi & Ohtakara 1988; Hashimoto et al. 1995a, 1995b; Eneyskaya et al. 1998; Scigelova & Crout 2000; Spangenberg et al. 2000; Tzortis et al. 2003; Yamashita et al. 2005), especially at high substrate concentrations. In transglycosidase activity the role of

acceptor is played by hydroxylic compounds other than water, for example, simple alcohols, hydrolysis products (Hashimoto et al. 1995a, b), saccharides or a second substrate molecule (substrate transglycosylation) (Eneyskaya et al. 1998; Weignerová 2001). Acceptor specificity and kinetics of transglycosidase activity of α-galactosidases has been the focus of research for many investigators (Savelév et al. 1996). Generally hexoses are found to be better acceptors of galactose molecule. The studies carried out so far showed that water and organic acceptors are bound at same site, hence hydrolysis and transfer reactions take place on the same site of the enzyme molecule, presumably by identical mechanism (Dey 1979). The α-galactosidases are also known to synthesize (*de-novo-synthesis*) oligosaccharides when incubated with high concentrations of monosaccharides (Clancy & Whelan 1967; Ajisaka & Fujimoto 1989), and this procedure has been used for the preparation of several glucose and galactose derivatives (Spangenberg et al. 2000). Recently an extracellular α-galactosidase with a unique transglycosylation potential was isolated from a filamentous fungus *Talaromyces flavus* CCF 2686 (Weignerová et al. 2001; Simerská et al. 2006, 2007). This enzyme showed an unusual regioselectivity in transglycosylation of *p*NP-α-Gal, with a selective preference for the formation of α-(1→3) bonds (Weignerová et al. 2001), and tolerates *p*-nitrophenyl-6-*O*-acetyl-α-D-galactopyranoside as an acceptor yielding *p*-nitrophenyl-α-D-galactopyranosyl-(1→3)-6-*O*-acetyl-α-D-galactopyranoside (Simerská et al. 2006). Besides its peculiar selectivity, this inducible enzyme was found to be active in the transfer of α-galactosyl residue onto sterically hindered acceptors like *tert*-butyl alcohol (Simerská et al. 2006, 2007), which is generally quite inert to hydrolases and specifically to glycosidases, and consequently used as a co-solvent in transglycosylation reactions to increase substrate solubility (van Rantwijk et al. 1999).

1.3.4.8. Protease-resistance

The scientific community is always in search for microbial enzymes with novel properties. With this aim several research groups have been searching for organisms endowed with a large repertoire of diverse enzymes with divergent properties. Recently Cao et al. (2007) reported the purification of a novel protease-resistant α-galactosidase

from *Rhizopus* sp. F78 ACCC 30795 which showed resistance to both neutral and alkaline proteases providing a basis for its possible application in the medical, food, animal feed and sugar-making industries. Following treatment with a variety of neutral proteases (including subtilisin A, proteinase K, collagenase, trypsin and α-chymotrypsin), the enzyme retained over 70% of its activity. Enzyme activity was a little activated by alkaline proteases (including proleather and alkaline protease).

1.3.5. Crystal structure of α-galactosidase

Despite the extensive studies on production, purification and characterization of exo-glycosidases from various sources, until 2002, there were no reports on the structure of any α-retaining exo-glycosidase like α-galactosidase, from clan GH-D (Garman & Garboczi 2004). The three dimensional structure has only recently been established, in both free and product-complexed forms, for two GH27 enzymes, α-*N*-acetylgalactosaminidase (E.C. 3.2.1.49) of chicken *Gallus gallus* (Garman et al. 2002) and α-galactosidase of rice *Oryza sativa* (Fujimoto et al. 2003), man (Garman & Garboczi 2004) and *Hypocrea jecorina* (née *Trichoderma reesei*) (Golubev et al. 2004). The first three dimensional structural co-ordinates for a member of GH36 family, α-galactosidase *Tm*GalA from *Thermotoga maritima* (Lesley et al. 2002) were deposited by the Joint Center for Structural Genomics (JCSG) in June 2005.

1.3.5.1. Structure of GH27 α-galactosidase

The first structure elucidation of α-galactosidase in rice *O. sativa* (Fujimoto et al. 2003) and the subsequent studies on *T. reesei* α-galactosidase (Golubev et al. 2004) and human α-galactosidase (Garman & Garboczi 2004) showed that all α-galactosidases have in common two domains, an *N*-terminal catalytic domain A, which forms a $(\beta/\alpha)_8$- barrel (TIM-barrel type structure), and a *C*-terminal β-jellyroll domain B, which consists of eight antiparallel β-strands (β-sandwich fold) with a Greek key motif (Fig. 1.6). The interface between the two domains mainly involves hydrophobic interactions. The *T. reesei* α-galactosidase possesses four *N*-linked branched oligosaccharides which makes multiple hydrogen bonds and hydrophobic interactions

with the residues in domain A and B, thus contributing to the stability of the molecule (Fig. 1.6b).

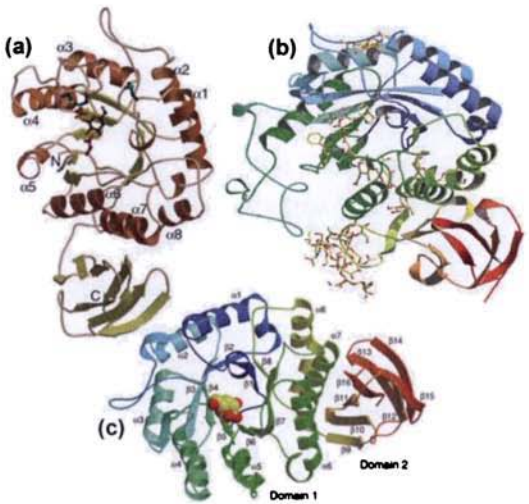


Fig. 1.6. (a) Stereo view of the ribbon model of rice α-galactosidase. The bound D-galactose, two catalytic residues, and two disulfide bonds are indicated by *ball2and2stick* drawings and shown in *black*, *red*, and *green*, respectively. (Fujimoto et al. 2003). **(b) The overall fold and the active site of α-galactosidase from *T. reesei*.** β-Strands and α-helices are represented, respectively, as arrows and coils; β-D-galactose, Asp132 and Asp226 participating in catalysis, two Trp residues, involved in important hydrophobic interactions with the substrate, and four *N*-binding sugar chains are represented as "sticks". The (β/α)8- barrel domain A and domain B, consisting of β-strands, are held together by a network of hydrogen bonds, mainly between α-helices 7 and 8 of domain A and two β-turns of domain B. Two long loops protruding from each domain interact by mutual hydrogen bonds between both, amino acid residues and mannose residues of the branched oligosaccharide linked to Asn157. (Golubev et al. 2004). **(c) The structure of human α-GAL monomer.** The monomer is colored from *N* (blue) to *C* terminus (red). Domain 1 contains the active site at the center of the β strands in the (β/α)8 barrel, while domain 2 contains antiparallel β strands. The galactose ligand is shown in yellow and red CPK atoms. (Garman & Garboczi 2004).

The human α-galactosidase is a homodimer with each monomer containing a $(\beta/\alpha)_8$ domain with the active site and an antiparallel β domain (Garman & Garboczi 2004) (Fig. 1.6c). Each monomer contains three *N*-linked carbohydrate sites, five disulfide bonds (C52–C94, C56–C63, C142–C172, C202–C223, and C378–C382), two unpaired cysteine residues (C90 and C174), and three *cis* proline residues (P210, P380, and P389). The most important residue in the dimer interface of human α-galactosidase is F273 which has a 130 $Å^2$ surface area buried per monomer upon formation of the dimer. This residue is a Phe or Tyr in most animal α-galactosidases and α-*N*-acetylgalactosaminidases, while in plant α-galactosidases, the equivalent residue is a Gly. Thus, this residue predicts the dimerization state of the enzyme in different species: Phe or Tyr indicates the enzyme is a dimer, while Gly indicates the enzyme remains a monomer (Garman & Garboczi 2004).

The known overall structures of GH27 enzymes are all highly conserved and the *N*-terminal domains are all closely super-imposable, with minor exceptions including the *T. reesei* α-galactosidase (Golubev et al. 2004) and the animal enzymes (Garman et al. 2002; Garman & Garboczi, 2004). The structural differences between the *T. reesei* α-galactosidase and the other GH27 structural homologues are mostly confined to the size and orientation of β-strands and α-helices in the $(\beta/\alpha)_8$-barrel of domain A and to the presence of two additional loops, residues Trp136–Ser205 and Glu367–Trp352, in the α-galactosidase structure. The human α-galactosidase shows 38% amino acid sequence homology with rice α-galactosidase and 51% homology with chicken α-*N*-acetylgalactosaminidase (Fig. 1.7), and differs from rice α-galactosidase in that it contains a short 10 residue insertion in the α1- β1 loop (Garman 2006). The C-terminal domains, although similar both at the primary and tertiary structural levels, are less well conserved (Garman 2006).

Further structural comparison reveals that both domains A and B of the α-galactosidase are very similar to those found in other carbohydrases. Structural conservation of the $(\beta/\alpha)_8$-barrel domain in carbohydrases has been related to its function as a scaffold for residues essential for catalysis (Coutinho & Henrissat 1999b). The role of domain B, present in many of the carbohydrate-binding enzymes in the form

of a β-barrel or β-sandwich, has not been clarified completely. In contrast to domain A, domain B varies in size, sequence and fold from enzyme to enzyme in many GH families (Jesperson et al. 1991; Tomme et al. 1995).

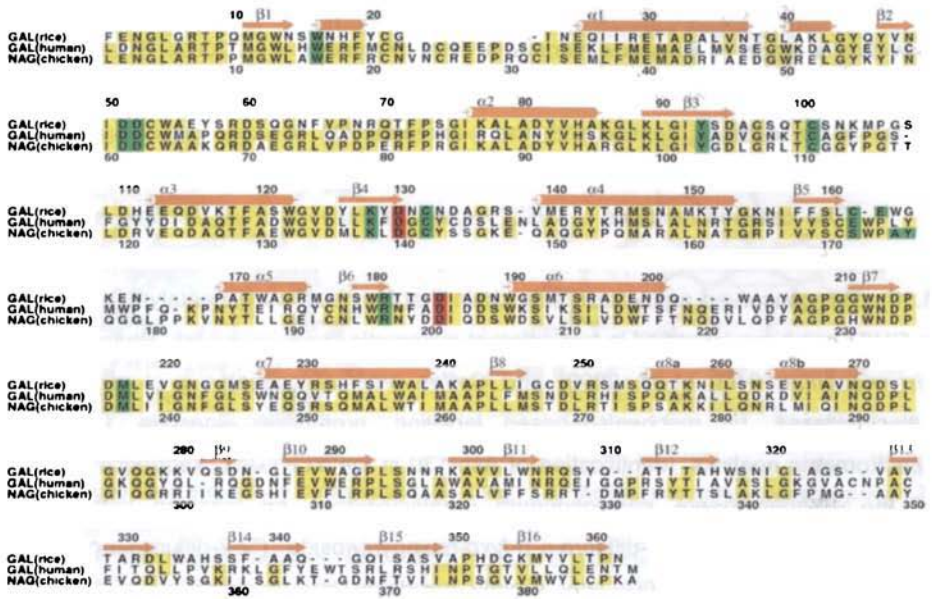


Fig. 1.7. Amino acid sequence alignment of rice α-galactosidase with those of human α-galactosidase and chicken α-*N*-acetylgalactosaminidase. The catalytic residues and the ligand-binding residues are indicated by *red* and *green* backgrounds, respectively (Fujimoto et al. 2003)

1.3.5.1.1. Active site and the catalytic residues

In keeping with the *exo* mode of action of α-galactosidases, which cleave α-galactose from the non-reducing terminals of their substrates, the active sites are pocket-shaped (Fujimoto et al. 2003; Golubev et al. 2004; Garman & Garboczi 2004). The catalytic pocket of α-galactosidase is similar to that of chicken α-N-acetylgalactosaminidase, whose three dimensional structure was first established among the enzymes of clan GH-D (Garman et al. 2002). The active sites of the

enzymes in the GH27 family are extremely well conserved (Fig.1.8, 1.9, 1.10). The active site pocket of α-galactosidase is found on the C-terminal side of the central β-barrel of the catalytic domain where the D-galactose molecule binds as the ligand. The structure of rice α-galactosidase was solved as a complex with D-galactose (Fig. 1.8), providing a mode of substrate binding in detail (Fujimoto et al. 2003). The galactose molecule is seen buried in the catalytic pocket on the C-terminal side of the central β-barrel of the catalytic domain with the α-anomeric O1 hydroxyl group open to the surface.

The catalytic residues of GH27 α-galactosidases are two aspartic acids, one acting as the nucleophile and the other acting as the catalytic acid/base (Fig. 1.8, 1.9, 1.10). The conserved amino acid side chain that functions as the catalytic nucleophile in GH27 has been identified in two different eukaryotic family members, *Phanerochaete chrysosporium* (Hart et al. 2000) and *Coffea arabica* (coffee) (Ly et al. 2000) α-galactosidases, by mechanism-based labelling, proteolytic digestion, and mass spectrometric analysis. Identification of Asp-130 in the YLKYDNC sequence fragment of the *Phanerochaete chrysosporium* α-galactosidase by labelling with 2',4',6'-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-*lyxo*-hexopyranoside ("2,2-difluoro-α-galactosyl picrate") (Hart et al. 2000) only slightly pre-dated the identification of the same conserved aspartate in the green coffee bean α-galactosidase (Asp-145 in the sequence LKYDNCNNN) using 5-fluoro-α-D-galactopyranosyl fluoride as a labelling agent (Ly et al. 2000). The analysis of three-dimensional enzyme-product complexes later facilitated identification of the conserved catalytic general acid/base residue (Garman et al. 2002; Fujimoto et al. 2003; Golubev et al. 2004; Garman & Garboczi, 2002, 2004).

In rice α-galactosidase, Asp-130 located at the end of strand β4 acts as the nucleophile and Asp-185 located after strand β6 acts as a catalytic acid/base (Fig. 1.8). These asparatates make hydrogen bonds to the galactose. Besides the catalytic residues, other residues in rice α-galactosidase that make hydrophilic/hydrophobic contacts to the ligand include Arg-181, Trp-164, Cys-62, Lys-128, Asp-51, Asp-52, Trp-16, Cys-101 and Met-217. Thus a total of 11 residues are involved in galactose binding.

The catalytic residues in *T. reesei* α-galactosidase are Asp-132 and Asp-226 (Fig. 1.9), of which Asp-132 acts as the nucleophile and Asp-226 plays the role of catalytic acid/base (Golubev et al. 2004). One of two water molecules found in the vicinity of Asp226 might participate in a second nucleophilic attack during breakdown of the glycosyl-enzyme intermediate.

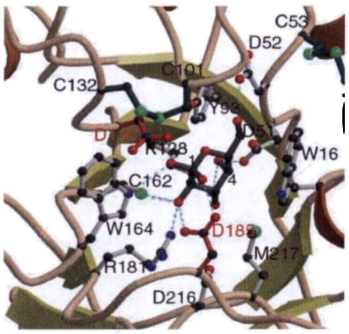


Fig. 1.8. Stereo view around the catalytic pocket of rice α-galactosidase. *A*, rice α-galactosidase. Bound sugar, two catalytic residues, surrounding residues, and disulfide bonds are indicated by *ball2 and2stick* drawings and are shown in *black*, *red*, *white*, and *green*, respectively. Coordinating hydrogen bonds are shown in *broken green lines* (Fujimoto et al. 2003.)

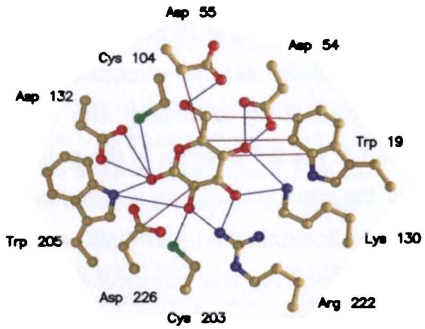


Fig. 1.9. Active site interactions between *T. reesei* enzyme and D-galactose. All five hydroxyl groups of galactose participate in hydrogen bonds with residues of the active site. The hydrogen bonds are shown as blue lines, van der Waals contacts are shown as brown lines (Golubev et al. 2004).

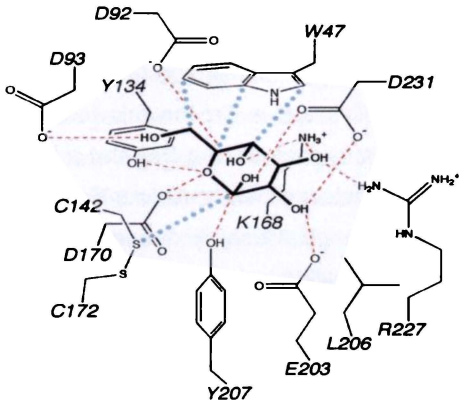


Fig. 1.10. Active site interactions of human α-galactosidase. The primary interactions between α-galactosidase and α-galactose are shown. The ligand is in bold and protein side-chains are labeled. Hydrogen bonds and polar interactions are in red and van der Waals interactions in blue. D170 and D231 act as the catalytic nucleophile and acid/base, respectively (Garman & Garboczi, 2004).

of the hexose ring. In the *T. reesei* α-galactosidase structure, the position of Asp54 is significantly different, which makes the binding of glucose impossible because of steric clashes between the Asp54 $O^{\delta 1}$ and the equatorial OH4 group of glucose (Golubev et al. 2004).

Another residue crucial for substrate recognition in *T. ressei* α-galactosidase is Trp19 (Golubev et al. 2004). It is stacked against the hydrophobic surface of the galactose sugar ring, which is composed of the C-4, C-5 and C-6 atoms. The hydrophobic interaction between Trp19 and a glucose ring would be weaker or impossible, due to the equatorial orientation of OH4. The packing of an aromatic residue against galactose rings is, however, rather common in many galactose-binding enzymes and lectins (Vyas et al. 1994; Weis & Drickamer 1996; Elgavish & Shaanan 1997, 1998; Kolatkar et al. 1998; Shabalin et al. 2002; Garman et al. 2002). These two structural features confer on α-galactosidase the ability to discriminate between galactose and glucose epimers. The α-anomeric hydroxyl group projects outwards, whereas the anomeric hydroxyl of β-D-galactose is directed towards Trp205 and the disulfide bridge between Cys104 and Cys134 in the catalytic pocket of *T. reesei* α-galactosidase. Therefore, steric hindrance would preclude β-galactooligosaccharides binding to the α-galactosidase catalytic pocket. This explains the strict specificity of the *T. reesei* α-galactosidase toward α-galactooligosaccharides.

The rice α-galactosidase, as well as the other eukaryotic α-galactosidases, can accommodate both terminal and side-chain α-galactosyl residues. The α-anomeric oxygen atom of the bound galactose molecule projects directly toward the solvent area, leaving enough space in the catalytic pocket so that the bound α-galactosyl residue in the catalytic pocket could be linked to either the terminal or the inner sugar of the manno-oligosaccharide through an α-1,6-glycosidic bond (Fujimoto et al. 2003). It is still unclear whether the enzyme recognizes the other sugars of the substrate besides the α-galactosyl moiety.

It has been shown that some bacterial and yeast α-galactosidases can cleave only terminal α-galactosyl or only side-chained α-galactosyl residues (Kaneko et al. 1991; Shibuya et al. 1995). For example, *M. vinacea* α-Gal I and *Saccharomyces*

cerevisiae α-Gal, which act on only the terminal α-galactosyl residues, have a 20-amino acid insertion before the helix α6, whereas *P. purpurogenum* α-Gal, which acts on only the side-chained α-galactosyl residues, has a 30-amino acid insertion before helix α4. Because the sequences of the core β/α-barrel are highly conserved among various α-galactosidases, these insertions could cause the extended loops to bulge out and possibly obstruct the catalytic pocket, thereby limiting accessibility to the side-chained or terminal α-galactosyl residues. Alternatively, they may cause more sub-sites against α-galactosyl oligosaccharides to be formed, resulting in multiple specificities against the α-galactosyl moiety.

1.3.5.2. Structure of GH36 α-galactosidase

In contrast to GH27 members, the molecular details governing catalysis by GH36 enzymes are less clear. Members of GH36 are particularly interesting enzymes with respect to their potential for carbohydrate synthesis through protein engineering, since both hydrolytic (α-galactosidase and α-*N*-acetylgalactosaminidase) and transglycosylation activities (raffinose and stachyose synthase) are represented in this group. Structural information on the GH36 α-galactosidase from *Thermotoga maritima* (*Tm*GalA) enzyme has recently been published (Lesley et al. 2002), facilitating efforts to compare catalytic mechanisms of GH27 and GH36.

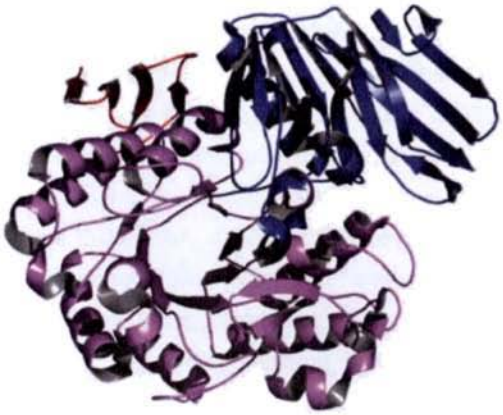


Fig. 1.12. Wall-eyed stereo overview of the α-galactosidase from *Thermotoga maritima* (*Tm*GalA). The *N*-terminal $(\beta/\alpha)_8$ -barrel, and *C*-terminal domains are coloured in blue, violet, and red, respectively (Comfort et al. 2007).

The tertiary structure of *Tm*GalA consists of three domains (Fig. 1.12): an *N*-terminal β-supersandwich domain (residues 1-179), a core $(\beta/\alpha)_8$ barrel (residues 180-482), and a *C*-terminal antiparallel β-sheet domain (residues 483-525) (Lesley et al. 2002). As expected, based on common clanship in GH-D (Henrissat & Bairoch 1993) (http://afmb.cnrs-mrs.fr/CAZY/), *Tm*GalA from GH36 shares a homologous $(\beta/\alpha)_8$ barrel catalytic domain with all of the known three-dimensional structures of GH27 enzymes.

1.3.5.2.1. Active site and catalytic residues

Detailed phylogenetic analysis of archaeal GH36 α-galactosidases within Clan GH-D originally highlighted likely candidates for the catalytic nucleophile and general acid/base residues in this family, based on protein sequence similarity with those identified in GH27 (Brouns et al. 2006). Mutagenesis of the corresponding residues in *Sulfolobus solfataricus* α-galactosidase GalS dramatically reduced enzyme activity: the D367G (nucleophile) and D425G (acid/base) mutant had $<1 \times 10^{-3}$ and 5×10^{-3} lower activity than the wild type enzyme when assayed against *p*-nitrophenyl-α-D-galactopyranoside (Brouns et al. 2006). Rescue of the catalytic function of both enzyme mutants was unsuccessful with both azide and formate anions (Brouns et al. 2006).

Indeed, a high degree of homology is observed between the active sites of *Tm*GalA and the *Oryza sativa* α-galactosidase (Fig. 1.13) (Comfort et al. 2007). Many active site residues are identical between the two enzymes, or are conservative substitutions, while in some cases more radical replacements are observed. The conserved catalytic nucleophile and general acid/base residues in GH27, represented by D130 and D185 in the *O. sativa* α-galactosidase, are clearly observed to have structural homologues in *Tm*GalA, namely, D327 and D387, respectively. This points to the shared common ancestry of GH27 and GH36 families (Rigden 2002). Site-directed mutation of Asp327 in *Tm*GalA to Gly yielded a variant that had a 200-800-fold lower rate on aryl galactosides compared with the wild type (WT) enzyme. Addition of azide was shown to rescue the ability of the enzyme to cleave *p*-nitrophenyl-α-D-galactopyranoside and resulted in formation of β-galactopyranosyl azide, confirming Asp327 as the nucleophilic residue. Mutation of the predicted acid/base residue,

Asp387, to Gly reduced activity 1500-fold on *p*-nitrophenyl-α-D-galactopyranoside, while addition of azide resulted in formation of α-galactopyranosyl azide by nucleophilic attack on the β-linked glycosyl enzyme (Comfort et al. 2007).

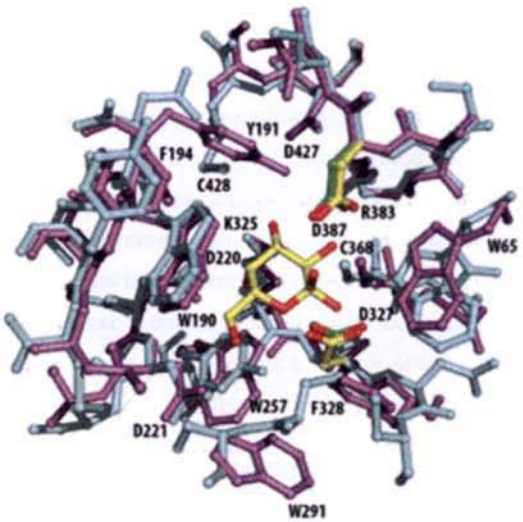


Fig. 1.13. Superposition of the active sites of GH36 *Tm*GalA (PDB 1ZY9, violet) and the GH27 *Oryza sativa* α-galactosidase (PDB 1UAS, cyan). Bound galactose, as well as the catalytic nucleophile (D130) and general acid/base (D185) residues, in the *O. sativa* structure is shown in yellow/red. The corresponding nucleophile and acid/base residues in the *Tm*GalA structure are D327 and D387, respectively (green/red). Residue numbering is shown for *Tm*GalA, following the numbering scheme in PDB 1ZY9 (Comfort et al. 2007).

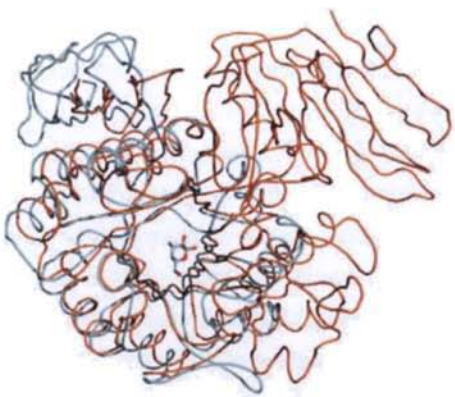


Fig. 1.14. Superposition of the CR traces of GH36 *Tm*GalA (PDB 1ZY9, orange) and the GH27 *O. sativa* α-galactosidase (PDB 1UAS, cyan) (Comfort et al. 2007).

As indicated by structural alignment, the *N*-terminal β-supersandwich domain of *Tm*GalA (residues 1-179) has no homologous structure in GH27 enzymes (Fig. 1.14). This domain, which is found in a number of other GH36 α-galactosidases, makes an

important contribution to the enzyme active site of *Tm*GalA located within the $(\beta/\alpha)_8$ barrel by providing the key substrate-binding residue W65. Deletion of this domain would likely produce an incorrectly folded and non-functional enzyme variant.

1.3.6. Chemical modification studies on α-galactosidases

The pH dependent inactivation and kinetic study of α-galactosidase from mung bean (Dey 1984), *Vicia faba* (Dey & Pridham 1969b), and sweet almond (Dey & Malhotra 1969) revealed participation of histidine and carboxylate at the active site. Further, the presence of these residues at the active site was also confirmed by photo-oxidation and chemical modification in mung bean (Dey 1984) and *Vicia faba* α-galactosidase (Dey & Pridham 1969b). More over the stoichiometric study of enzyme inactivation in mung bean α-galactosidase showed the probable presence of 12 carboxyl groups and 9 histidine imidazole groups per molecule of enzyme at the active site (Dey 1984). Chemical modification studies of coconut kernel α-galactosidase have indicated the probable presence of a tyrosine, tryptophan and two carboxyl groups at or near the enzyme's active site (Mathew & Balasubramaniam 1986). Site directed mutagenesis of coffee bean α-galactosidase suggested the presence of Trp-16 at the active site (Zhu et al. 1996). The potential role of this residue in substrate binding and in catalytic mechanism has also been reported (Maranville & Zhu 2000).

The role of methionine at the active site of *Trichoderma reesei* α-galactosidase has been shown by oxidation of methionine by H_2O_2 (Kachurin et al. 1995). Moreover oxidation induced activation of α-galactosidase was also observed by this modification, which gives 12-fold increase in the activity towards the substrate *p*-nitrophenyl-α-D-galactopyranoside (Golubev et al. 2004). Participation of tryptophan, lysine and carboxylate at or near the active site of α-galactosidase from thermophilic fungus *Humicola* sp. (Kotwal et al. 2000) and thermophilic bacteria *Bacillus stearothermophilus* (Gote et al. 2007) has also been suggested. The carboxylate and lysine residues take part in catalysis and only lysine residues are essential for substrate binding. Carbodiimide mediated chemical modification of the enzyme also supported that a

carboxylate residue located in the active site act as a nucleophile base in substrate cleavage. Acylation and reductive methylation of lysine residues suggested that four protonated lysine residues carrying positive charge on its ε-amino group provides the positive charge density for binding of the substrate. Additionally four tryptophan residues are found near to the active site and in a moderately hydrophobic environment. Kinetic and thermal inactivation study of modified enzyme indicated that these tryptophan residues might have a role in the catalytic site as well as in the thermal stabilization of active site conformation at higher temperature (Gote et al. 2007). Various studies have shown that *p*-chloromercuribenzoate and metal cations like Hg^{2+} and Ag^{2+} strongly inhibit α-galactosidase (Kim et al. 2002) by attacking the cysteine residues in the catalytic site and thereby interfere with substrate binding.

1.3.7. Mechanistic commonality of GH27 and GH36 α-galactosidases

The retention of anomeric stereochemistry for GH27 enzymes was first demonstrated by ^{1}H MNR in the main α-galactosidase from the white-rot fungus *Phanerochaete chrysosporium* (Brumer et al. 1999) establishing that enzymes in GH27 act through a double-displacement mechanism (Rye & Withers 2000). The global protein sequence analysis established the relationship of GH27 and GH36 in clan GH-D (Henrissat & Bairoch 1993), thus implying a conserved overall $(\beta/\alpha)_8$ tertiary structure and retaining catalytic mechanism. This issue was examined for GH27 and GH36 through biochemical analysis of GH36 α-galactosidase from *Thermotoga maritima* (*Tm*GalA) (Comfort et al. 2007). Structural homology of *Tm*GalA and GH27 members and also the site-directed mutagenesis and detailed kinetic analysis of *Tm*GalA have led to identification of the key catalytic and active site residues in GH36. The ^{1}H NMR studies also have showed the anomeric retention by GH36 enzymes through classical Koshland double displacement mechanism. These findings implied that the biochemical characteristics of GH36 *Tm*GalA are closely related to GH27 enzymes, confirming the mechanistic commonality of clan GH-D members (Comfort et al. 2007).

1.3.8. Phylogeny of α-galactosidases

The phylogenetic tree of 43 representative members of GH-D indicates that α-galactosidases of clan GH-D can be divided into at least three major types: the eukaryal type (GH27), the bacterial type (GH36b), and a type consisting of mainly plant enzymes (GH36p) (Fig. 1.15). The latter type additionally comprises uncharacterized sequences from the *Archaea*, intestinal bacteria, and a fungus. The two *Sulfolobus* α-galactosidase sequences form the deepest branch of this plant subfamily and, as yet, constitute the only archaeal sequences of clan GH-D. Moreover, the *Sulfolobus* sequences are clearly distinct from other thermostable α-galactosidases that are produced by thermophilic bacteria, such as those from the genera *Thermotoga* (Uniprot, O33835) (Liebl et al. 1998), *Geobacillus* (Q9LBD1) (Fridjonsson et al. 1999a) and *Thermus* (Q746I3) (Fridjonsson et al. 1999b). The phylogenetic tree reveals that vertebrate α-galactosidases and α-*N*-acetylgalactosaminidase cluster have evolved from a common precursor (Wang et al. 1990, 1998) while plant and other α-galactosidases segregate into distinct clusters. The 43 proteins share high amino acid sequence identity with the sequence conservation higher in domain 1, particularly among residues forming the active site (Garman & Garboczi 2004).

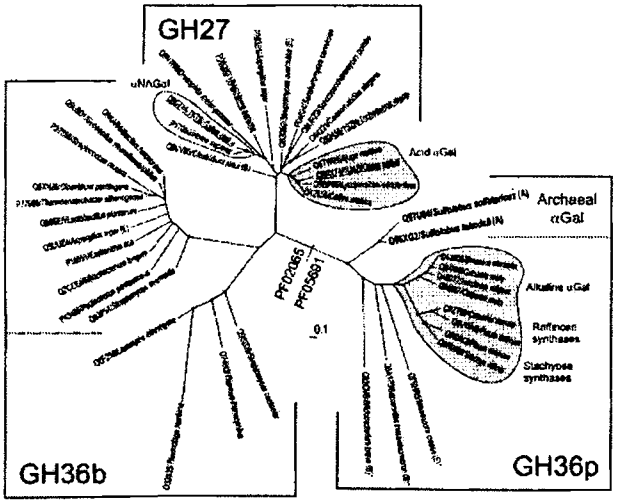


Fig. 1.15. Unrooted neighbor-joining tree of the catalytic domain of a representative set of α-galactosidases belonging to GH-D (Brouns et al. 2006).

1.3.9. Immobilization studies on α-galactosidases

Immobilization of enzymes on natural and synthetic supports is advantageous for industrial and biotechnological applications because; it extends the stability of the enzyme by protecting the active material from deactivation; it enables repeated use; it provides significant reduction in the operating costs; it facilitates easy separation and speeds up recovery of the enzyme; it enables continuous operation in a bioreactor. There are a large number of support materials and methods for the immobilization of enzymes. It is important that the choice of suitable support materials and immobilization method over the free enzyme should be well justified (Bakunina et al. 2006).

α-Galactosidase was first used in immobilized form as enzyme bound to mycelial pellets of *Morteirella vinacea* (Suzuki et al. 1969; Kobayashi & Suzuki 1976). This method is now employed industrially for decomposition of raffinose in sugar beet syrup, after extensive research, starting in the 1960s, at the Fermentation Research Institute, Inage, Chiba Prefecture, Japan.

Gels prepared from natural and synthetic polymers have been successfully used for entrapment of α-galactosidase (Prasanth & Mulimani 2005; Girigowda & Mulimani 2006; Naganagouda & Mulimani 2006; Naganagouda et al. 2007). The α-galactosidase from *Mortierella vinacea* (Thananunkul et al. 1976) and *Gibberella fujikuroi* (Thippeswamy & Mulimani 2002) were entrapped in polyacrylamide gel, packed in a fluidized bed reactor and used for continuous hydrolysis of raffinose and stachyose in soymilk. Polyacrylamide gel is a good matrix for enzyme immobilization especially when the substrate is of low molecular mass. α-Galactosidase substrates such as raffinose and stachyose have a molecular weight of 504 and 666 respectively, which can easily penetrate into the polyacrylamide gel. Despite these facts, polyacrylamide supports are not useful in food processing because of their toxicity. Particularly suitable matrix materials are natural polymers (alginate, κ-carrageenan, chitin and gelatin), since they are non-toxic and methods used for their gelation are very mild. α-Galactosidase from *Pycnoporus cinnabarinus* was immobilized on chitin dispersions with glutaric dialdehyde (Mitsutomi et al. 1985). Alginate (Prasanth & Mulimani 2005), κ-carrageenan (Girigowda & Mulimani 2006) and gelatin

(Naganagouda et al. 2007) were used effectively as immobilization matrices for the entrapment of α-galactosidase from *Aspergillus oryzae*. An inherent problem in enzyme immobilization is the leakage of enzyme from beads, which gradually leads to loss of enzyme activity. In order to avoid leakage glutaraldehyde is often used as a hardener. The immobilization of enzymes in gelatin requires freezing and thawing, which denatures the enzymes and lowers the immobilization efficiency. Naganagouda & Mulimani (2006) checked gelatin-alginate blended hydrogel fibers hardened with glutaraldehyde as an immobilization matrix, combining the advantages of alginate and gelatin. This immobilized enzyme remained very active over a long period of time and lost 70% of its activity only after 60 days of storage at 4 °C.

Covalent linking is one of the extensively used techniques for the immobilization of enzymes. Enzymes are covalently linked to the support through the functional groups in the enzymes, which are not essential for catalytic activity. It is often advisable to carry out the immobilization in the presence of its substrate or a competitive inhibitor so as to protect the active site. α-Galactosidase from *Aspergillus oryzae* was immobilized on Eupergit C (epoxy-activated acrylic beads) through covalent linking (Hernaiz & Crout 2000). Eupergit C is very stable and has good chemical and mechanical properties (simple immobilization procedure, high binding capacity, low water uptake, high flow rate in column procedures, excellent performance in stirred bath reactors, etc.) (Kramer et al. 1975, 1978). Epoxy-activated beads are bead polymers formed from a hydrophilic acrylamide with allyl glycidyl (epoxide) groups as the active components responsible for binding. These groups are convenient for the covalent binding of enzymes. The O-C and N-C bonds formed by the epoxide groups are extremely stable, so that the epoxide-containing polymers can be used for the immobilization of enzymes and proteins (Kramer et al. 1975). Silanized silica beads (silica gel chemically modified with 3-aminopropyltriethoxysilane) containing amino reactive groups were used for the immobilization of α-galactosidase from *Debaryomyces hansenii* UFV-1 (Viana et al. 2007).

Sol-gel technology, which involves no cross-linking agents, is another method reported for the immobilization of α-galactosidase (Shchipunov et al. 2004). The protein

macromolecules are encapsulated into the inorganic matrix formed *in situ* by polycondensation reactions. The macromolecules undergo minimal structural changes, and the enzymes can preserve their activity (Shchipunov et al. 2004).

There are several reports available in the literature on the use of microbial α-galactosidases for the removal of raffinose family sugars from soymilk (Prasanth & Mulimani 2005; Girigowda & Mulimani 2006; Naganagouda & Mulimani 2006; Naganagouda et al. 2007; Viana et al. 2007). Although satisfactory hydrolysis is obtainable by such processing, a single usage of free enzyme appears uneconomical and the processed milk has the possibility to contain foreign proteins.

1.3.10. Applications of α-galactosidases

α-Galactosidases find potential applications in various industries which use hemicelluloses or related polysaccharides as raw materials. These include the food and feed, pulp and paper, cosmetic and pharmaceutical industries. The primary characteristic, which determines the choice and application of a given enzyme, is the operational pH range. α-Galactosidases from several microbial and plant sources have been reported for use in various applications (Dey 1979; ; Ohtakara & Mitsutomi 1987; Ganter et al. 1988; Somiari & Balogh 1995; Mulimani & Ramalingam 1995; McCutchen et al. 1996; Bakunina et al. 1998; Chiba et al. 2002).

1.3.10.1. Legume food processing

Plant proteins are now identified as biologically active and functionally versatile dietary components and are cheaper substitutes than animal proteins (FAO, 1990). Dry legumes constitute one of the richest and least expensive sources of protein in many parts of the world. Legumes not only add variety to human diet, but also serve as an economical source of proteins, dietary fibers and a variety of micronutrients and phytochemicals (Messina 1999). Nevertheless, the food value of legumes is limited because of their flatulence-causing saccharide content. The flatulence-causing saccharides in legumes are primarily stachyose and raffinose (Fig.1.3), which together form the raffinose-family oligosaccharides (RFO). Human beings and monogastric

animals lack the ability to synthesize the enzyme α-galactosidase in their gastrointestinal tract. This α-galactosidase enzyme is essential for the hydrolysis of flatulence-causing oligosaccharides in legume-based food. Hence these oligosaccharides tend to remain unhydrolyzed in the upper intestine of human beings and animals. Thus, when the saccharide is fermented by bacteria in the large intestine, it produces gas, which results in substantial discomfort termed flatulence (Cristofaro et al. 1974). Before legumes or their derivatives are to be utilized as food, or food additives, it is desirable to remove these flatulence-causing factors.

Table 1.5. α-Galactosidases from various sources used for the treatment of legumes

Microbial source	Legume product	Reference
Aspergillus niger	Cowpea flour/meal	Somiari & Balogh 1993, 1995
	Chick pea flour	Mansour & Khalil 1998
Bifidobacterium breve 203	Soymilk	Sakai et al. 1987
Cladosporium cladosporioides	Soymilk	Cruz et al. 1981
Cladosporium cladosporides	Chick pea flour	Mansour & Khalil 1998
Aspergillus oryzae	Chick pea flour	Mansour & Khalil 1998
	Soymilk	Cruz & Park 1982; Prasanth & Mulimani 2005; Girigowda & Mulimani 2006
Gibberella fujikuroi	Soymilk	Thippeswamy & Mulimani 2002
Debaryomyces hansenii UFV-1	Soymilk/molasses	Viana et al. 2007
Humicola sp.	Soymilk	Kotwal et al. 1998

Microbial α-galactosidases were used to degrade RFOs in soymilk by Thananunkul et al. (1976), Cruz et al. (1981), Cruz & Park (1982), Mulimani & Ramalingam (1995), Kotwal et al. (1998), Scalabrini et al. (1998); Thippeswamy & Mulimani (2002), Prasanth & Mulimani (2005), Girigowda & Mulimani (2006), Naganagouda & Mulimani (2006), Naganagouda et al. (2007) and Viana et al. (2007). Mulimani et al. (1997) obtained promising results at removing RFOs from soybean flour by crude α-galactosidase from guar seed. Guimarães et al. (2001) reported that the soybean seed α-galactosidase or the corresponding genes can be used to establish a process to hydrolyze RFOs and improve the nutritional value of soymilk. The α-

galactosidases from various sources used for the treatment of various legumes are summarized in Table 1.5.

1.3.10.2. Animal feed processing

The high protein content coupled with its highly digestible, well-balanced amino acids makes soyabean meal a valuable protein source in diets for monogastric animals including pigs, cattle, and poultry. Other legume seed meals are also used extensively as protein sources in the diets of animals. Like humans, monogastric animals also lack the enzyme α-galactosidase in their digestive tract. This causes reduced digestibility of the nutrients and decreased energy utilization of the animal feed (Gdala et al. 1997). Enzyme supplementation has become a widespread technique to improve the nutritional value of food and feed since the enzymes can act on selected nutrients or non-nutritional compounds, without exerting any negative effect on the nutritional quality of the remaining components of the meal. Treatment with exogenous enzymes derived from microbial sources is of paramount importance in animal nutrition, and is usually carried out by direct supplementation of powder or liquid enzyme preparations during or after the process of diet mixing and pelleting. In diets for swine or poultry production, α-galactosidase supplementation has enabled a significant improvement in the overall performance of the animals and the nutritive utilisation of feed (Kocher et al. 2002; Kim & Baker 2003; Waldroup et al. 2005; Centeno et al. 2006; Urbano et al. 2007).

1.3.10.3. Sugar beet molasses processing

Raffinose is found in appreciable quantities in sugar beets. In the processing of sugar beet molasses, raffinose concentration increases and as it reaches a concentration of 4.5% or higher it seriously retards the crystallization of sucrose. These concentrations may be reached when molasses is treated with finely divided lime in a Steffen house and the precipitated di- and trisaccharides are recycled to concentration and crystallization steps. This considerably reduces the yield of sucrose in beet sugar industry. The raffinose level may be reduced by placing a α-galactosidase treatment step between the crystallizers and the Steffen house. Microbial α-galactosidases are

widely used for eliminating raffinose and thereby improving the yield of sucrose (Yamane 1971). Japanese workers have reported (Yamane 1971) on the use of α-galactosidase to hydrolyse unwanted raffinose in the Steffen process of sucrose recovery from beet molasses. The enzyme is present in the mold *Mortierella vinaceae* var. *raffinoseutilizer* and the washed mycelium is commercially used in the Melibiase process in pellet form.

Suzuki et al. (1966) used α-galactosidase from soil actinomycetes in an effort to decompose raffinose into galactose and sucrose. They found that not only were conditions for crystallization of sucrose from beet molasses improved, but also yields of sucrose were increased. Suzuki et al. (1966) described a method for estimation of raffinose by use of α-galactosidase. There are also many other reports that describe enzymatic process for the hydrolysis of raffinose by exploiting enzymes extracted from a number of microbial species of the genera *Absidia*, *Aspergillus*, Saccharomyces, *Bacillus*, *Circinella*, *Mortierella*, *Penicillium* etc, (Suzuki et al. 1972; Delente et al. 1974; Suzuki et al. 1974; Narita et al. 1975, 1976; Olivieri et al. 1984). The immobilized α-galactosidase preparations have also been extensively employed in the beet sugar industry for the elimination of raffinose (Reynolds 1974; Linden 1982; Ohtakara & Mitsutomi 1987).

1.3.10.4. Galactomannan processing

The most abundant polymers containing galactose are galactomannans, which occur in varying amounts in the endosperm of a wide range of leguminous seeds (Buckeridge et al. 1995). Locust bean gum can form gels both in the native stage and in combination with other polysaccharides such as agar, carrageenan and xanthan. Since guar galactomannan has a greater proportion of galactose side chains (38 % galactose and 62 % mannose), it is unable to bind with helices of xanthan or carrageenans to form synergistic gels (like locust bean gum) and can only produce an increase in viscosity (Dea & Morrison 1975). Hence, the potential applications of guar gum are limited. Since locust bean gum is rather expensive raw material, guar gum,

which has poorer gelling properties than locust bean gum, has been modified with α-galactosidases to produce galactomannans with improved gelling capacity.

To convert guar galactomannan into a locust bean galactomannan equivalent, some of the side chains α-(1-6)-linked-D-galactosyl residues need to be removed without significant cleavage of the galactomannan back bone (Pai & Khan 2002). This can be achieved by partial enzymatic hydrolysis of guar gum by α-galactosidase (Cronin et al. 2002). Many investigators have developed processes for the modification of galactomannan polymers by using plant and microbial α-galactosidases (Bulpin et al. 1990; McCutchen et al. 1996; Halstead et al. 2000; Joersbo et al. 2001). Studies with purified α-galactosidase have shown that modified guar galactomannan exhibits physical and functional properties similar to that of locust bean gum (Barry et al. 1981). The enzymatically modified galactomannans are commonly used in food to manipulate aqueous rheology. In addition, they have many industrial applications in pharmaceuticals, cosmetics, paper products, paints and plastics (Dey 1978). Thermostable α-galactosidases might also be used in the hydrolysis of guar gum for viscosity reduction of guar-based hydraulic fracturing fluids used in oil and gas well stimulation (McCutchen et al. 1996; Duffaud et al. 1997). Moreover, these galactomannans possess non-cytotoxic antitumor activity and act as inhibitors of viruses. Guar gum is also being tested for possible use in reducing the rate of glucose absorption by diabetics (Holt et al. 1979; Miranda & Horowitz 1978). The useful commercial properties of galactomannan are due to high viscosity in dilute aqueous solutions and co-gelation with other polysaccharides such as carrageenan, agar, xanthan gum etc.

1.3.10.5. Processing of pulp and paper

α-Galactosidases may be involved in the modification of wood-derived materials because galactomannans and galacto(gluco)mannans are the main groups of hemicelluloses in softwoods (Clarke et al. 2000). α-Galactosidases have application in pulp and paper industry, where it could enhance the bleaching effect of endo-β-1,4-mannanases on softwood Kraft pulp (Clarke et al. 2000; Puchart et al. 2000).

The mechanical processing of wood fibers and subsequent chemical treatment by the Kraft method produces a paper pulp which requires whitening by a multistage bleaching sequence. The enzymatic approach to aid bleaching of Kraft pulps relies on the removal of plant cell wall components that may trap residual lignin within the fiber matrix. Microbial degradation of arabinoxylans and galactoglucomannans, the principal hemicellulose components present in soft woods (Thompson 1983), relies on the interaction between a specific range of enzymes, such as xylanases, mannanases and side-chain-removing accessory enzymes (Hazlewood & Gilbert 1998). Mannan hydrolysis has been shown to further enhance xylanase-aided pulp bleaching (Viikari et al. 1994; Clarke et al. 1997), presumably as a result of improved xylanase accessibility to residual matrix xylan, which may in turn be mediated by improved mannanase accessibility to galactomannan and galactoglucomannan on the pulp matrix, through dispersal of reprecipitated xylan by xylanase. The galactosyl side-groups present in softwood mannans may also impair access of the mannanse (Tenkanen et al. 1997). These are removed by the action of α-galactosidase, and subsequent cleavage of the accessible mannan backbone may allow further xylan hydrolysis within the fibre matrix (Poutanen et al. 1991). Mannanase and α-galactosidase from *Aspergillus niger* have been shown to interact synergistically (Manzanares et al. 1998) and galactose release form softwood pulp is enhanced by the presence of mannanase in combination with α-galactosidase (Luonteri et al. 1998b).

1.3.10.6. Pharmacological applications

α-Galactosidases are gaining increased interest in human medicine especially in the treatment of Fabry's disease, prevention of xenorejection and blood group transformation.

1.3.10.6.1. Treatment of Fabry's disease

Fabry disease is an X-linked (locus Xq22) (Peters et al. 2001) lysosomal storage disorder caused by mutations in the α-galactosidase A gene resulting in the defective activity of this enzyme. This disease is characterized by accumulation of the

enzyme's substrate, ceramidetrihexoside, in the plasma and particularly in the vascular endothelial lysosomes of hemizygous male victims (Desnick et al. 1979). In affected males this leads to early death due to occlusive disease of heart, kidney and brain.

Until recently, treatment has been limited to symptomatic management of pain and other measures to alleviate the problems associated with end-stage complications from renal, cardiac and nervous system involvement. Recombinant human α-galactosidase has the ability to restore enzyme function in patients (Schiffmann et al. 2001; Eng et al. 2001). Enzyme replacement therapy represents a dramatic step forward in treating Fabry's disease (Pastores & Thadhani 2002; Martínez et al. 2004). It was approved in the United States in 2003 as a treatment for Fabry disease. α-Galactosidase became the second recombinant protein approved for the treatment of a lysosomal storage disorder, after β-glucosidase (treatment for Gaucher disease) (Beutler & Grabowski 2001), and α-galactosidase represents one of a small number of recombinant human proteins approved for the treatment of any disease. In addition to enzyme replacement therapy, gene replacement therapy using the α-galactosidase gene shows potential as a treatment for Fabry disease (Park et al. 2003).

The rationale for enzyme replacement in Fabry's disease was initially based on *in vitro* correction of the metabolic defect by addition of exogenous α-galactosidase A to the media of cultured fibroblasts obtained from hemizygotes with Fabry's disease (Dawson et al. 1973). Efforts have been made in Fabry patients and in cell culture to replace the defective enzyme with normal enzyme obtained from various human and recombinant DNA sources (Desnick et al. 1979; Brady et al. 1973; Mapes et al. 1970). Infusion of normal plasma or purified α-galactosidase A decreased the circulating substrate levels, and then concentrations of the substrate gradually returned to preinfusion values (Desnick et al. 1979). Further study of this approach has been limited until recently (Schiffmann et al. 2001) by the non-availability of sufficient quantities of the human enzyme. Technological progress has made it possible to obtain this enzyme from a human cell line using genetic engineering (Brady & Schiffmann 2000). Several trials conducted thus far have shown an improvement in gastrointestinal

manifestations, neuropathic pain, kidney function, myocardial mass and cardiac conduction (Schiffmann et al. 2003; Przybylska et al. 2004). In addition, cerebral perfusion has improved, thereby lowering the risk of cerebrovascular events. In general, this replacement therapy has been well tolerated, with a good safety profile in clinical trials. Enzyme replacement therapy mediated by gene transfer may become a promising alternative treatment strategy in the future (Breunig et al. 2003a; 2003b).

1.3.10.6.2. Prevention of xenorejection

The accelerating advances of organ transplantation have raised an imbalance between organ supply and demand. The availability of human organs meets only about 5% of the estimated demand (Evans et al. 1992; Lin et al. 2000; Jing-Lian et al. 2003). Xenotransplantation from pig to human beings is viewed as a potential solution for the acute organ shortage. However, consequent xenorejection prevents xenotransplantation from the clinical application. The carbohydrate epitope, Gal α-(1,3)-Gal (Gal epitope or G antigen), a terminal disaccharide on glycoproteins and glycolipids, abundantly expressed in pig, mouse, and new world monkey, induces most of the xenorejection by interacting with host xenoreactive natural antibodies (Sharma et al. 1996; Azimzadeh et al. 1997). The first approach to prevent xenorejection is designed from the treatment with soluble enzyme. *In vitro* treatment of porcine endothelial cells and lymphocytes with green coffee bean α-galactosidase dramatically decreased the binding of human xenoreactive natural antibodies (Cairns et al. 1994; Watier et al. 1996). Thus α-galactosidase treatment offers a most attractive alternative to prevent xenorejection.

1.3.10.6.3. Blood group transformation

The first major break-through in research relating to blood transfusion safety was the discovery of clinically most important ABO blood group system by Karl Landsteiner in 1900. This system is based on the presence or absence of blood group antigens A and/or B, found on the surface of erythrocytes, platelets and endothelial cells. The ABH blood group specificity is determined by the nature and linkage of

monosaccharides at the ends of the carbohydrate chains attached to peptide (glycoprotein) or lipid (glycolipid) backbones, which are attached to the cell membrane of the cells (Fig. 1.16). The immunodominant monosaccharide determining type A specificity is a terminal α-1-3 linked *N*-acetylgalactosamine (GalNAc), while the corresponding monosaccharide of B type specificity is an α-1-3 linked galactose (Gal). Group O cells lack either of these monosaccharides at the termini of oligo-saccharide chains, which instead are terminated with α-1-2 linked fucose (Fuc) residues and called the H antigen.

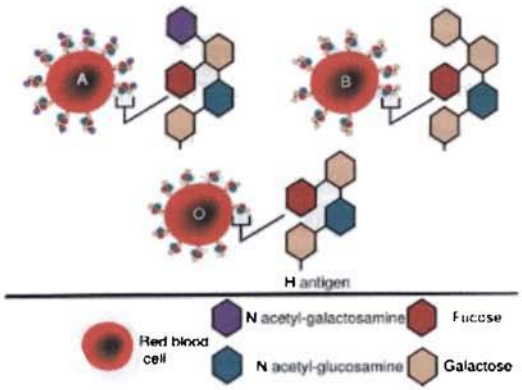


Fig. 1.16. The terminal monosaccharides responsible for ABO blood group specificity

The plasma of blood group A individuals contains naturally occurring antibodies to B antigen. Conversely, plasma of blood group B contains antibodies to A antigen. Blood of group AB has neither antibody, and blood group O has both. This is generally referred to as Landsteiner's rule (Landsteiner 1900). An individual whose blood contains either (or both) of the anti-A or anti-B antibodies cannot receive a transfusion of blood containing the corresponding incompatible antigen(s). Because group O blood contains no A or B antigens, it can be transfused into recipients with any blood group, *i.e.* A, B, AB or O. Thus type O blood is considered "universal" and may be used for transfusion in any individual of blood type A, B, AB and O. Accidental transfusion of

ABO-incompatible red blood cells (RBCs) is a leading cause of fatal transfusion reactions. Hence it is desirable for blood banks to maintain large quantities of type O blood. However, due to many reasons, there is a paucity of blood group O donors. Therefore, it is desirable and useful to remove the immunodominant A and B antigens on types A, B and AB blood.

The idea of converting blood group A and B antigens to H using specific exo-glycosidases capable of removing the immunodominant sugar residues was pioneered by Harpaz et al. in 1975. It was then adopted by Goldstein and colleagues at the New York Blood Center in the early 1980s. Enzymatic conversion of group B RBCs to O was initially carried out with α-galactosidase extracted from coffee beans (Harpaz et al. 1975; Goldstein et al. 1982; Goldstein 1989). This 'enzyme converted O' RBCs appeared to survive normally in all recipients independent of blood group. α-Galactosidase with activity against blood group B epitope has been also studied from *Glycine max* (Vosnidou et al. 1998) and micro-organisms, *Pseudoalteromonas* sp. (Bakunina et al. 1998) *Penicillium* sp. (Varbanets et al. 2001), *Streptomyces* sp. (Oishi & Aida 1971) etc.

1.3.10.7. Galacto-oligosaccharide synthesis

α-Galactosidases possess transglycosylation activities in addition to hydrolytic activity (Hara et al. 1994; Hashimoto et al. 1995a, b; Eneyskaya et al. 1998; van Laere et al. 1999; Spangenberg et al. 2000; Yamashita et al. 2005). By this mechanism α-galactosidases are capable of synthesizing α-galacto-oligosaccharides (Hashimoto et al. 1995a, b; Weignerová et al. 2001; Tzortis et al. 2003; Yamashita et al. 2005). α-Galactosidases have been used for the synthesis of novel heterogeneous branched cyclodextrins which are expected to have possible applications as drug carriers in the drug delivery systems (Kitahata et al. 1992; Hara et al. 1994; Koizumi et al. 1995). α-Galacto-oligosaccharides produced by transfer reaction of α-galactosidase can be used as a prebiotic in functional food (Rivero-Urgell & Santamaria-Orleans 2001). Functional food is defined as 'a dietary component that may exert physiological effects on the

consumer which may eventually lead towards justifiable health claims' (Roberfroid 1996).

Table 1.6. Functions of natural α-galactosides in animals

Linkage	Compound	Functions	Reference
α-1,6	Raffinose (Gal-Glc-Fru)	Alleviation of atopic dermatitis	Matsuda et al. 1998
		Immunopotenting activity	Kiyonobu et al. 1998; Nagura et al. 2002
		Prevention of allergic airway eosinophilia	Watanabe et al. 2004
	α-galactobiose	Sequence in glycosylphosphatidylinositol anchor of *Trypanosoma brucei*	Li et al. 1999
α-1,4	α-galactobiose	Prevention of infection of pathogenic *Escherichia coli* (O157 and p-fimbriated)	Ofek & Sharon 1990; Stromberg et al. 1990; Armstrong et al. 1995
α-1,3	α-galactobiose	Reduction of transplant rejection	Chen et al. 1999
		Inhibitor of toxin A produced by *Clostridium difficile* (enteric pathogen)	Glaser 1998
		Anti-infection factors against pathogenic organisms in newborn pig and bear (terminal moiety structure of milk oligosacchrides)	Urashima et al. 2001
α-1,2	α-galactobiose	Sequence in glycosylphosphatidylinositol anchor of *Trypanosoma brucei*	Ferguson et al. 1988
		Repeating unit of *Streptococcus pneumoniae* type 15 antigen	Wang et al. 2002
α-Galactosylceramide		Specific ligand of v α14 natural killer T cells	Taniguchi et al. 2003

Recent advances in glycobiology have high-lighted important roles the oligosaccharide epitopes play in living organisms. The oligosaccharides of globo-series bearing terminal α-Gal (1→4)β-Gal sequences illustrate a variety and significance of such interactions. They act as preferential binding sites for a multitude of pathogens (*Streptococcus suis*, uropathogenic *Escherichia coli*), toxins (*Shigella*, *E. coli*), and some viruses on the basis of molecular mechanisms for ligand-receptor interaction. Moreover, globo H antigen is associated with certain types of cancer (Scigelova & Crout 2000). This led to the notion of a role for α-galactooligosaccharides (Table 1.6) as

potential inhibitors of microbial adhesion, neutralization of toxins and regulation of immune system (Yamashita et al. 2005). A production of antibodies against human cancer cells was successfully achieved after immunisation of mice with fully synthetic globo H antigen (Ragupathi et al. 1997).

Even though there is a large demand for galacto-oligosaccharide derivatives, no methods for their industrial production have been reported. Chemical synthesis usually requires cumbersome and sophisticated protection and de-protection steps which render it unrealistic for industrial application (Scigelova & Crout 2000). Enzymatic synthesis using glycosyl hydrolases and glycosyl transferases could circumvent the drawbacks of the chemical methods.

1.3.10.8. Structure analysis of carbohydrates

Crystalline α-galactosidases may be used as a reagent for the determination of structures of polysaccharides and glycoproteins (McCleary & Matheson 1986). Several different purified enzymes with varying specificities are currently available. They can be used in the analysis of fine structures of galactomannans (McCleary 1994) and pectic polysaccharides (Lerouge et al. 1993). Improved understanding of the mode of action of the enzymes and the structures of various polysaccharides widens the possibilities for their utilization in different applications.

1.3.11. Commercial status of α-galactosidases

Industrial enzymes are a growing business worldwide worth about two billion US$ – with food enzymes capturing about half of it. Both the number of available food enzymes and their annual turnover have been steadily increasing for many years, so are the number and kind of applications. As of 2001 the major industry association AMFEP (Association of Manufacturers and Formulators of Enzyme Products) lists about 160 enzymes manufactured for use in food industry (Spök 2006).

The micro-organisms preferred for commercial production of α-galactosidase are *Circinella muscae*, *Absidia griseola*, *Absidia hyalospora* (Hokkaido Sugar Co. Ltd., Tokyo, Japan) (Narita et al. 1975, 1976), *Mortierella vinacea* (Suzuki et al. 1974) and

Bacillus stearothermophilus (Monsanto Company, St. Louis, Mo) (Delente et al. 1974). Recently it is being produced commercially from *Aspergillus niger* (Novo Nordisk A/S, Bagsvaerd, Denmark) (Knap et al. 2001).

Many α-galactosidase preparations are available commercially under the trade names Beano (Glaxo Smith Kline, USA), Gas-Zyme 3X, Terrainzyme, Jarro-Zymes-Plus, EZ-Gest, Bean-Zyme (Mikeska Products LLC, Santa Barbara, California, USA), Validase® AGS (Valley Research- Enzymes for the food industry, United States), Nutriteck alpha-galactosidase (Division of Ultra-Bio-Logics Inc. Rigaud QC, Canada), Alpha-gal® (Novozymes) etc, and are used as dietary supplements in humans diets. The enzymes from *Mortierella vinaceae* strain *raffinoseutilizer* (Suzuki et al. 1972) and *Aspergillus niger* (produced by Novo Nordisk A/S, Denmark) (Knap et al. 2001) are widely used in beet sugar industry and in food and feed processing. α-Galactosidase from *A. niger* is an enzyme supplement in many digestive enzyme products like Ω-zyme™, Enzalase™ Group #2 (Therabiotics Inc.). Highly purified Glyko® alpha(1-3, 4, 6)-galactosidase from green coffee bean without any β-galactosidse contamination (Glyko® Tools for Glycobiology) is also available commercially.

There are currently two recombinant glycoprotein products, Fabrazyme and Replagal, available for enzyme replacement therapy used in the treatment of Fabry disease (Schiffmann et al. 2001; Eng et al. 2001). Fabrazyme is produced and marketed by Genzyme Corporation (Cambridge, Massachusetts) and Replagal by Transkaryotic Therapies (Cambridge, Massachusetts.). These two glycoproteins have identical amino acid sequences but are produced in different cell lines, resulting in different glycosylation at the *N*-linked carbohydrate attachment sites. Fabrazyme is produced in a Chinese hamster ovary (CHO) cell line whereas Replagal is a human α-galactosidase A produced by genetic engineering technology in a human cell line. TKT's gene activation technology is a proprietary approach to the large-scale production of therapeutic proteins, which does not require the cloning of genes and their subsequent insertion into non-human cell lines. Replagal contains a greater amount of complex carbohydrate while Fabrazyme contains a higher fraction of sialylated and phosphorylated carbohydrate (Lee et al. 2003). Because the polypeptide

sequence of the two glycoproteins is identical, these differences in carbohydrate composition are solely responsible for the differences in tissue distribution and dose response of the two enzyme replacement therapies.

Genzyme Corporation stands at the centre of enzyme replacement therapy of Fabry disease by α-galactosidase. Recently, Genzyme Corporation has received US, Food and Drug Administration (FDA) approval for marketing of Fabrazyme. Genzyme estimates that only 2,000 to 4,000 people suffer from the disease worldwide. But even such a small number of patients can mean high revenue if there is no competition and the drug price is high. Genzyme's leading product, a drug called Cerezyme, which treats another rare inherited disorder called Gaucher disease, has annual sales of about $500 million. Even though only 3,000 patients are taking the drug, each one is paying about $170,000 a year. As of October 2002, Fabrazyme® was approved for use in 25 countries including all 15 countries of the European Union. Replagal has been approved for commercial use in 27 countries, including the 15 countries of the European Union. In the United States, Replagal is an investigational product. As per the reports of TKT, the sales of Replagal is about $77 million in 2004 and the estimated current market is $190 million in Western Europe which is higher than the US market.

Large Scale Biology Corporation (LSBC, Vacaville, California) has developed a new version of AGalA trademarked ENZAGAL™ using biomanufacturing in plants, that could potentially serve the needs of all segments of the Fabry population worldwide. In January 2003, LSBC received Orphan Drug designation for ENZAGAL™ by the FDA, clearing the way for market protection upon product launch. LSBC's ENZAGAL™ can be produced more efficiently and in higher abundance than competing products, potentially enabling substantial market expansion. Extensive preclinical and manufacturing R&D and regulatory assessments conducted by LSBC and its collaborators have shown that LSBC's new product could be commercialized rapidly and with significant competitive advantages (http://www.lsbc.com/thera.html#agala).

1.4. CONCLUSION

Enzymes hydrolyzing α-linked galactosidic bonds are widespread in micro-organims. Owing to its importance in many diverse applications, α-galactosidase has been extensively studied in order to understand the precise mechanism by which it hydrolyzes α-glycosidic bonds and to possibly improve its catalytic properties. There are numerous micro-organisms in nature that are yet to be isolated and identified. They remain untapped resources and their potential should not be overlooked. The end product inhibition is one of the many causes that impart a decrease in the catalytic efficiency of the enzymes. An enzyme with resistance to end product inhibition would be advantageous in this respect. Any attempt to identify a novel source of an enzyme like α-galactosidase with improved catalytic properties for versatile applications would be worthwhile since it can have a significant impact on the industrial processes that take advantage of the action of α-galactosidases.

1.5. OBJECTIVES AND SCOPE OF THE PRESENT STUDY

- Isolation and screening of actinomycetes for production of α-galactosidase.
- Identification and characterization of the selected actinomycete strain.
- Optimization of the cultural and nutritional parameters for production of α-galactosidase in submerged fermentation.
- Optimization of process parameters for α-galactosidase production in solid-state fermentation.
- Enhancement of α-galactosidase yield by employing packed bed bioreactor operating under solid-state fermentation conditions.
- Purification and characterization of thermostable α-galactosidases.
- Evaluation of the potentials of free and immobilized α-galactosidase in the reduction of non-digestible oligosaccharides in legumes
- Evaluation of the transglycosylation potential of α-galactosidase in the synthesis of α-galacto-oligosaccharides.

Chapter II

ISOLATION AND SCREENING OF ACTINOMYCETES FOR α-GALACTOSIDASE PRODUCTION

2.1. INTRODUCTION

Enzymes occur in every living cell, hence in all micro-organisms. Microbial enzymes are relatively more stable than corresponding enzymes derived from plants and animals. Furthermore, microbial enzymes provide a greater diversity of catalytic activities and can be produced more economically using submerged fermentation as well as surface culture methods. Microbial enzymes have been used in various industries for many centuries. Recently, with the advent of biotechnology, there has been a growing interest in the demand for enzymes with novel properties. The majority of enzymes currently used in industry are of microbial origin, and the vast majority of these are produced from fungi. Indeed, it has been estimated that only about 2% of the world's micro-organisms have been tested as enzyme sources (Wiseman 1985; Chandrasekaran 1997). Increased awareness of the use of biocatalytic capabilities of enzymes and micro-organisms has made possible the creation of a new generation of rationally developed biologically based processes and products. This has not only kindled interest in biotechnologists to improve the available strains but also to search for better and more efficient miroorganisms. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing, and metabolic in nature. But the absolute and relative amounts of the various individual enzymes produced vary markedly between species and even between strains of the same species. Hence, it is customary to select strains which have the capacity for producing highest amounts of the particular enzymes desired. Selection of a particular strain, however, is a tedious task, especially when commercially competent enzyme yields are to be achieved.

Microbes from terrestrial sources are currently extensively employed for industrial production of enzymes, although the potential for synthesis of several novel enzymes by marine and mangrove micro-organisms has been recognized. The marine

and mangrove environment harbours millions of species of micro-organisms having a diverse range of enzymatic activity and capable of catalyzing various biochemical reactions with novel enzymes. Among them, aerobic bacteria, including the filamentous actinomycetes, are capable of producing a wide spectrum of enzymes which includes amylases, lipases, proteases, galactosdiases, deoxyribonucleases etc (Chandrasekaran 1997). Thus marine and mangrove micro-organisms also can provide a wealth of enzymes.

Currently, there is a lot of interest in the scientific community around the world in exploiting novel micro-organisms for the production of industrially important enzymes and actinomycetes which have immense potential as source of exo-enzymes are yet to be harnessed as source of α-galactosidase for commercial application. Actinomycetes or Actinobacteria comprise an extensive and diverse group of aerobic, filamentous Gram-positive bacteria belonging to the order *Actinomycetales* (Lechevalier 1989), characterized by the formation of substrate mycelium and aerial mycelium on solid media, presence of spores and a high G+C content of the DNA (60-70 mol %). They can be separated into different genera on the basis of morphological, physical and chemical criteria. The physiological diversity of actinomycetes is demonstrated by the wide range of metabolic products, which they synthesize and secrete. Actinomycetes are noteworthy as producers of many different biologically active secondary metabolites such as antibiotics. In addition to antibiotics, the potential of actinomycetes to produce several industrially important enzymes is also well documented (Maheswari & Chandra 2000; Jang & Chen 2003). Although the existence of α-galactosidase in various organisms like plants (Bulpin et al. 1990), animals (Grossman & Terra 2001) and micro-organisms such as fungi (Wong et al. 1986; Zeilinger et al. 1993), yeast (Cavazzoni et al. 1987), bacteria (Jin et al. 2001) and actinomycetes (Suzuki et al. 1966; Lyons et al. 1969; Elshafei et al. 2001) have been known for a number of years, α-galactosidase from mangrove actinomycetes is not unravelled. This chapter explains the isolation and screening of actinomycete cultures from mangrove sources for production of α-galactosidase.

2.2. MATERIALS AND METHODS

2.2.1. Collection of soil samples

For the purpose of isolating novel actinomycetes, altogether 50 sediment soil samples were collected from different marine and mangrove regions along the West Coast of India. The soil samples were collected in sterile plastic containers.

2.2.2. Isolation and maintenance of actinomycetes cultures

Isolation of actinomycetes was performed by the dilution plate technique using starch casein agar (SCA) medium (Kuster & Williams 1964) (g/L: starch, 10.0; casein, 1.0; K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001; agar, 16.0). The starch casein agar medium (pH 7.0) containing 50% seawater was supplemented with 100 µg/mL of cycloheximide to inhibit the growth of fungal contaminants. Approximately 1.0 g of the collected soil samples were serially diluted in sterile distilled water pre-heated to a temperature of 55 °C for 6 min and 0.1 mL of the dilutions were plated on SCA plates for the isolation of actinomycete colonies. The plates were incubated at 30 °C for 7 days. The tough leathery colonies were isolated to purity by streak plate method. The isolates were grown on fresh SCA plates and morphology of the isolated cultures, colouration of aerial mycelia and substrate mycelia and the diffusible pigments were studied to ensure that the isolates are actinomycetes. The isolates were examined by light microscopy to study the nature of mycelial filaments and their response to Gram's staining. The gram positive filamentous cultures were selected for further screening experiments. The isolates were maintained on SCA slants at 4 °C and were sub-cultured fortnightly.

2.2.3. Screening of actinomycete cultures for α-galactosidase activity

2.2.3.1. Primary/qualitative screening

Primary screening of isolated actinomycete cultures for α-galactosidase activity was done qualitatively by plate assay method using bromocresol purple (BCP) agar medium (g/L: tryptone, 20; yeast extract, 5; NaCl, 4; sodium acetate, 1.5; bromocresol purple,

0.04 and agar 16) (Boucher et al. 2003; Labrie et al. 2005) supplemented with 0.5 g/L of melibiose. Melibiose was filter-sterilized (0.22 μm) and added to the autoclaved medium. The isolated actinomycete cultures were grown on the screening media at 30 °C for 7 days and the growth of the cultures and appearance of halo around the colonies were regularly monitored.

In another experiment the cultures were grown in the same medium but lacking BCP. In that case the plates (after 7 days of incubation) were flooded with the fluorescent substrate methyl umbelliferyl α-D-galactopyranoside (MU-α-gal) (1mM) in 0.1 M McIlvaine buffer (Citrate- Na_2HPO_4, pH 7.0) and the halo zones around the colonies were visualized as fluorescence in a UV transilluminator.

2.2.3.2. Secondary/quantitative screening

The α-galactosidase active cultures identified by primary screening were subjected to secondary screening by quantitative estimation of the α-galactosidase titre produced by the cultures in three different screening media under submerged fermentation (SmF). The media used and their compositions were as follows:

- Medium I- (g/L) Locust bean gum, 10; Yeast extract, 3; $(NH_4)_2HPO_4$, 3; KH_2PO_4, 1; $MgSO_4$, 0.5; and 1 mL of trace elements solution. The trace elements solution composed of (g/L): $FeSO_4.7H_2O$, 0.1; $MnCl_2.4H_2O$, 0.1 and $ZnSO_4.7H_2O$, 0.1.
- Medium II- (g/L) Soybean meal, 4; Tryptone, 4; KH_2PO_4, 0.5; $MgSO_4$, 0.3; $CaCl_2$, 0.2; $FeSO_4$, 0.01 and $MnSO_4$, 0.002 (Jin et al. 2001)
- Medium III- (g/L) Melibiose, 10; Yeast extract, 1; $(NH_4)_2SO_4$, 5; $MgSO_4$, 0.2 and K_2HPO_4, 1 (Cavazzoni et al. 1987)

The pH of the media was adjusted to 7.0. The 250-mL Erlenmeyer flasks containing 90 mL of the medium were inoculated with 10 % (v/v) of 48 h old inoculum grown under the same conditions and were incubated in a rotary shaker for 7 days at 30 °C and 150 rev/min. One mL of the inoculum contained 3 x 10^6 CFU. Aliquots were withdrawn at regular time intervals of 24 h and the culture supernatant obtained after centrifugation at 10,000 rpm for 20 min was used as the enzyme preparation.

2.2.4. α-Galactosidase assay

The activity of α-galactosidase was routinely determined according to the method of Dey & Pridham (1969a) using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) with minor modifications. The *p*NPG hydrolyzing activity was estimated by incubating 100 µL of enzyme with 50 µL of 2 mM pNPG and 850 µL of 0.1 M McIlvaine buffer (Citrate- Na_2HPO_4, pH 7.0) at 55 °C for 10 min. The reaction was terminated by addition of 2 mL of 1 M sodium carbonate. The α-galactosidase positive samples were bright yellow due to the release of *p*-nitrophenol (*p*NP) where as the α-galactosidase negative samples were virtually colourless. The *p*NP released was estimated spectrophotometrically by absorbance at 400 nm. One unit (U) of enzyme activity was expressed as the amount of α-galactosidase per mL of the enzyme preparation that liberated one µmol of *p*-nitrophenol per minute under the assay conditions.

The α-galactosidase activity was calculated as follows:

$$1\ U\ of\ \alpha\text{-galactosidase} = \frac{Abs_{test} - Abs_{control}}{V \times t} \qquad Eq.\ (1)$$

where, Abs_{test} = Absorbance of the test sample at 400 nm

$Abs_{control}$ = Absorbance of the control sample at 400 nm

V = Volume of enzyme

t = Incubation time in minutes

2.2.5. Enzyme profile of the selected strain

The enzyme profile of the culture was analyzed by carrying out the quantitative assays for different enzymes such as β-galactosidase, β-glucosidase, cellulase, xylanase, β-xylosidase, β-mannosidase, protease and invertase. The assays for β-galactosidase, β-glucosidase, xylosidase and α-mannosidase were done using *p*-nitrophenyl derivatives of β-D-galactopyranoside, β-D-glucopyranoside, β-D-xylopyranoside and α-D-mannopyranoside respectively. The assays were done at pH 7.0 (0.1 M McIlvaine buffer) and 55 °C for 10 min and the reaction was terminated by

addition of 2 mL of 1 M sodium carbonate. The amount of *p*NP released was estimated at 400 nm.

The cellulase and xylanase activities were determined by DNS method (Miller 1959) using carboxymethyl cellulose and oat spelt xylan respectively as the assay substrates. The amount of reducing sugar liberated was estimated at 540 nm.

The protease activity in the culture supernatant was determined according to the method described by Kunitz (1947) and Laskowski (1955). One unit of protease activity is defined as the amount of enzyme which releases 1 µmol of tyrosine per minute at 50 °C and pH 7.0 and 9.0 in 50 mM sodium phosphate and sodium carbonate buffer, respectively when 1% (w/v) casein was used as assay substrate. Protesase activity on haemoglobin was also determined similarly at pH 5.0 in 50 mM sodium acetate buffer.

Invertase activity in the culture supernatant was determined by incubating 100 µL of enzyme with 900 µL of 1% sucrose in 50 mM phosphate buffer (pH 7.0) at 55 °C for 1 h and the reducing sugar liberated was estimated at 540 nm by the method of Nelson (1944).

In all the cases the enzyme units were calculated using the equation 1 and one U of enzyme is defined as the amount of enzyme that liberates one µmol of the product (*p*NP or reducing sugar) under the assay conditions.

2.2.6. Analytical procedures

Total soluble protein and reducing sugars in the culture filtrate were determined by the method of Lowry et al. (1951) using Bovine serum albumin as standard and by the method of Miller (1959) using glucose as standard, respectively. The growth of the culture was observed by measuring the turbidity of the culture broth as increase in the absorbance at 600 nm. The pH of the culture filtrate was measured using Cyber Scan 1000 pH meter.

2.2.7. Scanning electron microscopy (SEM)

The filamentous nature of *S. griseoloalbus* was examined using a scanning electron microscope (JEOL JSM 5600LV, 115 Japan). The culture was mounted on a

brass stud followed by a mild gold coating (0.01 μm) and was subjected to electron microscopy at an accelerating voltage of 10 kV.

2.3. RESULTS AND DISCUSSION

2.3.1. Isolation of actinomycete cultures

In the course of screening for α-galactosidase producing actinomycetes, twelve different actinomycete cultures were isolated from the mangrove sediment soil samples collected from different regions along the West coast of India. The heat pre-treatment of the soil samples before plating was found to be very effective in preventing the other bacterial contaminants from growing on SCA plates. The incorporation of cycloheximide also could prevent the growth of fungal contaminants. From among the colonies that had grown on the SCA plates, those colonies, which appeared to be tough and leathery, were purified. The actinomycetes on the plates could be identified as coloured, dry, rough and generally convex colonies with irregular margin as described by Williams & Cross (1971). Such colonies are distinctly different from the other bacterial and fungal colonies. The isolated cultures were then subjected to Gram's staining which showed that they are gram-positive filamentous bacteria, confirming that they are actinomycetes. The gram positive filamentous bacterial cultures were selected for further studies.

Preliminary details regarding the morphological characteristics of the isolated cultures are shown in Table 2.1. The results showed that out of the twelve isolates, nine showed distinctive pigment production, which in some cases, diffused into the medium. Based on colony morphology, culture behaviour, and microscopic observation, most of the isolates were presumed to be of the genera *Streptomyces* as they showed good sporulation with compact, chalk-like dry colonies of different colours. Weyland (1986) also found that in high salinity mangrove areas *Streptomycetes* were the dominant actinomycete group. In a similar study, Takizawa et al. (1993) demonstrated a diverse actinomycete community in the Chesapeake Bay.

Table 2.1. Preliminary details of the morphological characteristics of isolated actinomycete cultures

Culture code	Colour of aerial mycelium	Pigmentation
AGP 40	Grey	Deep blue
AGP 41	Dirty white	Bluish green
AGP 42	Pink	Bright pink
AGP 43	Grey with white border	Nil
AGP 44	Dark grey	Nil
AGP 45	Bluish grey	Yellow
AGP 46	Dirty white	Nil
AGP 47	White	Brown
AGP 48	Grey	Pink
AGP 49	Pinkish white	Brown
AGP 53	Pink	Bright yellow
AGP 55	White with purple shade	Purple

In an actinomycete-based natural product screening program, the organisms must be isolated from the environment, grown in pure state, preserved, used for fermentation, and the fermentation products must be tested for biological activity. Essential to this process is knowledge of actinomycete ecology, taxonomy, and physiology, all of which contribute to the isolation of new strains with the potential of producing novel compounds. Actinomycetes are usually not in the focus of marine microbiology. They are in many respects regarded as boundary bacteria and they are known as terrestrial organisms. Strains isolated from aquatic habitats are usually considered as wash-in organisms living there in a dormant state (Cross 1981). However recent studies give evidences for the ubiquitous presence of actinomycetes in marine and mangrove sediments (Takizawa 1993; Niladevi & Prema 2005). The microbial diversity of the mangrove habitat is significant, representing different actinomycete species with diverse morphological and cultural characteristics.

2.3.2. Primary/qualitative screening of actinomycetes

Out of the twelve actinomycete cultures screened, three were judged to be active producers of α-galactosidase on the basis of their growth on screening plates and appearance of halo zones around the colonies. The cultures developed halo zones around the colonies due to the secretion of the enzyme outside the cells, indicating the extracellular localization of α-galactosidase. The results of the primary screening experiments are summarized in Table 2.2.

Table 2.2. Primary screening of actinomycete cultures for α-galactosidase activity on BCP agar plates and MU-α-gal

Culture code	Clear zone	
	BCP agar	MU-α-gal
AGP 40	-	-
AGP 41	-	-
AGP 42	+++	+++++
AGP 43	-	-
AGP 44	-	-
AGP 45	-	-
AGP 46	+	++
AGP 47	++	+++
AGP 48	-	-
AGP 49	-	-
AGP 53	-	-
AGP 55	-	-

The '-' symbol indicates the absence of halo zone.

The '+' symbol indicates the presence of halo zone; the number of '+' symbol indicates the intensity of α-galactosidase activity.

BCP medium is widely used for the detection of melibiose fermentation (Boucher et al. 2002, 2003; Labrie et al. 2005). Melibiose is one of the natural substrates for the enzyme α-galactosidase, which gives it the name melibiase. The BCP medium contains the pH indicator bromocresol purple which indicator gives yellow and blue/purple colours under acidic and alkaline conditions respectively. The tested actinomycete cultures produced colonies with purple/yellow halo zones indicating the

utilization of melibiose and hence production of α-galactosidase. Fig. 2.1a shows the purple halo zone on BCP medium with the culture coded AGP 42, which gave better results compared to the other α-galactosidase active cultures. MU-α-gal is the most sensitive substrate which can detect even traces of α-galactosidase. The hydrolysis of MU-α-gal by α-galactosidase liberates a fluorescent product methyl umbelliferone, which can be detected by its fluorescence under a UV transilluminator. Fig. 2.1b shows the fluorescent halo zone around the culture AGP 42 with MU-α-gal.

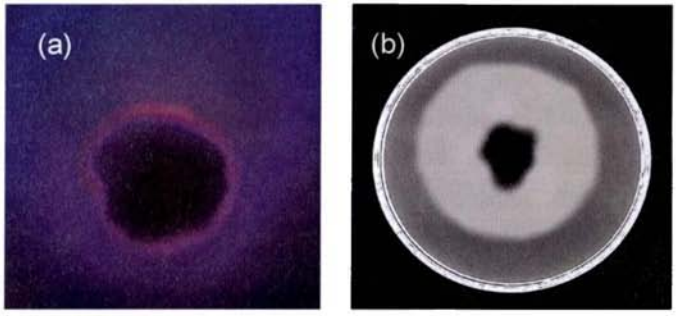


Fig. 2.1. The actinomycete culture coded AGP 42 showing (a) purple halo zone on BCP agar medium, and (b) fluorescent halo zone with MU-α-gal

2.3.3. Secondary/quantitative screening of actinomycetes

The α-galactosidase positive cultures were grown by submerged fermentation and quantitatively screened by *p*NPG assay method in order to identify the culture producing the highest enzyme titre. The *p*NPG assay for α-galactosidase presents the advantages of specificity of the enzyme for the substrate and product stability, as well as convenience of assay (Kew & Douglas 1976). Out of the three α-galactosidase active cultures, two cultures coded AGP 42 and AGP 47, which gave wider halo zones, were selected for quantitative screening by submerged fermentation to find out the best producer of α-galactosidase. Both the tested strains could produce α-galactosidase in submerged fermentation and AGP 42 gave the highest α-galactosidase titre than AGP 47 (Table 2.3). The α-galactosidase produced was secretory since the enzyme activity was obtained in the culture supernatant. This confirmed the extracellular localization of α-galactosidase in the tested cultures. Many of the reported α-galactosidases are either

intracellular or cell-bound. The extracellular nature of enzyme contributes towards process economy since it avoids the expenses related to extensive procedures for the extraction of intracellular enzymes.

Different levels of α-galactosidase were formed in different media and highest activity was obtained in Medium I, which contained 1% locust bean gum as the carbon source. Media II and III containing soybean meal and melibiose, respectively also showed good α-galactosidase titres (Table 2.3). The specific activity also was highest in Medium I in case of both the cultures. The highest production of α-galactosidase in Medium I occurred because of more profuse growth of the culture in it. The results suggested that more biomass was formed in Medium I and hence more α-galactosidase. It has already been established that the microbial production of α-galactosidase varies with growth rate (Giuseppin et al. 1993) and α-galactosidase activity increases with increase in biomass concentration (Zeilinger et al. 1993).

The α-galactosidase titre of AGP 42 was higher than that of *Monascus pilosus* (Wong et al. 1986), *Humicola* sp. (Kotwal et al. 1995) and *Streptomyces erythrus* (Elshafei et al. 2001). However the activity was less than that reported by Jin et al. (2001) from *Bacillus* sp. JF strain.

Table 2.3. Fermentation profile of the actinomycete cultures AGP 42 and AGP 47 in three different screening media under submerged fermentation

Parameter considered	AGP 42			AGP 47		
	Medium I	Medium II	Medium III	Medium I	Medium II	Medium III
α-Galactosidase yield (U/mL)	17.4	3.7	3.6	0.6	0.3	0.2
Total soluble protein (mg/mL)	0.94	1.4	0.4	0.5	1.6	0.6
Specific activity (U/mg protein)	18.6	2.6	8.3	1.2	0.2	0.3
Biomass ($OD_{600\,nm}$)	2.8	2.1	1.4	1.8	1.2	0.7

The values given correspond to the hour of maximum enzyme production

Actinomycetes have already been reported to produce α-galactosidase. Suzuki et al. (1966) reported a α-galactosidase from actinomycetes that could decompose raffinose. Lyons et al. (1969) tested α-galactosidase activity in twenty genera of the order *Actinomycetales* and found out that most of the producers of extra cellular α-galactosidase belong to the genus *Streptomyces*. He reported that members of the genera *Actinoplanes, Micromonospora and Promicromonospora* also exhibited α-galactosidase activity. Elshafei et al. (2001) reported *Streptomyces erythrus* as an active producer of α-galactosidase.

2.3.4. Identification and characterization of the selected strain

The actinomycete culture coded AGP 42 was evaluated as the best strain in terms of α-galactosidase yield and was used for subsequent studies. The culture was identified as *Streptomyces griseoloalbus* by, Institute of Microbial Technology, Chandigarh, India and deposited in Microbial Type Culture Collection (MTCC) with accession number 7447. The morphological, physiological and biochemical characteristics of *S. griseoloalbus* are summarized in Table 2.4. The morphological characteristics of *S. griseoloalbus* colonies on SCA plate is shown in Fig. 2.2a. The light microscopic (Fig. 2.2b) and scanning electron micrographic (Fig. 2.2c) studies of *S. griseoloalbus* revealed that it is a thin filamentous actinobacterium with chains of conidiospores being formed from the tip of the mycelia. In the growth cycle of a typical actinomycete, the spores bud into long filamentous hyphae which grow in and on the nutrient surface (say a plate of agar). The hyphae undergo branching and a dense mycelium is gradually formed. This phase is usually referred to as the vegetative growth phase. A typical streptomycete filament, such as *S. coelicolor*, is less than 1 μm in diameter and can grow to lengths of 50–100 μm in this phase. The filamentous growth of actinomycetes is strongly conditioned by both the elastic properties of cellular walls and the physical properties of the environment (Goriely & Tabor 2003).

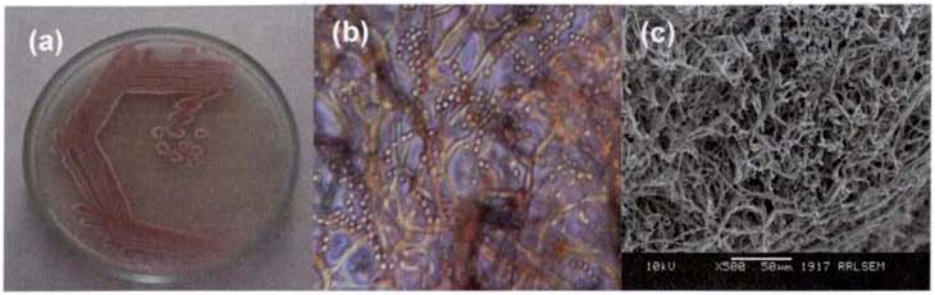


Fig. 2.2 (a) Morphology of the growth of *Streptomyces griseoloabus* on SCA plate, (b) Light microscopic view of *S. griseoloalbus* showing chains of conidiospores being formed from the tip of branching filamentous hyphae, (c) Scanning electron micrograph showing the filamentous nature of *S. griseoloalbus*

Table 2.4. Morphological, physiological and biochemical characteristics of *Streptomyces griseoloalbus*

Characteristics studied	Observation
Morphology of colony	Gram positive filaments with bright pink substrate mycelium and pink aerial mycelium forming chains of spores
Growth temperature	30-37 °C
Growth pH	4-12
Growth on NaCl	2-4%
Growth under anaerobic condition	Nil
Biochemical characteristics	Catalase positive, hydrolyses xanthine, casein, starch and citrate
Carbohydrate utilization	Utilizes L-arabinose, galactose, mannitol, raffinose, rhamnose, xylose

2.3.5. Enzyme profile of *Streptomyces griseoloalbus*

In addition to α-galactosidase *S. griseoloalbus* is a treasure source of other commercially important enzymes like β-galactosidase, xylanase, β-xylosidase, β-mannosidase, protease and invertase. But the culture did not produce β-glucosidase and cellulase. Significant reduction in the viscosity of the locust bean gum-based fermentation medium was visually noticed after 24 h of incubation. Since β-

mannosidase was secreted into the medium along with α-galactosidase, complete hydrolysis of locust bean gum could be possible by the synergistic action of these enzymes. Concerted action of mannosidase and α-galactosidase in the hydrolysis of polymeric galactomannans was previously reported by other workers (Zeilinger et al. 1993; Duffaud et al. 1997).

Table 2.5. Enzyme profile of *Streptomyces griseoloalbus*

Enzyme	Yield (U/mL)
β-Galactosidase	0.26
β-glucosidase	0
Cellulose	0
Xylanase	15.3
β-Xylosidase	4.2
β-Mannosidase	16.9
Acidic protease	1.2
Alkaline protease	0.89
Neutral protease	0.32
Invertase	3.2

2.4. CONCLUSION

S. griseoloabus isolated from mangrove sediment soil sample was evaluated as the best among the twelve actinomycete cultures screened in view of its high α-galactosidase titre. Marine and mangrove micro-organisms, by virtue of their unique nature, differ very much in many aspects from their terrestrial counterparts and hold immense potential to produce diverse spectra of useful substances including enzymes. In this context, the results obtained during the course of this study indicate the scope for utilization of mangrove actinomycetes for extracellular α-galactosidase production.

Chapter III

OPTIMIZATION OF α-GALACTOSIDASE PRODUCTION BY *STREPTOMYCES GRISEOLOALBUS* IN SUBMERGED FERMENTATION

3.1. INTRODUCTION

The production and characterization of an enzyme are necessary for its industrial application. The first step in achieving this goal is the establishment of a suitable enzyme production technology. The majority of microbial enzymes in current industrial use are produced under conventional aerobic submerged fermentation (SmF), owing to its advantages such as purity of the product, greater control of growth conditions and scale-up processes (Wiseman 1985; Chandrasekaran 1997). SmF is the cultivation of organisms in liquid culture media. Although SmF process appears to be identical for the production of all enzymes, there is a need for optimization of process parameters such as pH, temperature, nutrients, incubation time etc. This is mainly due to the variation in the performance of the strain and the nature of the enzyme desired.

The conventional method of medium optimization involves changing one independent variable at a time while fixing all the others at a certain level. This method is not only very tedious, but also unreliable as it overlooks the interactive effects among the variables (Dey et al. 2001). Statistical optimization by response surface methodology (RSM) is preferable because it is helpful in evaluating the interactions among the possible influencing parameters with limited number of experiments (Francis et al. 2003). It involves a specific design of experiments, which minimizes the error in determining the effect of parameters, and the results are achieved in an economical manner. However, 'one-variable-at-a-time' approach can be useful in determining the suitable operational intervals for the significant variables prior to response surface studies.

Immobilization of microbial cells is yet another approach widely used in industrial biotechnology for enhancing the production of desired products. Self-immobilization of micro-organisms as films, flocs and pellets is a widespread

phenomenon in nature. The intentional use of immobilized cells in bioprocesses, however, is a relatively recent development. Biochemical processing with immobilized microbial cells offers a number of unique advantages over traditional fermentation processes with free cells, such as relative ease of cell mass separation from the bulk liquid for possible re-use, prevention of washout, reduced risk of contamination and operational stability. Furthermore, using the entrapment technique, a dense cell culture can be established leading to improved productivity (Kourkoutas et al. 2004). This has triggered a surge of research activity in this exciting and rapidly growing field. The biotransformational and biosynthetic abilities of immobilized growing microbial cells for the production of diverse valuable products like antibiotics, organic acids, enzymes and alcohols have been well demonstrated. Proper selection of immobilization techniques and supporting materials is needed to minimize the disadvantages of immobilization (Ramakrishna & Prakasham 1999). One of the most suitable methods for cell immobilization is entrapment in calcium alginate, because this technique is simple and cheap. Sodium alginate is a readily available non-toxic biological material and is therefore suitable as an immobilization matrix for bio-molecules and micro-organisms (Mattiasson et al. 1984). Beads of calcium alginate prepared under mild conditions have been used extensively for microencapsulating and entrapping cells (Jamuna & Ramakrishna 1992).

Optimization of α-galactosidase from different microbial sources in SmF by conventional (Kotwal et al. 1995; Li et al. 1997; Elshafei et al. 2001; Jin et al. 2001; Rezessy-Szabo et al. 2003; Gote et al. 2004) as well as response surface methodology (Liu et al. 2007a) has been reported by several workers. Although much interest has been focused on the use of immobilized *Streptomyces* sp. for the production of extracellular hydrolytic enzymes such as xylanases (Beg et al. 2000) and pectinases (Kuhad et al. 2004), no reports have appeared in the literature documenting the use of cell immobilization technique for the production of α-galactosidase. This chapter describes the optimization of process parameters for the production of α-galactosidase from *Streptomyces griseoloalbus* in SmF by applying statistical methods. The

enhancement of α-galactosidase production by employing whole cell immobilization is also reported.

3.2. MATERIALS AND METHODS

3.2.1. Inoculum preparation

Inoculum was prepared by transferring a loopful of culture from fresh SCA slants into sterile medium (100 mL in 250-mL Erlenmeyer flask) composed of (g/L): locust bean gum, 10; yeast extract, 3; $(NH_4)_2SO_4$, 3.03; $MgSO_4.7H_2O$, 0.49; and 1 mL of trace elements solution. The trace elements solution composed of (g/L): $FeSO_4.7H_2O$, 0.1; $MnCl_2.4H_2O$, 0.1 and $ZnSO_4.7H_2O$, 0.1. The flasks were incubated at 30 °C on a rotary shaker at 150 rev/min. A 48 h old culture containing 3 x 10^6 CFU/mL was used as the inoculum.

3.2.2. Medium and fermentation conditions

The medium used for SmF was the one selected (Medium I) based on the preliminary studies mentioned in Chapter II. The 250-mL Erlenmeyer flasks containing 90 mL of the medium were inoculated (10%, v/v, 3 x 10^7 CFU) and were incubated in a rotary shaker for 7 days at 30 °C and 150 rev/min. Aliquots were withdrawn at regular time intervals of 24 h and the culture supernatant obtained after centrifugation at 10,000 rpm for 20 min was used as the enzyme preparation.

3.2.3. Evaluation of carbon sources

Various carbon sources such as Locust bean gum (LBG), Guar gum (GG), Gum arabic (GA), Soymeal (SM), Soyflakes (SF), Soy flour (SFlr), Chick pea flour (CPF), Wheat bran (WB) (1%, w/v), were supplemented in the basal medium to study their effects on α-galactosidase production.

3.2.4. Screening of physico-chemical factors using Plackett-Burman design

Plackett-Burman experimental design (Plackett & Burman 1946) consisting of a set of 12 experiments was used to determine the relative significance of 11 factors that influenced α-galactosidase production by *S. griseoloalbus* in SmF. The complete

experimental design is shown in Table 3.1. The factors or independent variables considered for study included 6 physical factors (X_1 to X_6, representing pH, temperature, inoculum size, inoculum age, incubation period and agitation respectively) and 5 nutritional factors (X_7 to X_{11}, representing carbon source, yeast extract, $MgSO_4$, $FeSO_4$ and salinity respectively). All variables except the carbon source were numerical factors and were investigated at two widely spaced levels designated as –1 (low level) and +1 (high level). The carbon source was studied as a categorical factor and the two carbon sources (1%, w/v) studied were locust bean gum (LBG) and chick pea flour (CPF). The level of salinity was varied by varying the amount of sea water (having a salinity of 35 g/L) and distilled water in the medium. The operational intervals of pH, temperature, inoculum size, agitation and salinity were selected based on the previous experience with 'one-variable-at-a-time' approach.

Table 3.1. Plackett-Burman experimental design matrix with α-galactosidase production by *S. griseoloalbus* in SmF

Trial no.	Variables											α-Galactosidase yield (U/mL)[a]
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
1	+1	+1	-1	+1	-1	-1	LBG	+1	+1	+1	-1	0.96
2	-1	-1	-1	+1	+1	+1	LBG	+1	+1	-1	+1	2.75
3	+1	+1	-1	+1	+1	-1	CPF	-1	-1	-1	+1	23.7
4	-1	+1	+1	-1	+1	-1	LBG	-1	+1	+1	+1	14.1
5	-1	-1	-1	-1	-1	-1	LBG	-1	-1	-1	-1	2.3
6	-1	+1	-1	-1	-1	+1	LBG	+1	-1	+1	+1	25.08
7	-1	-1	+1	+1	+1	-1	CPF	+1	-1	+1	-1	3.06
8	-1	+1	+1	+1	-1	+1	CPF	-1	+1	-1	-1	6.2
9	+1	+1	+1	-1	+1	+1	CPF	+1	-1	-1	-1	13.1
10	+1	-1	+1	+1	-1	+1	LBG	-1	-1	+1	+1	24.4
11	+1	-1	-1	-1	+1	+1	CPF	-1	+1	+1	-1	4.6
12	+1	-1	+1	-1	-1	-1	CPF	+1	+1	-1	+1	8.9

-1, low level; +1, high level; LBG, locust bean gum; CPF, chick pea flour; The variables X_1, X_2, X_3,.....X_{11} respectively represent pH (5 and 7), temperature (27 °C and 33 °C), inoculum size (4.5 x10^5 CFU and 1.5 x10^6 CFU), age of inoculum (48 h and 96 h), incubation period (72 h and 120 h), agitation (125 rev/min and 175 rev/min), carbon source as a categorical variable, yeast extract (2 g/L and4 g/L), $MgSO_4$ (0 g/L and 0.98 g/L), $FeSO_4$ (0 g/L and 2 x10^{-5} g/L), salinity (9.6 g/L and 19.2 g/L)

[a]Values are the mean of three replicate experiments.

The design matrix (Table 3.1) for Plackett-Burman design was obtained using the software Design-Expert® (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA). A statistical analysis was performed to find the impact of each parameter on the process. The effect of parameters was estimated as the difference between the mean responses at high and low levels of each variable as follows:

$$E = (\Sigma M_+ - \Sigma M_-)/N \qquad \text{Eq. (1)}$$

where E is the effect of parameter under study and M_+ and M_- are the responses (α-galactosidase activities) of trials at which the parameter was at its higher and lower levels respectively and N is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on α-galactosidase production. Significant variables were then identified using a regression analysis.

3.2.5. Effect of different concentrations of LBG

The influence of different concentrations of LBG, which was found out as the best substrate for α-galactosidase production in the preliminary screening of substrates as well as in Plackett-Burman design, was examined by varying its concentration from 0.1 to 5.0% (w/v).

3.2.6. Optimization of significant variables using Box-Behnken design

Response surface methodology using Box-Behnken design (Box & Behnken 1960) was adopted to find the optimum levels of the significant variables (salinity, $MgSO_4$ and temperature) and the effects of their mutual interactions on enzyme production. A total of 17 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as –1, 0 and +1 respectively). The center point of the design was replicated five times for the estimation of error. The experimental design used for the study is shown in Table 3.2. Each run was performed in triplicate and the average of α-galactosidase yield obtained was taken as the experimental values of the dependent variable or response (Y), while predicted

values of the response were obtained from quadratic model fitting techniques. A multiple regression analysis of the data was carried out to define the response in terms of the independent variables. The behavior of the system was explained by a second order polynomial equation. The model equation used for the analysis is given below:

$$Y = \beta_0 + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_i X_j \qquad \text{Eq. (2)}$$

where, Y is the predicted response; β_0 is the offset term; β_i is the linear effect; β_{ii} is the squared effect and β_{ij} is the interaction effect.

For a three variable system the model equation is given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \qquad \text{Eq. (3)}$$

Table 3.2. Box-Behnken design matrix with experimental and predicted values of α-galactosidase production by *S. griseoloalbus* in SmF

Trial no:	Variables			α-Galactosidase yield (U/mL)[a]	
	Salinity (g/L)	$MgSO_4$ (g/L)	Temperature (°C)	Experimental	Predicted
1	19.2 (0)	0 (-1)	27 (-1)	27.8	28.5
2	19.2 (0)	0.13 (0)	30 (0)	35.6	35.6
3	19.2 (0)	0.25 (+1)	33 (+1)	33.8	33.1
4	24.9 (+1)	0.25 (+1)	30 (0)	35.9	36.1
5	19.2 (0)	0.13 (0)	30 (0)	35.8	35.6
6	24.9 (+1)	0.13 (0)	27 (-1)	28.6	28.7
7	13.4 (-1)	0.13 (0)	27 (-1)	28.1	27.5
8	19.2 (0)	0.13 (0)	30 (0)	35.7	35.6
9	19.2 (0)	0.13 (0)	30 (0)	35.5	35.6
10	13.4 (-1)	0.13 (0)	33 (+1)	31.4	31.2
11	13.4 (-1)	0 (-1)	30 (0)	34.3	34.1
12	19.2 (0)	0.13 (0)	30 (0)	35.2	35.6
13	24.9 (+1)	0.13 (0)	33 (+1)	33.4	34.0
14	19.2 (0)	0 (-1)	33 (+1)	32.6	33.0
15	24.9 (+1)	0 (-1)	30 (0)	35.2	34.3
16	13.4 (-1)	0.25 (+1)	30 (0)	31.4	32.4
17	19.2 (0)	0.25 (+1)	27 (-1)	29.05	28.6

[a]Values are the mean of three replicate experiments.

The coded values of variables are given in parentheses.

The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum α-galactosidase production. The software Design-Expert® (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building.

The statistical model was validated with respect to all three significant variables within the design space. A random set of six experimental combinations under the optimized conditions was used for validation of the statistical model.

3.2.7. Whole cell immobilization

The preparation of inoculum was the same as mentioned above in Section 3.2.1. The mycelial cells in 50 mL of inoculum (approximately 5.8 g of cells, wet wt.) were aseptically harvested by centrifugation (8000 rpm, 10 min). The pellet was washed and re-suspended in 5 mL of saline solution (0.85% NaCl, w/v) and used for immobilization. The cells were mixed thoroughly with 10 mL of sodium alginate solution (final concentration 3%, w/v) and the mixture obtained was extruded drop-wise through a syringe into 500 mL of 0.1 M $CaCl_2$ solution. The beads were allowed to harden in this solution for 2 h at 4 °C and were then washed with saline solution to remove excess of $CaCl_2$ and free cells. The average diameter of beads was 4 mm. The calcium alginate beads with immobilized cells of *S. griseoloalbus* were then used for SmF. The whole procedure of immobilization was carried out under sterile conditions. Unless otherwise specified, the parameters of immobilization were kept constant.

3.2.7.1. Optimization of immobilization parameters

For the preparation of beads with proper permeability and rigidity, parameters such as sodium alginate concentration and the molarity of the calcium chloride need to be optimized. For this purpose, varying concentrations of sodium alginate (1- 4%, w/v) and $CaCl_2$ (0.03- 0.15 M) were used. The initial biomass that can be loaded in the beads was investigated by incorporating different amounts of cells in the aqueous sodium alginate solution. For this purpose different volumes of inoculum were used for

immobilization, such as 10, 20, 30, 40, 50 and 60 mL. The wet weight of cells in 10 mL of inoculum was approximately 1.1 g and wet-to-dry weight ratio for the cells was approximately 1: 0.1. The beads were cured for varying periods (2- 24 h) at 4 °C to check the time requirement for proper curing of the beads.

3.2.8. Comparison of α-galactosidase production by free and immobilized cells

The immobilized cells obtained as above were used to inoculate 250-mL Erlenmeyer flasks containing 90 mL of the production medium. The composition of production medium was the same as that optimized for α-galactosidase production with free cells, except that 0.5 g/L of $CaCl_2$ was added in order to keep the beads intact during prolonged operation. The sea water was excluded from the medium since the phosphates present in the sea water may destabilize the calcium alginate beads. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also conducted. Batch fermentations with free and immobilized cells were carried out in a rotary shaker for 120 h at 30 °C and 175 rev/min. Aliquots were withdrawn at regular time intervals of 24 h and the culture supernatant obtained after centrifugation at 10,000 rpm for 20 min at 4 °C was used as the enzyme preparation.

The effectiveness factor of the immobilized system was defined as the ratio of α-galactosidase activity of the immobilized system to that of the free cells:

$$\text{Effectiveness factor} = C_{imm}/C_{free} \qquad \text{Eq. (4)}$$

where C_{imm} is the α-galactosidase yield produced by immobilized cells and C_{free} is the α-galactosidase yield produced by free cells.

3.2.9. Repeated batch fermentation with immobilized cells

Optimized conditions of immobilization were selected for effective cell encapsulation in repeated batch fermentation. Repeated batch fermentation was conducted by running each fermentation cycle up to 72 h. At the end of each fermentation cycle, the beads collected from the spent medium were washed with

sterile saline solution and transferred aseptically to fresh production medium to continue the fermentation.

The operational stability of the immobilized system was determined by the following equation:

$$\text{Operational efficiency (\%)} = 100 \times (C_x/C_1) \qquad \text{Eq. (5)}$$

where C_1 is the α-galactosidase yield produced in the 1st fermentation cycle and C_x is the α-galactosidase yield produced in the xth fermentation cycle.

3.2.10. Cell growth and cell leakage

The biomass was estimated by measuring the turbidity of the culture broth as increase in the absorbance at 600 nm. The corresponding cell dry weight (CDW) was also determined after it had been dried to constant mass in an oven at 80 °C. The CDW values were plotted as a standard curve against optical density at 600 nm. Both cell growth in freely suspended cultures and cells leaked from the gel matrix of immobilized cell beads were determined as CDW by measuring the optical density at 600 nm. The optical density value was then converted into mg CDW/mL using a standard curve.

3.2.11. Analytical procedures

The α-galactosidase assay was carried out as mentioned in Section 2.2.4 of Chapter II. The salinity of sea water was measured either using salinometer or by weighing the salts in 1 mL of sea water after evaporating it to dryness.

3.3. RESULTS AND DISCUSSION

3.3.1. Evaluation of carbon sources

A number of different carbon sources were tested and α-galactosidase production was monitored during the fermentation. The results are summarized in Fig. 3.1. Among the different substrates tested LBG gave the highest α-galactosidase activity. Micro-organisms cannot transport macromolecular substrates across the cell membrane, so the macromolecule must be hydrolyzed externally into soluble units,

which can be transported into the cell. This requires synthesis and secretion of the necessary extra-cellular enzymes. Since polymeric substrates cannot themselves enter the cell they cannot be as such the inducers of the enzyme responsible for their hydrolysis. The enzymes may be produced constitutively at very low levels and produce low concentrations of low molecular weight degradation products which then act as inducers (Singh et al. 2003; Moreira & Filho 2008). LBG is a polymeric galactomannan consisting of a straight chain β-mannan with galactose side chains on every fourth mannose residue. The action of α-galactosidase on LBG releases galactose which serves as an inducer of further α-galactosidase biosynthesis. Analogous results were reported by Zeilinger et al. (1993) with *Trichoderma reesei* RUT C-30. It is also reported that galactose is a good inducer of α-galactosidase production (Wong et al. 1986).

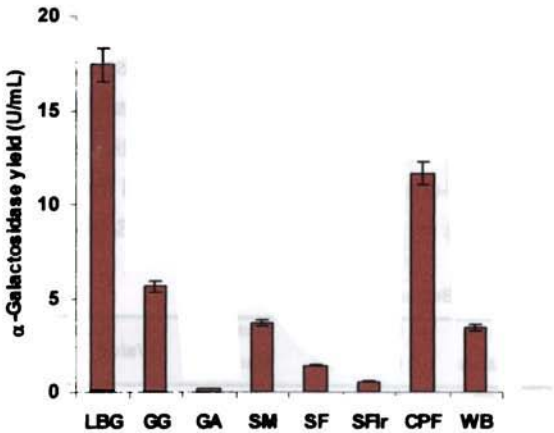


Fig. 3.1. Screening of different carbon sources for α-galactosidase production in SmF. LBG- Locust bean gum,GG- Guar gum, GA- Gum arabic, SM- Soymeal, SF- Soyflakes, SFlr- Soy flour, CPF- Chick pea flour, WB- Wheat bran

Other legume-based substrates like CPF and soy derivatives also could support substantial α-galactosidase formation. These legume-based substrates are good sources of galactose containing oligosaccharides like raffinose and stachyose,

the hydrolysis of which liberates galactose. These results suggested that carbohydrates containing α-galactosidic linkages are good inducers of α-galactosidase production. The induction of α-galactosidase was most effective with LBG as the growth substrate. The comparison revealed that the α-galactosidase synthesis on LBG was 1.5 times higher than on CPF. Though GG contains more galactose residues than LBG, α-galactosidase formation on this substrate was considerably less. This was because the length and more complex nature of this polymeric galactomannan hindered the complete hydrolysis of the galactosyl residues. It was reported that α-galactosidase of *Bacteroides ovatus* (Gherardini et al. 1985) could not hydrolyze soluble guar gum but were active toward the species of smaller molecular weight which were found after its cultivation on GG.

3.3.2. Screening of physico-chemical factors using Plackett-Burman design

The Plackett-Burman experimental design used for the screening of physico-chemical factors influencing α-galactosidase production by *S. griseoloalbus* along with the corresponding experimental and predicted values of response is shown in Table 3.1. The Plackett-Burman experimental design is a two-level fractional factorial design, which identifies the critical physico-chemical parameters required for elevated enzyme production by screening *n* variables in *n*+1 experiments (Plackett & Burman 1946).

Table 3.3. ANOVA for Plackett-Burman design

Source	Sum of squares	Degree of freedom	Mean square	F-Value	*Prob>F*
Model	875.63	6	145.93	13.96	0.0055
pH (X_1)	40.64	1	40.64	3.88	0.1057
Temperature (X_2)	115.72	1	115.72	11.07	0.0208
Agitation (X_6)	44.75	1	44.75	4.28	0.0933
Yeast extract (X_8)	38.64	1	38.64	3.69	0.1125
$MgSO_4$ (X_9)	243.30	1	243.30	23.27	0.0048
Salinity (X_{11})	392.55	1	392.55	37.55	0.0017

The adequacy of the model was tested and the parameters with statistically significant effects were identified using the Fischer's test for ANOVA (Table 3.3). The

ANOVA for the selected fractional factorial model showed that the model was significant with F-value of 13.96. The *Prob*>F value is used as a tool to check the significance of each variable. A *Prob*>F ≤ 0.0500 indicates that the effect of the parameter in question can be considered as significant at 95% confidence level. The *Prob*>F value of the model was 0.0002, which also confirmed that the model was highly significant. The variables temperature (X_2), $MgSO_4$ (X_9) and salinity (X_{11}) were found to be statistically significant in influencing α-galactosidase production, whereas all the other variables were insignificant.

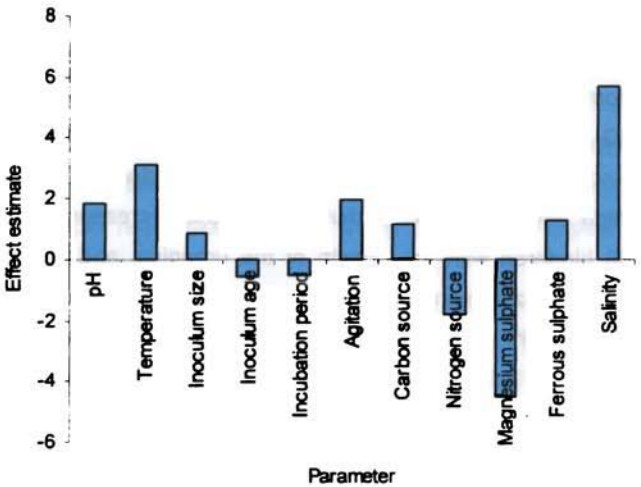


Fig. 3.2. Effect of different operational variables on α-galactosidase production in SmF

The coefficient estimate for the different variables is shown in Fig. 3.2. The magnitude of the coefficient of each variable indicated the intensity of its effect on the studied response. The greater the magnitude, the higher was the significance of that variable. Thus salinity had the highest influence on α-galactosidase production compared to the other factors. This is probably because higher salinity is essential for the normal growth of the actinomycete *S. griseoloalbus*, which was originally isolated

from mangrove soil sample. Salinity of the medium was adjusted by varying the amount of sea water and distilled water rather than adding NaCl because the sea water is rich in numerous micronutrients other than NaCl, which can promote the growth of mangrove and marine micro-organisms. The positive or negative sign of the coefficient of a tested variable indicates whether an increase in the level of that tested variable enhanced or inhibited α-galactosidase production within the tested limits. The variables pH, temperature, inoculum size, agitation, carbon source, $FeSO_4$ and salinity had a positive effect on α-galactosidase production, while the rest had a negative influence.

3.3.3. Effect of different concentrations of LBG

To get a more detailed information about the effect of concentration of LBG on α-galactosidase formation, enzyme activity was tested in media with 0.1, 0.5, 1, 2, 3, 4 and 5% (w/v) LBG. The results are shown in Figure 3.3. As seen in the graph, 0.1 and 0.5% LBG were not sufficient to result highest enzyme production. At concentrations above 1%, the high viscosity of the medium due to the decreased solubility of LBG (Glicksman 1969) hindered with the growth of the organism and hence the growth sufficient for enhanced enzyme production was delayed. The results suggested that 1% LBG was most suitable for highest α-galactosidase production by *S.griseoloalbus*.

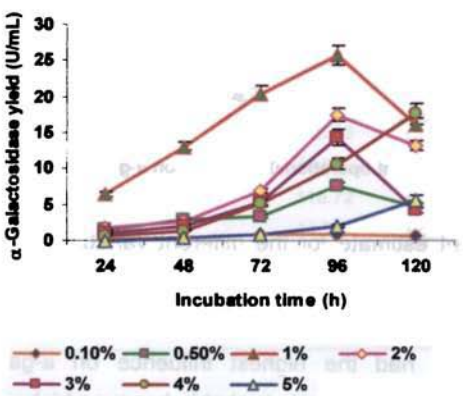


Fig. 3.3. Effect of different concentrations of LBG on α-galactosidase production

3.3.4. Optimization of significant variables using Box-Behnken design

The Plackett-Burman design does not consider the interaction effects among the variables. The variables that are found significant in this initial screening can be further optimized using RSM. Based on the results of screening experiments by Plackett-Burman design, those variables with *Prob*>F value lower than 0.0500 (Salinity, $MgSO_4$ and temperature) were selected and further optimized using Box-Behnken design. All the non-critical factors were fixed at a particular level mostly that which has produced the maximum response in the screening experiment or sometimes at their median values. The optimum values thus fixed were locust bean gum 1% (w/v), pH 6.0, incubation temperature 30 °C, inoculum size 1.5 x 10^6 CFU, inoculum age 48 h, agitation 175 rev/min, yeast extract 4 g/L, $FeSO_4$ 2 x10^{-5} g/L and incubation period 96 h. The Box-Behnken design along with the corresponding experimental and predicted values of the α-galactosidase yield and biomass is given in Table 3.2. The data were analyzed by multiple regression analysis using the Design-Expert software and the following equation was obtained:

$$Y = 35.6285 + 0.9827\,x_1 + 0.0297\,x_2 + 2.2344\,x_3 - 0.9096\,x_1^2 - 0.4694\,x_2^2 - 4.298\,x_3^2 + 0.8991\,x_1x_2 + 0.3866\,x_1x_3 - 0.0113\,x_2x_3 \quad \text{Eq. (6)}$$

where Y is the predicted value of α-galactosidase yield and x_1, x_2 and x_3 are the coded values for salinity, $MgSO_4$ concentration and temperature, respectively.

The experimental data were statistically analyzed by analysis of variance (ANOVA) and the results are shown in Table 3.4. The ANOVA of the quadratic regression model indicated that the model was highly significant, as the F-value for the model was 24.67. There was only 0.02% chance that the 'model F-value' this large could occur due to noise. The *Prob*>F value of the model was 0.0002, which also confirmed that the model was highly significant. The *Prob*>F values (Table 3.4) suggested that among the independent variables studied, salinity and temperature as well as the squared terms of these two variables had a significant effect on α-galactosidase production by *S. griseoloalbus*.

Table 3.4. ANOVA for the response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	*Prob>F*
Model	137.1738	9	15.24153	26.01593	0.0002
x_1	7.7261	1	7.726184	13.1879	0.0095
x_2	0.0070	1	0.007057	0.012045	0.9179
x_3	39.9407	1	39.94079	68.17534	< 0.0001
x_1^2	3.4836	1	3.483672	5.946315	0.0493
x_2^2	0.9278	1	0.927831	1.583724	0.2600
x_3^2	77.7820	1	77.78204	132.7669	< 0.0001
$x_1 x_2$	3.234	1	3.234063	5.520254	0.0560
$x_1 x_3$	0.5979	1	0.597993	1.02072	0.3580
$x_2 x_3$	0.0005	1	0.000518	0.000883	0.9777

The coefficient of variation (CV), indicative of the degree of precision with which the treatments are compared, had a lower value (2.38%), showing greater reliability. Also, the multiple regression coefficient (R^2) had a value of 0.9694, indicating that the model could explain up to 96.94% of the variability of response. The value of R^2 (0.9694) indicated a good agreement between the experimental and predicted values of α-galactosidase yield. The signal to noise ratio (adequate precision) for the model was higher than 4 (14.21), indicating a good fit.

The effect of the interaction of various physico-chemical parameters on α-galactosidase production by *S. griseoloalbus* was investigated by plotting the response surface curves against any two independent variables while keeping the third independent variable at the '0' level. Thus three response surfaces were obtained by considering all the possible combinations. The interactive roles of salinity, $MgSO_4$ concentration and temperature on α-galactosidase production by *S. griseoloalbus* are illustrated in the three-dimensional curves of the calculated response surface shown in Figs. 3.4a, b and c.

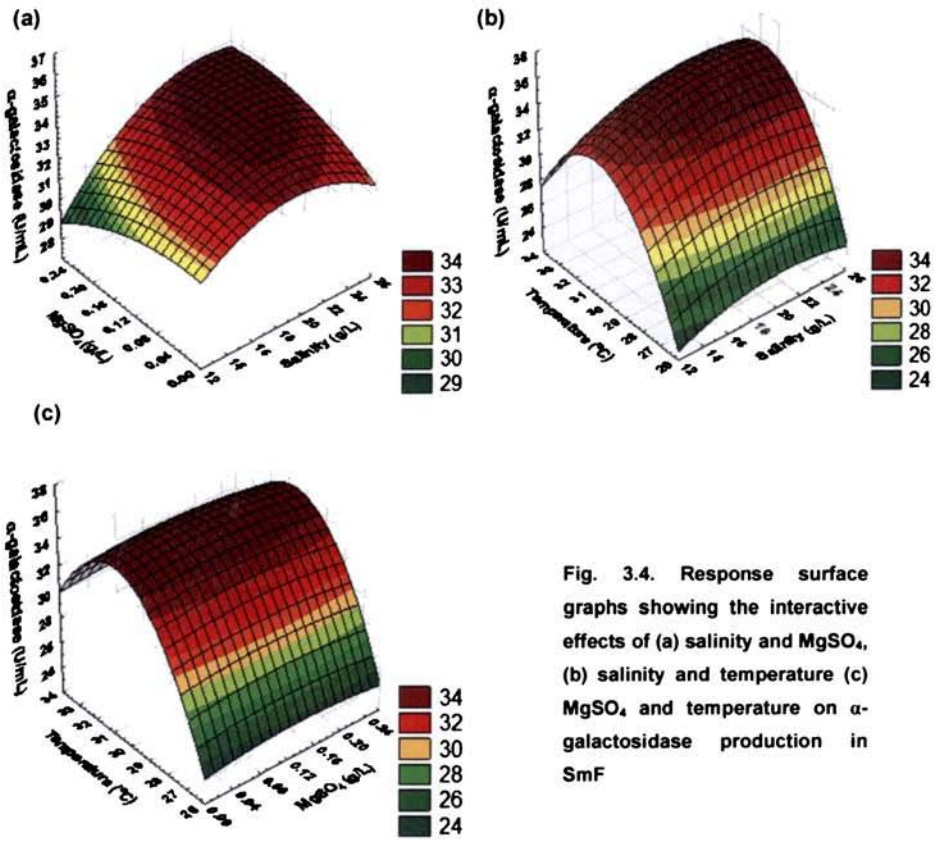


Fig. 3.4. Response surface graphs showing the interactive effects of (a) salinity and $MgSO_4$, (b) salinity and temperature (c) $MgSO_4$ and temperature on α-galactosidase production in SmF

An increase in α-galactosidase production was observed when the salinity was increased together with an increase in the concentration of $MgSO_4$ (Fig. 3.4a), and the highest enzyme activity (35.9 U/mL, Table 3.2) was obtained when the salinity and $MgSO_4$ concentration were 24.9 g/L and 0.25 g/L respectively. Within the tested limits, when the salinity was low, the α-galactosidase yield was also low regardless of the $MgSO_4$ concentration and *vice versa*. In the initial screening experiments using Plackett-Burman design, $MgSO_4$ was found to exhibit a negative effect on α-galactosidase production at the higher tested level (0.98 g/L). The results of final optimization with RSM suggest that a concentration of at least 0.25 g/L of $MgSO_4$ is

necessary for enhanced α-galactosidase production, as the enzyme activity decreased in the absence of $MgSO_4$. In the present study the α-galactosidase production was found to be growth associated since higher activities correlated with higher biomass. Thus it was further emphasized that a relatively higher salinity was essential for the better growth of *S. griseoloalbus*. However using sea water for enzyme production in large scale fermenters is not appreciable as it may have a corroding effect.

The response surfaces in Figs 3.4b and c show the effect of temperature on α-galactosidase production in combination with the other two variables, where it can be seen that the influence of temperature is independent from the other two variables. Growth of the organism and α-galactosidase production increased with the increase in temperature up to the optimum level, after which growth declined and the associated enzyme production also dropped. The effect was the same at all concentrations of sea water and $MgSO_4$. The results indicate that the independent influence of temperature on α-galactosidase production masked the effects of the other two variables.

From the response surface graphs and Table 3.2 it could be concluded that the optimum values of salinity, $MgSO_4$ and temperature for the maximum production of α-galactosidase by *S. griseoloalbus* were 24.9 g/L, 0.25 g/L and 30 °C respectively.

Table 3.5. Validation of response surface quadratic model for α-galactosidase production in SmF

Trial no.	Salinity (g/L)	$MgSO_4$ (g/L)	Temperature (°C)	α-Galactosidase yield (U/mL)[a]	
				Experimental	Predicted
1.	20.5	0.17	30	50.3	36.0
2.	21.0	0.09	30	48.6	36.0
3.	23.8	0.22	31	35.3	36.4
4.	21.6	0.15	30	32.1	36.2
5.	23.4	0.19	30	31.6	36.3
6.	21.9	0.11	30	49.8	36.0

[a]Values are the mean of three replicate experiments.

In order to validate the adequacy of the model, a total of six verification experiments were conducted under various fermentation conditions within the experimental range. The design matrix showing the uncoded values of the independent

variables along with the experimental and predicted values of α-galactosidase yield are given in Table 3.5. The results indicate that the model was satisfactory and could enhance the enzyme yield considerably. Statistical optimization of α-galactosidase production by *S. griseoloalbus* resulted in 194% increase compared to the un-optimized medium.

3.3.5. Optimization of whole cell immobilization in calcium alginate

Optimizing the parameters of immobilization offers the advantage of improving the bead characteristics, such as permeability and rigidity. The effect of sodium alginate concentration on α-galactosidase production by *S. griseoloalbus* and cell leakage is shown in Fig. 3.5a. The medium and fermentation conditions optimized with free cells were used for α-galactosidase production by immobilized *S. griseoloalbus*. The highest α-galactosidase yield was obtained with beads prepared using 3% (w/v) sodium alginate. At higher concentration of sodium alginate the pellet rigidity was improved but the α-galactosidase yield decreased due to diffusional limitations that limited the transfer of nutrients into the gel and enzyme out of the gel into the medium (Ellaiah et al. 2004). On the other hand, beads prepared using lower concentrations of sodium alginate were fragile and the cell leakage into the medium was high. The property of the beads depends upon the concentration and type of alginate as it contains different proportions of mannuronic acid and guluronic acid (Zhang et al. 2000). The optimum concentration of alginate may differ with respect to the organism and the product of interest (Nampoothiri & Pandey 1998).

The effect of $CaCl_2$ concentration on the rigidity of beads is shown in Fig. 3.5b. As in the case of sodium alginate, lower concentration of $CaCl_2$ resulted in increased leakage of cells into the fermentation medium owing to the decreased rigidity of beads. On the other hand, an increase in $CaCl_2$ concentration from 0.03 M to 0.1 M minimized the cell leakage and consequently increased the α-galactosidase yield. A still higher concentration of $CaCl_2$ decreased cell leakage still further, but the enzyme yield was remarkably declined. Considering minimum cell leakage and maximum enzyme yield,

0.1 M $CaCl_2$ was chosen as the optimum for the formation of beads with suitable rigidity and permeability. The mechanical strength of alginate beads is highly dependent on $CaCl_2$ concentration of the gelation solution and the use of concentrated $CaCl_2$ solutions has a higher effect on the efficiency of immobilized system (Konsoula & Liakopoulou-Kyriakides 2006).

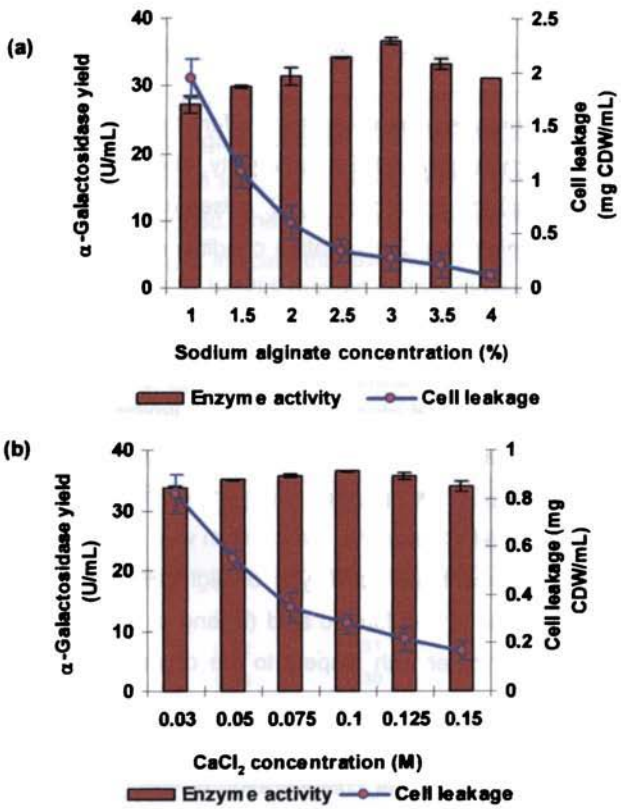


Fig. 3.5. Effect of (a) sodium alginate concentration and (b) $CaCl_2$ concentration on cell leakage and α-galactosidase production by immobilized *S. griseoloalbus*.

Another factor to be considered in the efficiency of immobilized system is the initial biomass that can be loaded in the beads. As the cell loading in the gel beads was increased, the cell leakage into the fermentation medium also increased correspondingly (Fig. 3.6). The α-galactosidase yield increased when the initial biomass in the beads was lowered from 5.8 to 2.2 g wet weight in gel. Though immobilized cell systems are reported to enable the maintenance of high cell densities resulting in higher overall reaction rates and higher product yields, which is not possible with suspension cultures (Brodelius & Vandamme 1987; Konsoula & Liakopoulou-Kyriakides 2006), in the present study the initial biomass of 5.8 g was too high to support highest enzyme yield. The decreased enzyme activity with increase in bead inoculum might be due to competition between cells because of which the nutrient concentration available in the medium might not have been sufficient for optimal growth. Further, the competition between cells would have led to rapid use of substrate resulting in smaller duration of enzyme activity.

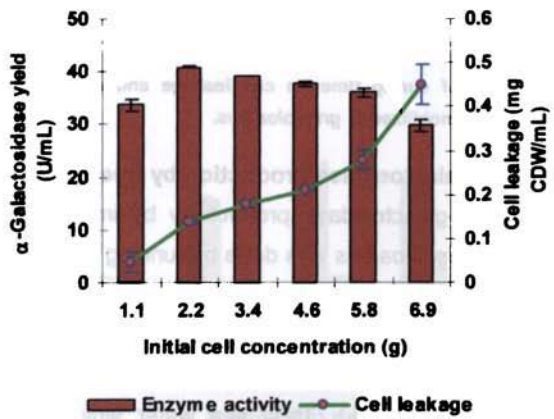


Fig. 3.6. Effect of initial cell biomass on cell leakage and α-galactosidase production by immobilized *S. griseoloalbus*.

Fig. 3.7 shows the effect of curing time on the stability of gel beads. The prolongation of hardening time from 2 h to 10 h led to improved stability of beads, which resulted in decreased cell leakage. The improvement of bead stability and subsequent decrease in cell leakage resulted in 50% more α-galactosidase yield. Further extension of curing period resulted in only slight increase in α-galactosidase production.

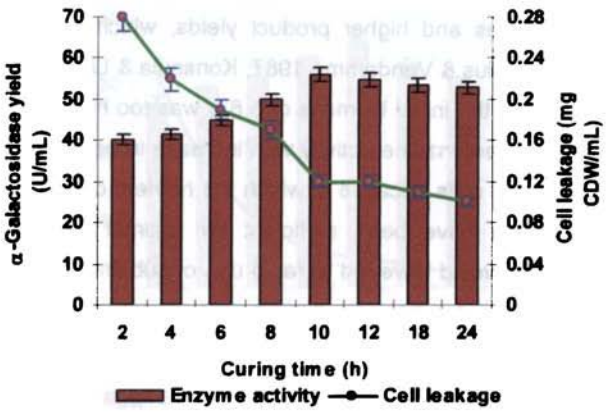


Fig. 3.7. Effect of curing time on cell leakage and α-galactosidase production by immobilized *S. griseoloalbus*.

3.3.6. Comparison of α-galactosidase production by free and immobilized cells

A comparison of α-galactosidase productivity by immobilized and an equal amount of free cells of *S. griseoloalbus* was done by running both the fermentations till 120 h. The calcium alginate entrapment technique led to an increased α-galactosidase yield in comparison to free cell cultures (Fig. 3.8). With alginate-entrapped cells there was a two-fold increase in the enzyme yield (55 U/mL) compared to the highest yield obtained with free cells (23.6 U/mL). Thus sea water which was identified as a statistically significant parameter for α-production by free cells, could be excluded from the fermentation medium without any decrease in enzyme yield adopting the advantage of whole cell immobilization. *S. griseoloalbus* was isolated from a soil sample collected

from mangrove region, where the organisms thrive in a habitat in which the environmental conditions including salinity changes frequently. Mangrove species show different salinity tolerance limits. The results of the present study showed that *S. griseololabus* had a facultative nature with respect to salinity.

A difference in the course of α-galactosidase biosynthesis was observed between immobilized biocatalysts and free cells. More specifically, cell immobilization resulted in higher production rates giving the highest enzyme yield at 72 h of incubation, where as in the case of freely suspended cells the time required for highest enzyme yield was 96 h (Fig. 3.8). With immobilized cells there was an increase in enzyme yield up to 72 h after which it declined. The decrease in enzyme production might be due to the depletion of nutrients. Moreover, the accumulation of metabolites in the fermentation medium led to an increase in medium pH to alkaline condition (pH 7.9) which also decreased the stability of enzyme. The time of maximum enzyme production is significant because it is considered to be the cycle time for reusability transfer in repeated batch fermentation and in this case a period of 24 h could be saved in each fermentation cycle which also added to the advantage of whole cell immobilization.

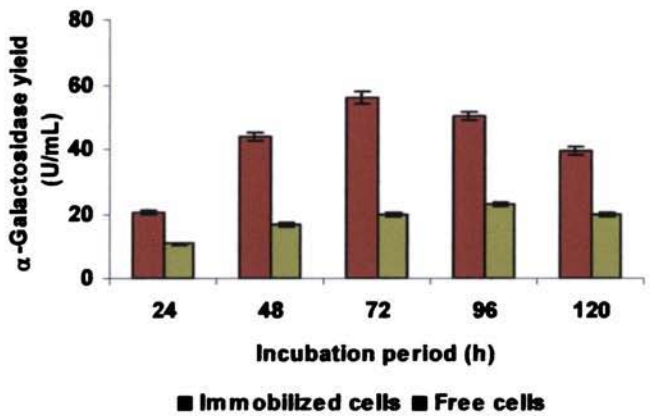


Fig. 3.9. Time course of α-galactosidase production by immobilized and free cells of *S. griseoloalbus*.

Fig. 3.10a shows the calcium alginate beads with immobilized *S. griseoloalbus*. The constraints created inside the polysaccharide gels by the immobilization procedure leads to changes in the micro environmental conditions which can modify the physiological and morphological behavior of a micro-organism compared to that of the free cell cultures. By only adjusting the conditions of immobilization, it is possible to substantially influence the ratio in which the individual metabolites are synthesized. The enhanced production of enzymes by immobilized cells can be explained by the stabilization of biosynthetic factors involved in metabolism due to the immobilization constraints (Ivanova et al. 1995; Jamuna & Ramakrishna 1992; Mamo & Gessesse 1997). It could also be possible that the cell wall membrane system interacts strongly with the gel matrix network. Since this network can present different structural presentations, unconventional three-dimensional adjustments between the cell wall and the matrix molecules are possible; therefore, these altered spatial organizations at discrete molecular levels induce the cells at determined specific conditions to enhance the biosynthesis of metabolites and increase the permeability mechanisms (Karel et al. 1985; Teruel et al. 1997). The SEM photograph showing the inside appearance of calcium alginate bead and *S. griseoloalbus* immobilized inside calcium alginate bead are shown in Fig. 3.7b and c respectively.

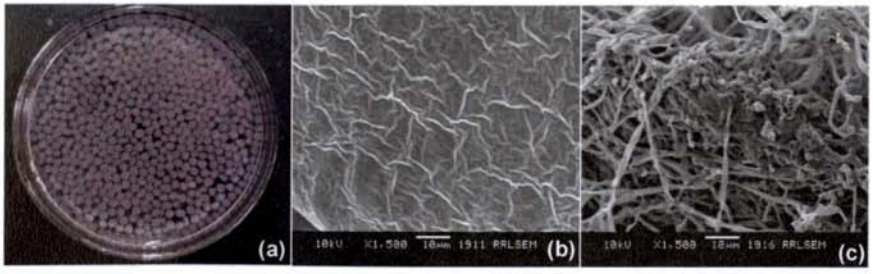


Fig. 3.10. (a) Calcium alginate beads with immobilized *S. griseoloalbus*. (b) Scanning electron micrograph showing the inside view of calium alginate bead and (c) immobilized *S. griseoloalbus*.

3.3.7. Operational stability of immobilized cells

The possibility of re-using immobilized cells of *S. griseoloalbus* for α-galactosidase production was studied over a period of 24 days, which corresponded to 8 cycles of fermentation. The duration of a fermentation cycle selected for repeated batch fermentation was 72 h. The immobilized beads could be reused effectively for α-galactosidase production three times without any apparent loss of enzyme yield (Fig. 3.11). After the third cycle, there was about 3% loss in activity with each consecutive use till the sixth cycle. Even after 8 successive and efficient fermentation operations, the alginate beads had good stability and maintained 75% of the enzyme yield obtained in the first cycle. The decrease in enzyme yield with successive fermentation might be due to the loss of cell vialbility.

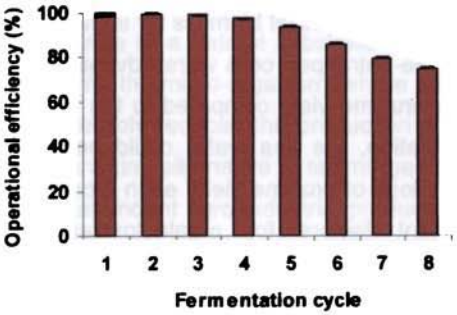


Fig. 3.11. Operational efficiency of calcium alginate-entrapped cells of *S. griseoloalbus* for the production of α-galactosidase. The duration of a single fermentation cycle was 72 h.

3.4. CONCLUSION

There is a growing acceptance for the use of statistical experimental designs in biotechnology. The application of statistical design for screening and optimization of process parameters allows quick identification of important factors and interactions between them. In the present study screening of variables to find their relative effect on α-galactosidase production was done using Plackett-Burman design. Out of the eleven

factors screened, salinity, magnesium sulphate and temperature were found to influence the enzyme production significantly. The optimal levels of these variables and the effect of their mutual interactions on enzyme production were determined using Box-Behnken design. The interaction between salinity and magnesium sulphate concentration was found to enhance α-galactosidase production, whereas temperature exhibited an influence independent of the other two factors. Using this statistical optimization method, the α-galactosidase production was increased from 17 to 50 U/mL.

Streptomyces griseoloalbus was immobilized in calcium alginate gel and the optimal immobilization parameters for the enhanced production of α-galactosidase were determined. The immobilization was most effective with 3% sodium alginate and 0.1 M calcium chloride. The optimal initial biomass for immobilization was approximately 2.2 g (wet wt.). The alginate-entrapped cells were advantageous because there was a two-fold increase in the enzyme yield compared to the free cells. By taking advantage of whole cell immobilization, the sea water could be excluded from the fermentation medium without any loss of enzyme yield, even though it was identified as one of the statistically significant factors for α-galactosidase production. Moreover, with immobilized cells the maximum yield was reached after 72 h of incubation in batch fermentation under optimal conditions, whereas in the case of free cells the maximum enzyme yield was obtained only after 96 h of incubation. The alginate beads had good stability and also retained 75% ability of enzyme production even after 8 cycles of repeated batch fermentation.

Chapter IV

OPTIMIZATION OF α-GALACTOSIDASE PRODUCTION BY *STREPTOMYCES GRISEOLOALBUS* IN SOLID-STATE FERMENTATION

4.1. INTRODUCTION

Reducing the costs of enzyme production by optimization of the fermentation conditions is a goal of basic research targeting industrial enzymology. An important point to consider, from the industrial perspective, is the selection of the suitable mode of fermentation and the culture medium in relation to cost and efficiency. Solid-state fermentation (SSF) holds tremendous potential for the production of microbial enzymes. SSF is defined as any fermentation process occurring in the absence or near absence of free water, using an inert substrate or a natural substrate as a solid support. The former only functions as an anchor for the micro-organism, where as the latter also acts as a carbon source, which considerably reduces the production costs (Pandey et al. 2001). SSF continues to be an interesting alternative to submerged fermentation due to the possibility of using cheap and abundant agro-industrial products as substrates and the metabolites so obtained are more concentrated and purification procedures are less costly. Filamentous micro-organisms are most exploited in SSF processes because of their ability to grow on complete solid substrate and produce a wide range of extra-cellular enzymes.

The successful development of commercial scale SSF processes is limited due to the lack of well established knowledge regarding how to design and operate large-scale bioreactors for SSF. The simplest bioreactor design for SSF, next to trays, involves the packed bed bioreactors, which typically have static substrate beds and are suited for SSF processes in which mixing is deleterious. Though there has been considerable interest in producing α-galactosidase by SSF (Cruz & Park 1982; Annunziato et al. 1986; Shankar et al. 2006; Liu et al. 2007b, c), information about α-galactosidase production by SSF bioreactors is limited to the few studies dealing with tray fermentation (Kotwal et al. 1998; Wang et al. 2004; Shankar & Mulimani 2007).

This chapter describes the identification of process conditions conducive for enhanced production of α-galactosidase by *Strepomyces griseoloalbus* under SSF in flask as well as in packed bed bioreactor.

4.2. MATERIALS AND METHODS

4.2.1. Solid-state fermentation in flask

Ten grams of solid substrate taken in 250-mL Erlenmeyer flask was moistened with mineral salt solution (g/L: KH_2PO_4, 1; $MgSO_4$, 0.4; pH 7.0), thoroughly mixed and autoclaved at 121 °C for 30 min. The cooled medium was inoculated with 2.25 x 10^6 CFU/g of initial dry substrate and incubated at 30 °C for 5 days. The preparation of inoculum was done as reported in Section 3.2.1 of Chapter III. One mL of inoculum contained 3 x 10^6 CFU. The moisture content of the medium after inoculation was 50%. Unless otherwise specified, these fermentation conditions were maintained throughout the experiment.

4.2.1.1. Evaluation of solid substrates

Different agro-industrial materials such as soymeal, soybean, chick pea, guar seeds, maize, sorghum, wheat bran, rice bran, groundnut oil cake and coconut oil cake were obtained from the local market, ground to flour and used as solid substrates to evaluate their suitability in sustaining good production of α-galactosidase by *S. griseoloalbus*. The best solid substrate chosen was used for further optimization experiments.

4.2.1.2. Optimization of process parameters

The significant process parameters that influence the production of α-galactosidase in SSF were identified by 'one-varaible-at-a-time' approach and they were optimized by multi-factorial approach using RSM.

4.2.1.2.1. 'One-variable-at-a-time' approach

Experiments were conducted using the selected solid substrate at four different ranges of particle size and a combination of all of them (< 300 µm, 300-500 µm, 500-1000 µm, 1000-2000 µm and a combination of all the particle sizes in 1:1:1:1 ratio) and

six different moisture levels (30, 40, 50, 60, 70 and 80%). Care was taken to keep the amount of mineral salt solution constant while varying the moisture level. The effects of initial pH (pH 4.0-10.0), incubation temperature (25-40 °C) and inoculum size (0.3×10^6 - 2.4×10^6 CFU/g initial dry substrate) on α-galactosidase production were studied. Different carbon supplements (glucose, galactose, melibiose, lactose, raffinose, xylan and locust bean gum; 1%, w/w), organic nitrogen supplements (yeast extract, tryptone, corn steep solid and urea; 0.3%, w/w) and inorganic nitrogen supplements ($(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$ and KNO_3; final concentration of nitrogen 0.004%, w/w) were incorporated in the medium to investigate their effects on α-galactosidase production. In each step of optimization, the process conditions resulting highest enzyme yield was chosen as the control for next experiment.

4.2.1.2.2. Multi-factorial approach

The individual effects as well as the interactive effects of the three crucial parameters- galactose concentration, inoculum size and initial moisture level- on α-galactosidase yield was statistically evaluated by response surface methodology (RSM) using Central Composite design (CCD). The Box-Wilson Central Composite Design (Box & Wilson 1951) is a type of statistical design that has been widely used in the optimization experiments. The three variables were studied at five levels coded as –1.68, –1, 0, +1 and +1.68. The levels of the variables were set on the basis of the previous experience with 'one-variable-at-a-time' approach. A total of 20 experimental runs (Table 4.1) were performed in triplicate and the average of α-galactosidase yield obtained was taken as the experimental values of dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting techniques. The center point of the design was replicated six times for estimation of error. The software Design-Expert® (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building.

Table 4.1. Central Composite design matrix with experimental and predicted values of α-galactosidase production by *S. griseoloalbus* in SSF

Trial No.	Variables			α-Galactosidase yield (U/gds)	
	Galactose (%, w/w)	Inoculum size (x 10^6 CFU/ g Initial dry substrate)	Moisture (%)	Experimental*	Predicted
1	1 (0)	1.9 (0)	6 (-1.68)	12.7	27.49
2	0.1 (-1.68)	1.9 (0)	40 (0)	27.2	47.01
3	1 (0)	1.9 (0)	40 (0)	110.2	109.74
4	1 (0)	0.18 (-1.68)	40 (0)	4.9	47.36
5	1 (0)	3.7 (+1.68)	40 (0)	106.5	92.9
6	0.5 (-1)	0.9 (-1)	20 (-1)	51.6	25.5
7	0.5 (-1)	0.9 (-1)	60 (+1)	41.1	18.6
8	1 (0)	1.9 (0)	40 (0)	111.3	109.7
9	1 (0)	1.9 (0)	40 (0)	110.6	109.7
10	1 (0)	1.9 (0)	74 (+1.68)	13.6	27.7
11	1.5 (+1)	0.9 (-1)	20 (-1)	87.1	68.9
12	0.5 (-1)	3 (+1)	20 (-1)	46.0	46.8
13	1 (0)	1.9 (0)	40 (0)	109.5	109.7
14	1.5 (+1)	3 (+1)	60 (+1)	94.1	99.8
15	1.5 (+1)	3 (+1)	20 (-1)	90.7	92.7
16	1.8 (+1.68)	1.9 (0)	40 (0)	117.1	126.2
17	1 (0)	1.9 (0)	40 (0)	111.6	109.7
18	1 (0)	1.9 (0)	40 (0)	110.2	109.7
19	1.5 (+1)	0.9 (-1)	60 (+1)	88.3	67.0
20	0.5 (-1)	3 (+1)	60 (+1)	51.2	49.0

The coded values of variables are given in parentheses.

*Values are the mean of three replicate experiments

A multiple regression analysis of the data was carried out to fit a second order polynomial equation (1) in order to define the response in terms of the independent variables.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC \quad \text{Eq. (1)}$$

where, Y = response variable, β_0 = intercept, β_1, β_2, β_3 = linear co-efficients, $\beta_1\beta_1$, $\beta_2\beta_2$, $\beta_3\beta_3$ = squared co-efficients, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ = interaction co-efficients and A, B, C = coded values of independent variables.

The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels. The statistical model was validated with respect to all three significant variables within the design space. A random set of five experimental combinations suggested by the software under the optimized conditions was used for validation of the statistical model.

4.2.2. Solid-state fermentation in packed bed bioreactor

Packed bed bioreactor studies were carried out under the process conditions optimized at flask-level. The heat-sterilized vertical glass column reactor (length, 22 cm; inner diameter 5 cm) was aseptically filled with 50 g of pre-inoculated soybean flour leaving a head space of about 5 cm at the top of the column. Air from an aerator pump was filtered through a glass column filter filled with glass wool before entering the humidification flask. In the humidification flask air was saturated and the saturated moist air was then continuously supplied through the bottom of the column. The outlet air from the top of the column was directed to the air exit unit. The diagrammatic representation of the experimental set up is shown in Fig. 4.1. The aeration rates were varied from 1 to 3 vvm (vessel volume/min) and fermentation was carried out for 120 h at 30 ± 1°C. For comparison a similar glass column filled with the pre-inoculated substrate but without aeration and a 250 mL Erlenmeyer flask containing 10 g of inoculated substrate were incubated at 30 ± 1°C for 120 h.

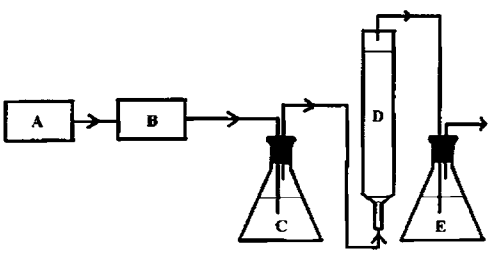


Fig. 4.1. Diagrammatic representation of packed bed bioreactor. (A) aerator pump, (B) air sterilization filter filled with glass wool, (C) air humidification unit, (D) packed bed column, (E) air exit unit. The arrow marks show the direction of air flow.

4.2.3. Enzyme extraction

The fermented matter was thoroughly mixed with distilled water (1:5, w/v) on a rotary shaker at 200 rev/min for 1 h. It was then filtered through muslin cloth and the filtrate obtained was centrifuged at 10,000 rpm for 20 min at 4 °C. The resultant supernatant was used as the enzyme preparation.

4.2.4. Enzyme assay

The activity of α-galactosidase was routinely determined using *p*NPG as described in Section 2.2.4 of Chapter II. α-Galactosidase yield under SSF was expressed as U/g dry fermented substrate.

4.2.5. Biomass estimation

The biomass was estimated by determining the *N*-acetyl glucosamine released from the cell wall of the actinomycete by acid hydrolysis (Sakurai et al. 1977) and was expressed as mg of glucosamine/g dry fermented substrate.

4.2.6. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the average of triplicate determinations and the standard deviation for all the values were <± 5%.

4.2.7. Scanning electron microscopy

Growth distribution of *S. griseoloalbus* on soybean flour was examined using a scanning electron microscope (JEOL JSM 5600LV, 115 Japan). The fermented sample (96 h) was adequately dried and mounted on a brass stud followed by a mild gold coating (0.01 μm) and was subjected to electron microscopy at an accelerating voltage of 10 kV.

4.3. RESULTS AND DISCUSSION

4.3.1. Solid-state fermentation in flask

Laboratory scale studies of SSF at flask-level can be useful in determining the optimum process conditions prior to bioreactor studies.

4.3.1.1. Evaluation of solid-substrates for α-galactosidase production

Selection of a suitable substrate for the production of enzyme is a primary factor and an extremely significant step in SSF. The capability of a micro-organism to produce a product in large amount is correlated with the nature and nutrient-availability of the substrate (Pandey et al. 2001). Of the various solid substrates used in the present study, soybean flour proved to be most suitable for the colonization of *S. griseoloalbus*, as indicated by the maximum visible growth on the surface of substrate and the highest enzyme yield (Fig. 4.2). All the other tested legume-based substrates such as soymeal, guar flour, and chickpea flour; and oil cakes such as groundnut oil cake and coconut oil cake supported good α-galactosidase yield. Wheat bran, rice bran, maize flour and sorghum flour gave only poor results (Fig. 4.2).

The increased α-galactosidase yield obtained in the present study is attributed to the presence of α-galactosides in the soybean flour. It is also reported that manninotriose, the main constituent of soybean carbohydrate, is the best inducer for α-galactosidase synthesis (Cruz & Park 1982). Soy flour and soy beans are considered as the most ideal substrates for α-galactosidase production in SSF as an enhancement in enzyme yield was recorded with these substrates (Cruz & Park 1982; Annunziato et al. 1986; Kotwal et al. 1998; Shankar et al. 2006). Unlike the reports of Cruz & Park (1982), using ground soy products directly as the solid substrate is much more economical than extracting, fermenting and recovering the oligosaccharides and then adding them to the SSF system. Developing a α-galactosidase production process based upon soybean flour as the solid substrate is very attractive, since it is a readily available source of carbon.

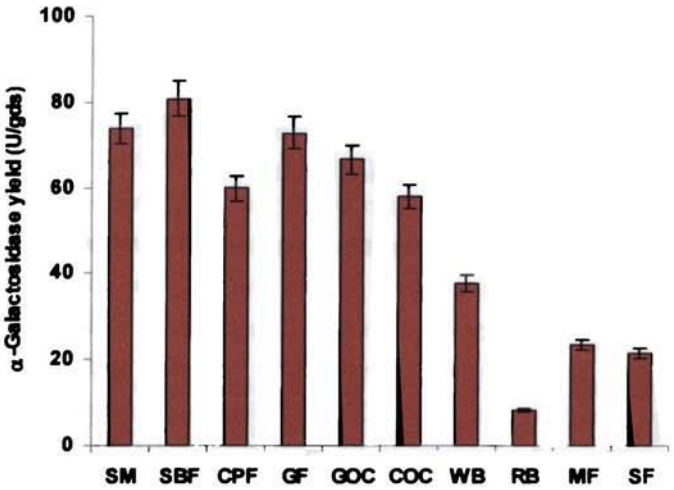


Fig. 4.2. Screening of solid substrates for α-galactosidase production by *S. griseoloalbus* in SSF. SM- Soymeal, SBF- Soybean flour, CPF- Chick pea flour, GF- Guar flour, GOC- Groundnut oil cake, COC- Coconut oil cake, WB- Wheat bran, RB- Rice bran, MF- Maize flour, SF- Sorghum flour

4.3.1.2. Optimization of process parameters

The growth of micro-organisms and metabolite production in SSF is governed by a large number of physical factors which include mainly particle size of the substrate used, initial moisture level of the medium, pH, incubation temperature and inoculum size (Pandey et al. 2001). Numerous nutrients, which includes mainly carbon and nitrogen supplements also influence growth and metabolite production by micro-organisms (Krishna 2005).

4.3.1.2.1. 'One-variable-at-a-time' approach

Soybean flour in the particle size range of 500-1000 µm (Fig. 4.3) supported highest α-galactosidase yield by *S. griseoloalbus*. A decrease in α-galactosidase yield was observed at levels higher or lower than the optimum. There are two opposing effects of particle size on SSF at any given moisture content. The first effect is the

increase in surface area for the growth of micro-organism with decreasing particle size. The second is the reduction in void volume, and hence gas phase oxygen transfer with decreasing particle size. Superimposed on these two effects is the reduction of both surface area and void volume due to the growth of micro-organism during the course of fermentation (Valera et al. 2005). Hence too small and too large particle sizes are not suitable for the growth of micro-organisms and it would be necessary to arrive at a compromised particle size for a particular process (Pandey et al. 2001).

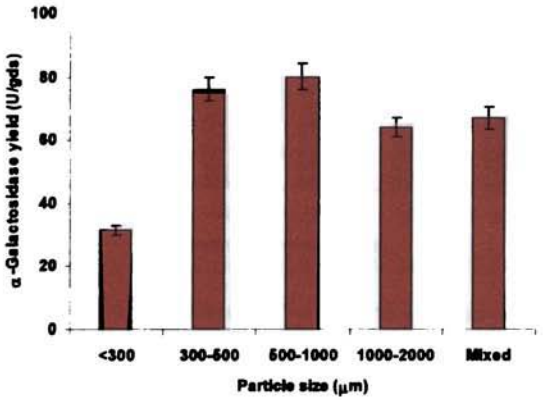


Fig. 4.3. Effect of substrate particle size on α-galactosidase production

The optimum initial moisture content was 40% for the highest α-galactosidase yield obtained in the present study (Fig. 4.4). The moisture content of the substrate in SSF greatly depends on water activity of the substrate, which in turn is highly dependent on the water binding properties of the substrate (Krishna 2005). For a substrate like soybean flour the water activity is very high and hence even a slight increase in water content by addition of mineral salt solution can lead to agglomeration of the substrate. The importance of moisture and water activity implies that it is necessary to consider the exact quantities to be added to the substrate, while preparing a substrate (Gowthaman et al. 2001).

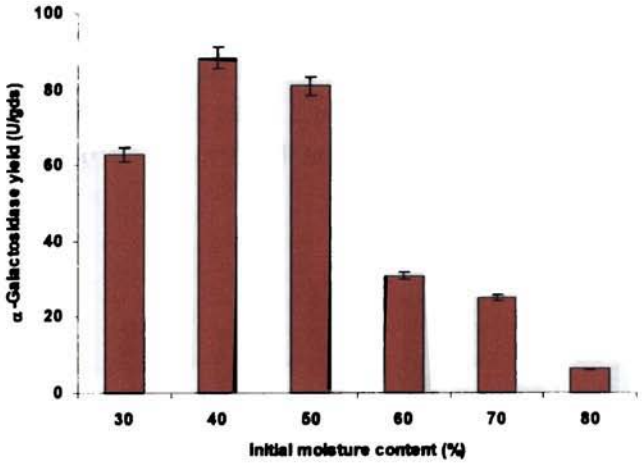


Fig. 4.4. Effect of initial moisture content of the medium on production of α-galactosidase. Care was taken to keep the amount of mineral salt solution constant while varying the moisture level.

The α-galactosidase yield was highest when the pH of the medium was 7 (Fig 4.5). Each micro-organism possesses a pH range for its growth and activity with an optimum value within the range. The α-galactosidase yield was comparatively higher in the acidic range than in the alkaline range, similar to the previous results obtained in submerged fermentation. At pH 10.0 the enzyme yield was drastically reduced.

The optimum incubation temperature for highest α-galactosidase yield was 30 °C (Fig 4.6). α-Galactosidase yield increased with increase in incubation temperature from 25 to 30 °C and higher temperatures were detrimental to the growth and α-galactosidase production by *S. griseoloalbus*, which may be attributed to the mesophilic nature of the microbe.

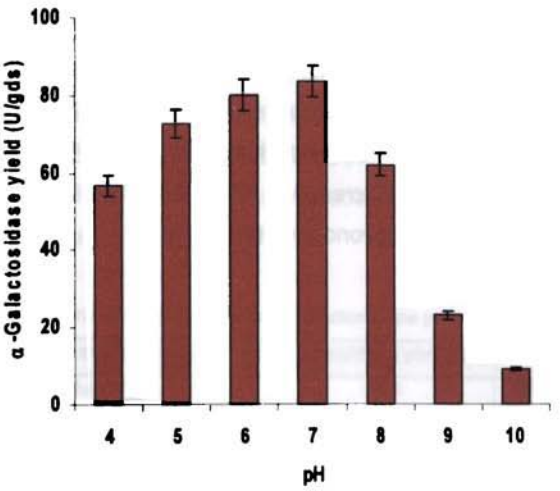


Fig. 4.5. Effect of initial pH on production of α-galactosidase

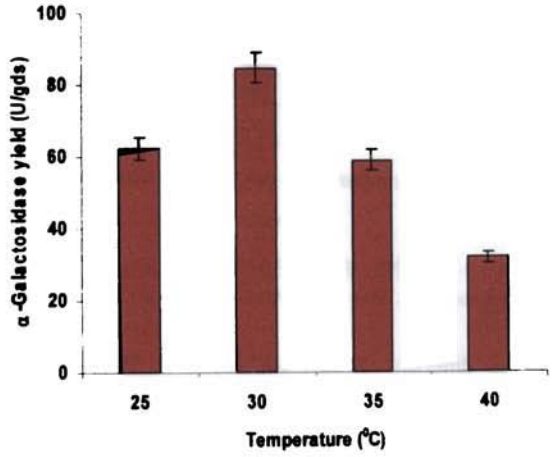


Fig. 4.6. Effect of incubation temperature on production of α-galactosidase

Optimization of inoculum size is yet another important step in any fermentation process. The optimum inoculum size for α-galactosidase production by *S. griseoloalbus* was 1.8 x 10^6 CFU/g initial dry substrate (Fig. 4.7). Since actinomycetes are slow growing organisms they require considerably higher inoculum compared to their fungal and bacterial counterparts. A decrease in enzyme production was observed when the inoculum size was increased beyond the optimum level.

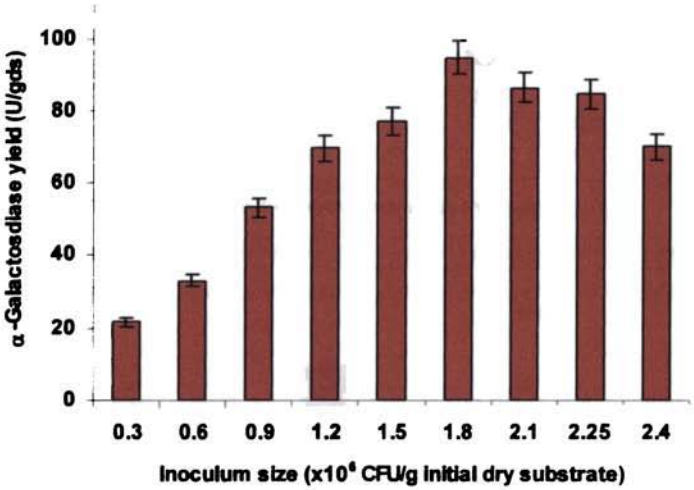


Fig. 4.7. Effect of inoculum size on production of α-galactosidase. One mL of inoculum contained 3x10^5 CFU.

Among the different carbon supplements tested, galactose had the highest inducing effect on α-galactosidase synthesis by *S. griseoloalbus* in SSF (Table 4.2). Generally SSF systems are resistant to catabolite (Viniegra-González & Favela-Torres 2006) even at high sugar concentration, whereas in conventional submerged fermentation strong inhibition of inducible enzymes occurs in presence of simple

sugars. This is because, thin layers of microbial cells growing on a solid support exhibit changes in cell permeability to sugars, thereby creating a microenvironment where sugar concentration is optimal for growth and enzyme production (Viniegra-González & Favela-Torres 2006). Glucose and melibiose also showed slight inducing effect whereas raffinose and lactose were not effective as inducers of the enzyme production. This was in contradiction to the reports of Srinivas et al. (1993) who reported lactose as the most effective inducer of

α-galactosidase.

Table 4.2. Effect of different carbon supplements on α-galactosidase production by *S. griseoloalbus*.

Carbon supplement	α-Galactosidase yield (U/gds)
Glucose	83.6
Galactose	110.2
Melibiose	96.8
Lactose	60.1
Raffinose	84.3
Xylan	47.8
Locust bean gum	62.6
Control	93.6

Table 4.3. Effect of different nitrogen supplements on α-galactosidase production by *S. griseoloalbus*

Nitrogen supplement		α-Galactosidase yield (U/gds)
Organic nitrogen supplement (0.3%, w/w)	Yeast extract	83.4
	Tryptone	75.2
	Corn steep liquor	55.6
	Urea	59.1
Inorganic nitrogen supplement (0.004% N)	$(NH_4)_2SO_4$	65.3
	NH_4Cl	75.7
	$(NH_4)_2HPO_4$	89.6
	KNO_3	67.8
	$NaNO_3$	64.1
Control		110.8

None of the nitrogen supplements enhanced α-galactosidase production in comparison to the control without any nitrogen supplement (Table 4.3). The soybean flour used in the study is a rich source of nitrogen in the form of protein, which accounts for about 40% of dry soybeans by weight. This was sufficient to support the highest α-galactosidase yield by *S. griseoloalbus.*

4.3.1.2.2. Multi-factorial approach

By 'one-variable-at-a-time' approach three variables- galactose concentration, inoculum size and initial moisture level- were identified to have a predominant influence on production of α-galactosidase by *S. griseoloalbus.* It is worthwhile to identify the optimum levels of these variables and to study how these variables interact with each other in enhancing the α-galactosidase yield. Hence these crucial parameters were optimized by RSM using CCD. Central composite design (CCD) combines a two-level full or fractional factorial design with additional points and at least one point at the center of the experimental region in order to fit quadratic polynomials. For fitting quadratic response models, CCD is a better alternative to full factorial three-level design because its performance is comparable at a lower cost. Therefore, it has been the most accepted experimental design for optimization of biological processes.

The data obtained was analyzed by multiple regression analysis using the software and the following equation was obtained:

$$Y = 109.74 + 23.56\,X_1 + 13.54\,X_2 + 0.075\,X_3 + 8.17\,X_1^2 - 14.00\,X_2^2 - 29.04\,X_3^2 + 0.61\,X_1X_2 + 1.24\,X_1X_3 + 2.24\,X_2X_3 \qquad \text{Eq. (2)}$$

where, Y is the predicted value of α-galactosidase yield and X_1, X_2 and X_3 are the coded values for galactose concentration, inoculum size and moisture level respectively.

The experimental data was statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Table 4.4. The ANOVA of the quadratic regression model indicated that the model was highly significant, as the F-value for the model was 5.52. There was only 0.67% chance that the 'model F-value' this large could occur due to noise. The *Prob*>F value of the model

was 0.0067, which also confirmed that the model was highly significant. The coefficient estimate and the corresponding *Prob*>F values (Table 4.4) suggested that among the independent variables studied, galactose concentration and inoculum size and also the squared terms of inoculum size and moisture content had a significant effect on α-galactosidase production by *S. griseoloalbus*. The co-efficient of determination (R^2) was calculated as 0.8323 indicating that the statistical model can explain 83.23% of variability in the response. For a good statistical model R^2 value should be close to 1.0 and a value >0.75 indicates the aptness of the model. The model recorded an adequate precision of 6.869 which indicated an adequate signal to noise ratio to navigate the design space, since a ratio greater than 4.0 is desirable.

Table 4.4. ANOVA for the response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	*Prob*>F
Model	24355.84	9	2706.20	5.52	0.0067
X_1	7577.67	1	7577.67	15.44	0.0028
X_2	2504.29	1	2504.29	5.10	0.0474
X_3	0.077	1	0.077	1.578×10^{4}	0.9902
X_1^2	962.56	1	962.56	1.96	0.1916
X_2^2	2825.00	1	2825.00	5.76	0.0374
X_3^2	12149.85	1	12149.85	24.76	0.0006
X_1X_2	2.98	1	2.98	6.067×10^{3}	0.9395
X_1X_3	12.20	1	12.20	0.025	0.8778
X_2X_3	40.23	1	40.23	0.082	0.7805

The individual effects and interactive roles of galactose concentration, inoculum size and moisture content on α-galactosidase production are illustrated in the three-dimensional curves of the calculated response surface shown in Fig. 4.8. The interactive effect of galactose concentration and inoculum size showed that the α-galactosidase yield was improved when inoculum size was increased together with an increase in galactose concentration (Fig. 4.8a). An increase in inoculum size beyond the optimum did not increase enzyme yield still further, regardless of the increase in galactose concentration. The interactive effects of moisture content with galactose

concentration (Fig. 4.8b) and inoculum size (Fig. 4.8c) showed that higher moisture content was detrimental to the growth and α-galactosidase production by *S. griseoloalbus*. The α-galactosidase production increased with increase in moisture up to the optimum level (40%), after which it declined despite the increase in galactose concentration and inoculum size. The highest α-galactosidase yield was obtained with 1.5% galactose (w/w), 1.9 x 10^6 CFU/g initial dry substrate and 40% moisture, indicating the optimum levels of these variables.

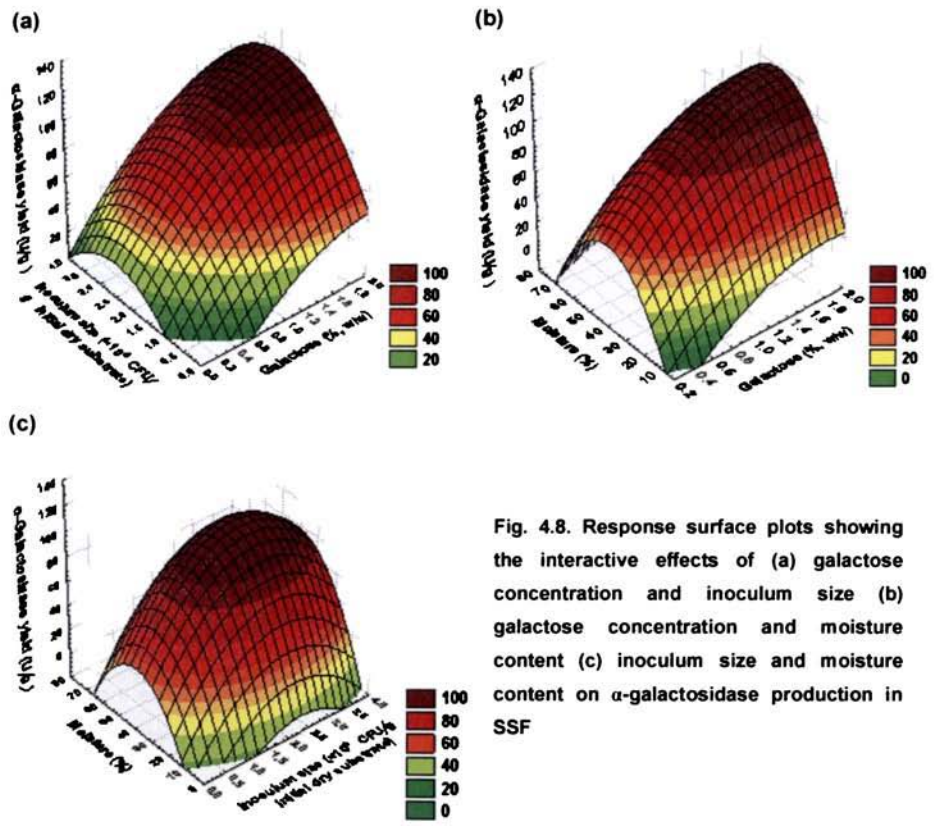


Fig. 4.8. Response surface plots showing the interactive effects of (a) galactose concentration and inoculum size (b) galactose concentration and moisture content (c) inoculum size and moisture content on α-galactosidase production in SSF

Inducing effect of galactose in the biosynthesis of α-galactosidase in SSF was previously reported by Shankar et al. (2006). Usually, only the substrates of an enzyme or its structural analogs are able to act as inducers. Dey & Pridham (1972) showed that the rate of hydrolysis of a substrate or its binding with α-galactosidase depends upon the substrate having a pyranoid ring structure and that the configuration of carbon atoms 1-4 must be similar to that in D-galactose.

For fast growth of mycelium and product formation, an adequate inoculum is required which also reduces the growth of contaminants. Enzyme production attains its peak when the nutrients available to biomass are balanced. Under conditions when there is misbalance between nutrients and proliferating biomass, it results in decreased enzyme synthesis, which is true with all the micro-organisms (Ramachandran et al. 2005).

Moisture content is one of the crucial factors that strongly influence the growth of micro-organisms and metabolite production in SSF. Moisture is reported to cause swelling of the solid substrate thereby facilitating its better utilization by micro-organisms (Pandey et al. 2001). There is an optimum range of moisture content for each micro-organism beyond which it may not function to produce the metabolites. Though filamentous micro-organisms are generally reported to grow best when the substrate moisture content is between 50 and 75% (Pandey et al. 2001), Annunziato et al. (1986) reported 35% initial moisture to be the optimum for highest α-galactosidase yield from *Aspergillus oryzae*. The decrease in moisture level is advantageous since the chance of contamination in the fermentation medium is reduced. However, decrease in moisture level below the optimum results in minimized heat exchange and oxygen transfer, a lower degree of substrate swelling, reduced solubility and low availability of nutrients to the culture and higher water tension. Higher initial moisture in SSF decreases porosity, changes the particle structure and promotes development of stickiness due to agglomeration of the substrate. This reduces mass transfer process and gas exchange subsequently restricting the supply of oxygen for the growth of micro-organism and leading to suboptimal product formation (Narahara et al. 1982; Pandey et al.2001).

In order to validate the adequacy of the model, a total of five verification experiments were conducted under various fermentation conditions within the experimental range. The design matrix showing the actual values of the independent variables along with the experimental and predicted values of α-galactosidase yield are given in Table 4.5. The results indicated that the model was satisfactory and could result in considerably enhanced enzyme yield.

Table 4.5. Validation of response surface quadratic model for α-galactosidase production in SSF

Trial No.	Galactose (%, w/w)	Inoculum size (x10^6 CFU/g initial dry substrate)	Moisture content (%)	α-Galactosidase yield (U/gds)	
				Experimental [a]	Predicted
1	1.4	1.9	37	120.2	117.6
2	1.2	2	46	119.8	121.9
3	1.3	1.8	48	123.1	125.4
4	1.3	1.9	49	123.2	124.5
5	1.5	1.9	41	123.3	122.7

[a] Values are mean of triplicate experiments

The use of statistical methods for the optimization of α-galactosidase production in SSF system was previously reported by Srinivas et al. (1994) and a 73% increase in enzyme production was achieved. A remarkable enhancement in α-galactosidase yield from the thermotolerant *Absidia* sp. (Li et al. 2006) and *Aspergillus foetidus* (Liu et al. 2007c) in SSF using response surface methodology are reported.

4.3.1.3. Time course of α-galactosidase production

The growth of *S. griseoloalbus* as determined by the glucosamine content of fermented matter (Fig. 4.9) showed a lag phase up to 24 h, after which there was the exponential growth phase from 24 to 96 h with rapid increase in the biomass, followed by a stationary phase. The initial trend of α-galactosidase biosynthesis by *S. griseoloalbus* closely followed the growth profile of the culture. The organism colonized well on the solid substrate and exhibited a good growth on the surface after 36 h of incubation, but enzyme production reached the maximum at 96 h when the organism had established itself well in the deeper layers of the solid medium. After 96 h the

enzyme titer declined due to the depletion of nutrients that were then utilized mostly for cell multiplication and compensation of cell death in the stationary phase. Moreover, the already synthesized enzyme molecules might have undergone proteolytic degradation. Similar results of growth associated α-galactosidase production in SSF system have been reported by Srinivas et al. (1993) from *Aspergillus niger* NCIM 839.

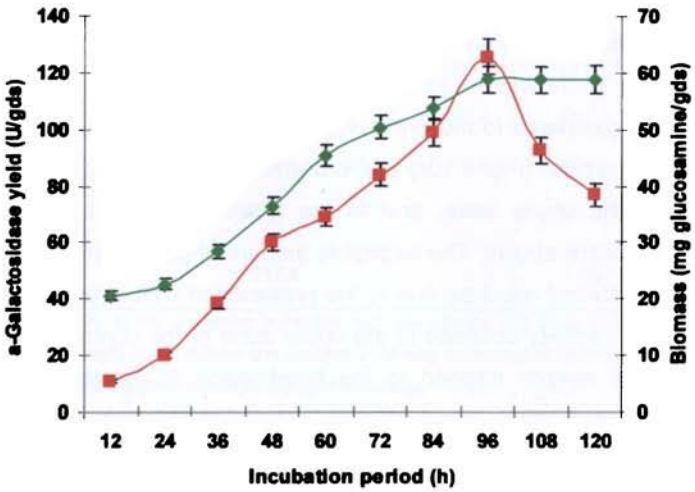


Fig. 4.9. Fermentation profile of *Streptomyces griseoloalbus* showing α-galactosidase yield (■) and growth kinetics (◆) at different hours of incubation.

4.3.2. Solid-state fermentation in packed bed bioreactor

In a packed–bed reactor consisting of a cylindrical column, the substrate is generally loosely packed to larger heights and air is forced through the bed from the bottom. Fig. 4.10a shows the experimental set up of packed bed bioreactor used for the present study. Different air-flow rates were evaluated in order to identify the best condition for α-galactosidase production by *S. griseoloalbus* in a vertical glass column bioreactor. For enzyme extraction and assay the entire substrate bed was divided into three equal zones, i.e. upper, middle and lower, and growth of the culture and enzyme yield was monitored in each segment. The results (Table 4.5) showed that growth and

α-galactosidase yield was highest in the upper zone and lowest in the lower zone, irrespective of the aeration rate provided. Forced aeration of 2 vvm resulted in highest α-galactosidase yield of 197.2 U/gds which was approximately twice the yield obtained in flasks.

Fig. 4.10b and c evidently shows the necessity of oxygen supply for the growth of *S. griseoloalbus*. There was an immense growth of the microbe in the column with forced aeration as compared to the un-aerated column. In the control column without any aeration, slight growth (Fig. 4.10c) and enzyme yield was noted (Table 4.5) which was restricted to the upper zone, and in the lower zones visible growth and α-galactosidase yield were absent. The negligible amount of glucosamine recorded in the lower zones of the control could be due to the presence of culture added as inoculum. The α-galactosidase activity obtained in the upper zone of the control could be due to the less amount of oxygen trapped in the head-space at the top of the column, emphasizing the significance of aeration in enhancing the growth and metabolic activities of this strictly aerobic micro-organism.

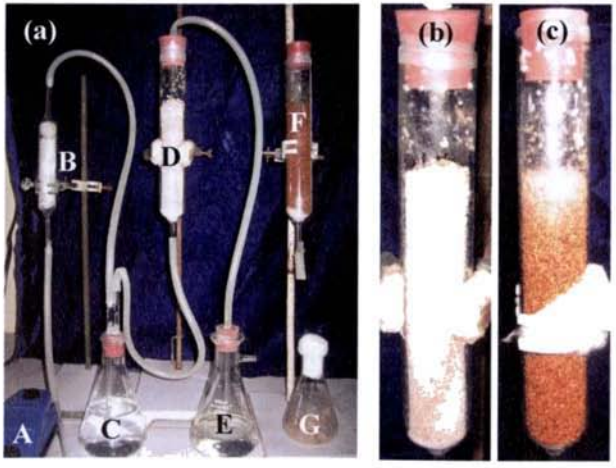


Fig. 4.10a Experimental set up of packed bed bioreactor for α-galactosidase production in SSF. (A) Aerator pump, (B) air sterilization filter, (C) air humidification unit, (D) aerated packed bed column, (E) air exit unit, (F) un-aerated packed bed column, G- Control flask. Fig. 4.10b and c respectively shows the growth of *S. griseoloalbus* in aerated (2 vvm) and un-aerated packed bed columns after 96 h of incubation.

Table 4.5. Growth and α-galactosidase production by *S. griseoloalbus* in packed bed bioreactor with different rates of aeration

Aeration rate (vvm)[a]	α-Galactosidase yield (U/gds)			Glucosamine (mg/gds)		
	Upper zone	Middle zone	Lower zone	Upper zone	Middle zone	Lower zone
1	117.6	106.7	99.4	63.2	27.3	21.4
1.5	148.9	119.1	101.3	81	42.5	36.1
2	197.2	139.4	126.3	83	47.7	40.7
2.5	158.1	124.2	111.8	82.4	42.5	40.3
3	127.2	108.9	88.7	78.6	28.7	23
Control column without aeration	7.3	ND[b]	ND[b]	10.9	2.6	2.3
Control (Flask)		117.8			56.7	

For enzyme extraction and assay the entire substrate bed was divided into three equal zones, i.e. upper, middle and lower, and growth of the culture and enzyme yield was monitored in each segment. The data are mean of three independent samples with triplicate determinations

[a]vessel volume/min

[b]not detected

Enhancement of enzyme productivity in traditional SSF by oxygen enrichment has been reported previously by many workers (Pandey et al. 1996; Archana & Satyanarayana 1997; Milagres et al. 2004). Lower enzyme yields with aeration lower than 2 vvm could be due to inadequate oxygen supply. At higher rates of aeration, though the oxygen supply was higher, the reduction in α-galactosidase yield could be due to reduction in water content of the fermented matter below the critical level, which adversely affected the growth and microbial activity. As a result of end-to-end aeration, axial gradients of oxygen distribution are impossible to prevail in packed bed bioreactors and temperature gradients have a greater potential to limit reactor performance than oxygen gradients (Mitchell et al. 2000, 2003; Krishna 2005). These axial temperature gradients promote evaporation even if saturated air is used to aerate the column because the water carrying capacity of the air increases as it heats up. This

evaporation can remove 65% of the waste metabolic heat but is undesirable since drying out of the substrate will inhibit growth, while replenishment of water is not practical within an unmixed bed (Mitchell et al. 2003). In packed bed bioreactor, most of the metabolic heat and CO_2 released from the fermented mash could be removed by forced aeration with humidified air, thus minimizing the rise in temperature of the fermenting substrate. The packed bed bioreactor offers several advantages over the tray fermentation previously reported by several workers for α-galactosidase production in SSF. It allows better control of fermentation parameters than is possible in trays.

4.3.3. Scanning electron microscopic studies

The degree of substrate transformation in SSF depends upon the capability of micro-organism to penetrate deep into the intracellular space. The mycelial morphology of actinomycetes is well suited for invasive growth on solid substrate. Fig. 4.11 shows the scanning electron micrograph (SEM) elucidating the knitted extensive growth of *S. griseoloalbus* on soybean flour. As observed from SEM, the average diameter of the filaments was between 1 and 2 μm. The filaments of *S. griseoloalbus* grown on soybean flour are slender compared to the fungal counterparts such as *Aspergillus niger* which are having filaments of diameter around 3 μm. It is advantageous because slender cells have faster reaction rates without having diffusion problems. It is very significant that this information is relevant for future studies of modeling the diffusion and consumption of oxygen across the network of filamentous microbial growth.

Fig. 4.11. Scanning electron micrograph showing extensive growth of *Streptomyces griseoloalbus* on soybean flour

4.4. CONCLUSION

Soybean flour was the best solid substrate for α-galactosidase production by *S. griseoloalbus* in SSF, which was possibly due to the presence of various suitable nutrients in soybean flour and/or due to its most suitable particle size and consistency required for anchorage, colonization and enzyme secretion by *S. griseoloalbus*. Optimization of process parameters at flask level by 'one-variable-at-a-time' approach resulted in a highest enzyme yield of 110.8 U/gds. Galactose was found to have an inducing effect on enzyme production whereas none of nitrogen sources tested was found to be necessary in enhancing the enzyme yield, as soybean flour itself was effective in supporting the nitrogen demand of the culture. RSM was adopted to derive a statistical model for the optimization of fermentation conditions and it increased the maximum α-galactosidase yield to 125 U/gds. Packed bed bioreactor performed well in enhancing the enzyme yield still further and resulted in a highest yield of 197.2 U/gds with a forced aeration of 2 vvm. The packed bed bioreactor offers several advantages over the tray fermentation and allows better control of fermentation parameters than is possible in trays. The results obtained during the course of this study indicate the scope for utilization of actinomycetes in SSF systems for harnessing their immense potential as source of exo-enzymes.

Chapter V

PURIFICATION AND CHARACTERIZATION OF MULTIPLE THERMOSTABLE α-GALACTOSIDASES

5.1. INTRODUCTION

Purification and characterization of any enzyme are of paramount importance in revealing the novel biochemical and catalytic properties suitable for its excellent industrial application. The enzyme recovery process is considered to begin once the fermentation has achieved peak yield. In purification of enzymes there is often a dramatic reduction in the overall yield, which follows as a direct consequence of the number of steps involved in the recovery process. Generally it may be assumed that the higher the product specification then the more numerous and sophisticated are the stages involved in its preparation. Therefore, to achieve maximum yield and minimize production costs the recovery process must be capable of attaining the desired specification by the most direct route.

Purification and characterization of α-galactosidases from thermophilic (Gote et al. 2006) and hyperthermophilic micro-organisms (Miller et al. 2001; King et al. 1998) have been reported recently. Production and characterization of α-galactosidases from *Streptomyces olivaceus* (Suzuki et al. 1966) and *S. eryhtrus* (Elshafei et al. 2001) are reported. Reports are available in the literature documenting cloning and expression of the gene encoding α-galactosidase in *S. coelicolor* A3(2) (Kondoh et al. 2005). Crystal structure of α-galactosidases from *Trichoderma reesei* (Golubev et al. 2004) and rice (Fujimoto et al. 2003) has been elucidated. Competitive inhibition by galactose is a common characteristic of most α-galactosidases (Ademark et al. 2001; Gote et al. 2004). This chapter describes the purification and characterization of multiple α-galactosidases from *S. griseoloalbus* and the presence of a novel galactose-tolerant α-galactosidase.

5.2. MATERIALS AND METHODS

5.2.1. Production and extraction of α-galactosidases

α-Galactosidase production by *S. griseoloalbus* was carried out under solid-state fermentation conditions as described in Chapter IV. Enzyme extraction was carried out by mixing the fermented matter with 50 mM sodium phosphate buffer (pH 7.0; 1:5, w/v) on a rotary shaker at 200 rev/min for 1 h. The thoroughly agitated fermented matter was then filtered through muslin cloth and the filtrate obtained was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was used as the enzyme preparation for further purification.

5.2.2. Purification of α-galactosidases

The steps involved in the purification of α-galactosidases are shown as a flow chart in Fig.5.1. The crude enzyme preparation was concentrated five times at 4 °C by an Amicon Ultra-filtration unit using a 10 kDa cut-off membrane and was then applied to Phenyl Sepharose CL 4B hydrophobic interaction chromatographic column (10 cm x 2.8 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1.25 M ammonium sulphate. The enzyme was eluted at a flow rate of 0.5 mL/min with 50 mM sodium phosphate buffer (pH 7.0) with a linear decreasing gradient of ammonium sulphate (1.25-0 M). The fractions collected were screened for protein content, α-galactosidase activity and conductivity. The α-galactosidase active fractions were pooled, concentrated by ultrafiltration as described above and desalted by dialyzing against 50 mM sodium phosphate buffer (pH 7.0). The resultant enzyme sample was then applied to Sephadex G-100 (30 cm x 3 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. Three peaks with α-galactosidase activity were pooled separately, concentrated by ultra-filtration and stored at 4°C until further use.

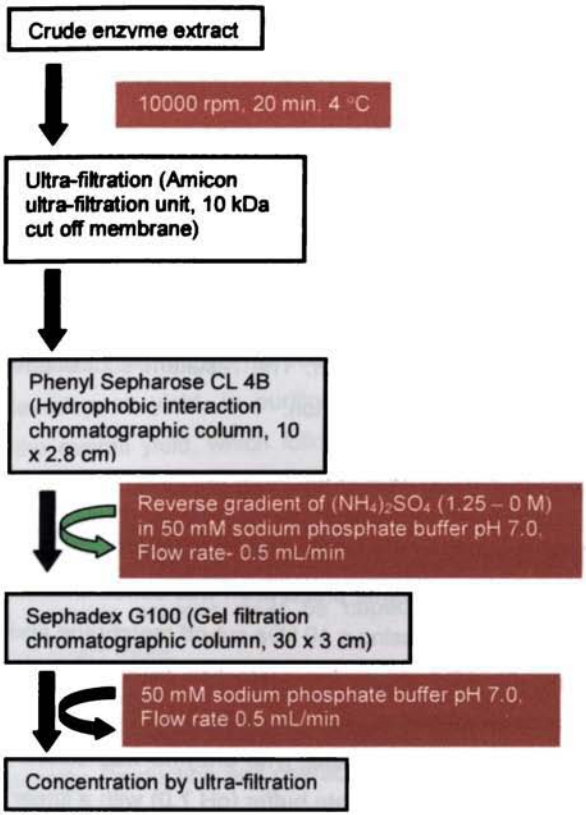


Fig. 5.1. The steps involved in the purification of α-galactosidases from *S. griseoloalbus*. The curved arrows indicate the elution of proteins.

5.2.3.α-Galactosidase assay

The activity of α-galactosidase was routinely determined using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) as reported earlier in Section 2.2.4 of Chapter II. When oligosaccharides like raffinose and stachyose and polysaccharides like locust bean gum and guar gum were used as substrates, the amount of galactose liberated was

estimated as reducing sugar by the method of Nelson (1944). When melibiose was used as substrate, the amount of galactose liberated was determined by galactose oxidase method using the galactose assay kit (Biovision Research Products, USA) following the manufacturer's directions. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated one μmol of the product (*p*-nitrophenol or galactose) per min under the assay conditions.

5.2.4. Protein estimation

The total soluble protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. All chromatographic fractions were monitored for protein by absorbance at 280 nm.

5.2.5. Relative molecular mass (M_r) and zymogram analysis

The native M_r of the purified α-galactosidases was determined by native PAGE (10%, w/v) in a vertical slab gel apparatus. For calibration of the gel standard molecular mass markers (catalase, 240 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; trypsin soybean inhibitor, 20.1 kDa and lactoglobulin, 18.4 kDa; Genei™, Bangalore) were also electrophoresed along with the purified α-galactosidases. The half of the gel was stained with Coomassie Brilliant Blue R-250 (0.25%, w/v) and destained with methanol: acetic acid: water (1:1:8, v/v) to visualize the protein bands. For zymogram analysis, α-galactosidase activity in the other half of the gel was visualized as fluorescent band under UV transilluminator after incubating the gel at 55 °C for 10 min in 0.1 M citrate phosphate buffer (pH 7.0) containing the fluorescent substrate, 4-methyl-umbeliferyl-α-D-galactopyranoside (MU-α-gal, 1 mM).

The M_r of the subunits of purified α-galactosidases was calculated based on the relative mobility of standard molecular mass markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soyabean trypsin inhibitor, 20.1 kDa and lysozyme, 14.3 kDa; Genei™, Bangalore) in SDS PAGE (12%, w/v), which was done in a vertical slab gel apparatus according to

the method of Laemmli (1970). The protein bands were visualized by silver staining (0.2%, w/v) (http://www.proteinchemist.com/tutorial/silverst.html).

The relative mobility of molecular mass markers and purified α-galactosidases in native PAGE and SDS PAGE was calculated according to the equation given below.

$$\text{Relative mobility} = \frac{\text{Distance of protein migration x Length of gel before staining}}{\text{Distance of dye migration x Length of gel after staining}} \quad \text{Eq. (1)}$$

The molecular mass of the α-galactosidase was determined by interpolation from a linear semi logarithmic plot of log molecular mass of standard markers versus the relative mobility.

5.2.6. *N*-terminal sequencing

The region of gel corresponding to the main α-galactosidase protein band was cut from an unstained SDS PAGE gel and thoroughly mixed with 1 ml Tris buffer (pH 7.6). It was then kept overnight at -20 °C, centrifuged and the supernatant containing dissolved protein was dialyzed (12 h) against distilled water which was frequently replaced with fresh distilled water to ensure complete removal of Tris. The first 10 amino acid residues at the *N*-terminus of the enzyme were sequenced using Shimadzu Protein Sequencer at Rajiv Gandhi Centre for Biotechnology, Trivandrum, India and its sequence homology with α-galactosidases from other sources was determined using the BLASTP search of the Non-redundant Peptide Sequence Database at the National Centre for Biotechnology Information *via* the World Wide Web Interface (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

5.2.7. Glycoprotein analysis

The total carbohydrate content of purified α-galactosidases was determined by the method described by Dubois et al. (1956).

5.2.8. Isoelectric focusing

Isoelectric pH of the purified α-galactosidases was determined using rotofor (Biorad) according to manufacturer's instructions. The ampholyte used was in the pH range of 3.0-10.0. Focusing was carried out at a constant power of 12 W for 3 hrs. The pH and enzyme activity of individual fractions were assayed.

5.2.9. Effect of pH and temperature on enzyme activity and stability

The optimum pH for α-galactosidase activity was determined in the assay mixture over a pH range of 3.0-9.0, using different buffers (0.1 M) such as citrate phosphate buffer (pH 3.0-7.0), sodium phosphate buffer (pH 6.0-8.0) and Tris-HCl buffer (pH 7.5-9.0). For pH stability determination, enzyme preparations were incubated at pH 3.0-9.0 for 2 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

The optimum temperature for enzyme activity was determined by incubating the assay mixture at 40-75 °C. Thermostability of α-galactosidases was determined by studying the time-dependent thermal inactivation of enzyme at temperatures in the range of 50-75 °C for 2 h at optimum pH.

5.2.10. Substrate specificities and kinetic parameters

The relative substrate specificities of the three α-galactosidases towards various synthetic and natural substrates were determined under standard assay conditions. For kinetic studies the initial rate of hydrolysis of various glycosides at different concentrations (0.5-5 mM) was measured under standard assay conditions and the kinetic constants K_m, and V_{max} were determined from Lineweaver-Burk plot using the Enzyme Kinetics module of Sigmaplot (Systat software Inc. Version 1.2.0.0).

5.2.11. Hydrolysis of polymeric galactomannans

The polymeric galactomannan, locust bean gum (0.1%; w/v) was incubated with 0.2 U of each α-galactosidase at its optimum pH and temperature for 2 h. Aliquots (100 μL) were taken at regular time intervals of 1 h, boiled to stop the reaction and 5 μL was applied on silica gel G plates (20 x 20 cm) and developed by ascending thin layer

chromatography using *n*-propanol: ethyl acetate: water (6:1:3, v/v) as the solvent system (Tanaka et al. 1975). The hydrolysis products were located by keeping the plates in an oven at 140 °C for 5 min after spraying with 1% α-naphthol in absolute ethanol containing 10% of *ortho*-phosphoric acid (Albon & Gross 1950).

5.2.12. Stereochemical analysis of hydrolysis products by ^{1}H NMR

The stereochemistry of hydrolysis by α-galactosidases was determined by ^{1}H NMR spectroscopy using *p*NPG as substrate. Prior to analysis by NMR, the reaction mixture was concentrated to dryness using Eppendorf concentrator 5301 and then dissolved in ^2H_2O. The ^{1}H NMR spectra of the hydrolysis products were recorded using Bruker Avance DPX Spectrometer operating at 300 MHz. The anomeric resonances of α- and β-anomers were determined relative to the chemical shift δ of HO^2H at 4.79 ppm.

5.2.13. Effect of various metal ions, sugars and inhibitor reagents

The enzyme was incubated with various metal ions, sugars and inhibitor reagents at room temperature for 10 min and enzyme assay was carried out under standard conditions. Relative activity was calculated as the percentage of activity obtained in the absence of these chemicals.

5.2.14. Kinetics of enzyme inhibition

To determine the type of inhibition, kinetic constants K_m and V_{max} of hydrolysis of *p*NPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations of inhibitor (10-100 mM). For the determination of inhibition constant (K_i) for competitive inhibitors enzyme assay was carried out under standard assay conditions at two different substrate concentrations after incubating the enzyme in different concentrations of inhibitor (10-100 mM). The K_i was determined by Dixon method using the Enzyme Kinetics module of Sigmaplot (Systat software Inc. Version 1.2.0.0).

5.2.15. Galactose tolerance studies

The galactose tolerance of the enzyme was investigated by determining relative activity under standard assay conditions after incubating the enzyme with different concentrations of galactose (10-100 mM) for 30 min at room temperature.

5.2.16. Effect of amino acid group specific reagents

The residual activity of enzyme samples was determined by standard assay method after incubating (30 min at room temperature) the purified α-galactosidases with chemical reagents (*N*-ethyl maleimide (NEM), *p*-chloromercuribenzoate (PCMB), 2,2-dithiobisnitrobenzoic acid (DTNB), diethylpyrocarbonate (DEPC), *N*-acetylimidazole (NAI), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N*-bromosuccinimide (NBS), phenylmethyl-sulfonylfluoride (PMSF), 2,3-butanedione, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and Woodward's reagent K (WRK); Sigma) specific to various amino acid functional groups at specific concentration.

5.2.17. Statistical analysis

The data presented here are the average of triplicate determinations and the standard deviation for all the values were <± 5%.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification and biochemical properties of α-galactosidases

The crude enzyme extract obtained after solid-state fermentation was subjected to ultra-filtration, hydrophobic interaction chromatography and gel filtration chromatography. Preliminary zymogram analysis of the crude enzyme extract with MU-α-gal indicated the presence of three proteins with α-galactosidase activity (Fig. 5.2). But all the three α-galactosidases co-eluted during hydrophobic interaction chromatography (Fig. 5.3a) with a reverse gradient of ammonium sulphate (0.8-0.45 M) in 50 mM sodium phosphate buffer (pH 7.0). Complete purification of the three α-galactosidases was achieved by gel filtration chromatography in which they were resolved into three peaks (Fig. 5.3b). The three purified α-galactosidases were

designated α-Gal I, α-Gal II and α-Gal III. α-Gal I which showed the highest enzyme activity and specific activity was identified as the main α-galactosidase. The summary of purification steps is shown in Table 5.1.

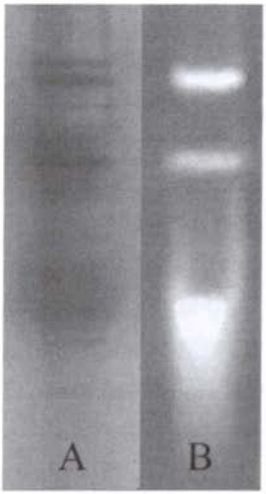


Fig. 5.2. (A) Native PAGE and (B) zymogram analysis for α-galactosidase activity of the crude enzyme extract from *S. griseololabus*. α-Galactosidase activity was visualized by incubating gel at 55 °C for 10 min in 0.1 M citrate phosphate buffer (pH 7.0) containing fluorescent substrate, MU-α-gal (1 mM)

Table 5.1. Purification of α-galactosidases from *S. griseololabus*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme extract	1114.7	1431.5	0.78	100	1
Ultrafiltration	1090.4	356.1	3.1	97.8	3.9
Phenyl Sepharose CL 4B	860.5	20	43	77.2	55.1
Sephadex G100					
α-Gal I	353.7	2	176.9	31.7	226.8
α-Gal II	184.8	1.1	168	16.6	215.4
α-Gal III	197.8	3.2	61.8	17.7	79.2

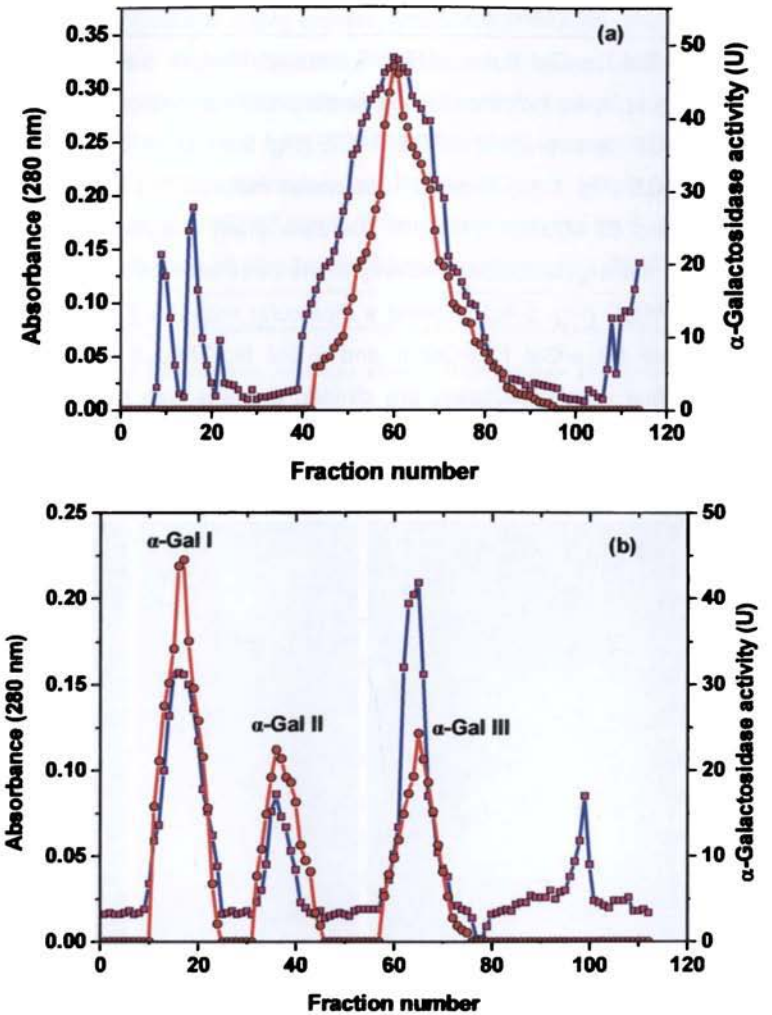


Fig.5.3. Elution profile of proteins ( ) and α-galactosidases ( ) in (a) hydrophobic interaction chromatography with Phenyl Sepharose CL 4B and (b) gel filtration chromatography with Sephadex G 100.

α-Gal I, α-Gal II and α-Gal III were purified to homogeneity with gel filtration chromatography as indicated by the single protein bands obtained with each sample in native PAGE, zymogram and SDS PAGE (Fig. 5.4a, b, c). The native M_r determined by native PAGE (Fig. 5.4a) showed a molecular mass of 141 kDa for α-Gal I, 113 kDa for α-Gal II and 89 kDa for α-Gal III. The zymogram analysis with MU-α-gal (Fig. 5.4b) confirmed the α-galactosidase activity of the three purified proteins. The M_r determined by SDS PAGE (Fig. 5.4c) showed a molecular mass of 72 kDa, 57 kDa and 35 kDa respectively for α-Gal I, α-Gal II and α-Gal III. Thus it could be possible that *S. griseoloalbus* α-galactosidases are dimeric proteins with monomers of approximately same molecular masses.

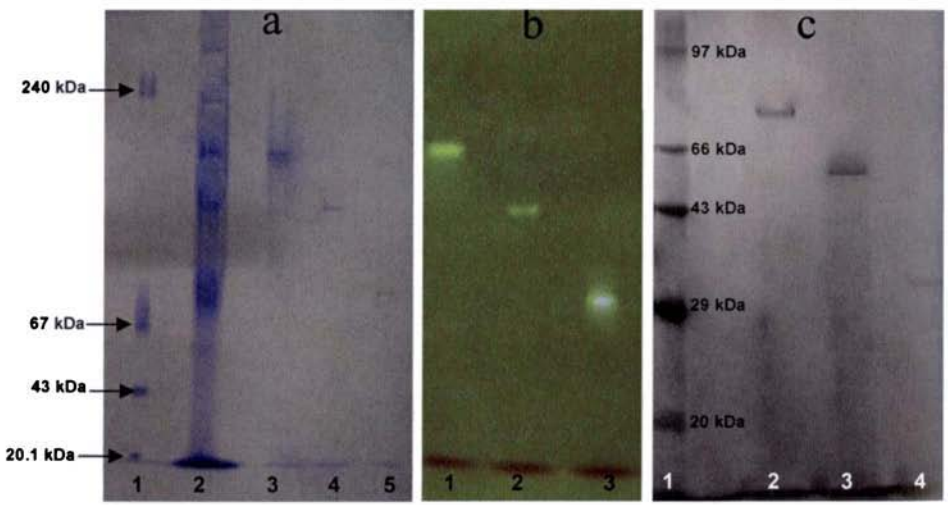


Fig. 5.4. Electrophertic patterns of *S. griseoloalbus* α-galactosidases in (a) Native PAGE after each chromatographic step of purification, Lane 1: Fraction from hydrophobic interaction chromatography, Lanes 2, 3 and 4: α-Gal I, α-Gal II and α-Gal III from gel filtration chromatography (b) Zymogram analysis of (Lane 1) α-Gal I, (Lane 2) α-Gal II and (Lane 3) α-Gal III from gel filtration chromatography (c) SDS PAGE of the fractions from gel filtration chromatography, Lane 1: Molecular mass markers, Lanes 2, 3 and 4: α-Gal I, α-Gal II and α-Gal III respectively.

The *N*-terminal sequence of first 10 amino acid residues of the purified α-Gal I, the main α-galactosidase active protein, was MAHLPMRAPR. The BLASTP search of the Non-redundant Peptide Sequence Database using the confirmed 10 *N*-terminal amino acid sequence of α-Gal I showed that it was 80% homologous (Fig. 5.5) to the *N*-terminus of α-galactosidase from *S. coelicolor* A3(2), accession number CAB54169 (Bentley et al. 2002) belonging to family 27 of glycosyl hydrolases (GH) (http://www.cazy.org/fam/GH27.html). This sequence similarity suggested that α-Gal I from *S. griseoloalbus* could also be classified as a member of GH27 family.

```
>  ref|NP_624611.1|  probable secreted alpha-galactosidase [Streptomyces coelicolor
A3(2)]
 emb|CAB54169.1|  probable secreted alpha-galactosidase [Streptomyces coelicolor
A3(2)]
Length=680

 GENE ID: 1095706 SCO1654 | probable secreted alpha-galactosidase
[Streptomyces coelicolor A3(2)] (10 or fewer PubMed links)

 Score = 29.1 bits (61),  Expect =    40
 Identities = 8/10 (80%), Positives = 8/10 (80%), Gaps = 0/10 (0%)

Query  1  MAHLPMRAPR  10
          M HLPMR PR
Sbjct  1  MRHLPMRVPR  10
```

Fig. 5.5. BLASTP search result for the confirmed 10 *N*-terminal amino acid sequence of *S. griseoloalbus* α-Gal I

The total carbohydrate contents of the three α-galactosidases were 15.3%, 19.1% and 8% respectively. The pI of α-Gal I, α-Gal II and α-Gal III were 4.41, 5.6 and 6.13 respectively indicating that they are acidic in nature. The isoelectric point of the α-galactosidases reported here are in well agreement with the values reported for other α-galactosidases (Hashimoto et al. 1993; Puchart et al. 2000; Gote et al. 2006).

5.3.2. Effect of pH and temperature on enzyme activity and stability

The optimum pH for highest enzyme activity was 5.0, 6.5 and 5.5 respectively for α-Gal I, α-Gal II and α-Gal III (Fig. 5.6a). All the three enzymes showed more activity towards acidic range than towards alkaline range where only negligible activity was detected. α-Gal I was active over a wider range of pH (3.0-9.0) than α-Gal II (pH 4.0-

8.0) and α-Gal III (pH 4.0-9.0). The pH stability studies also gave similar results (Fig. 5.6b), with α-Gal I having stability over a wider pH range (5.0 to 7.0) for 2 h than α-Gal II and α-Gal III which remained stable only in a narrow range of pH (5.5-7.0). Moreover, α-Gal I could retain more than 85% of its original activity at pH 4.0 and 8.0 after 2 h. Most bacterial α-galactosidases show narrow pH optima and are stable over an alkaline pH range (Gote et al. 2004). *S. coelicolor* A3(2) family 36 α-galactosidase is reported to be most active at pH 7.0 and is stable between pH 7.0 and 9.5 for 1 h (Kondoh et al. 2005). *S. griseoloalbus* α-Gal I is unusual in this respect with broad pH activity and stability profile. This is an appreciable character for diverse industrial applications since it eliminates the need for pH adjustment for enzyme action.

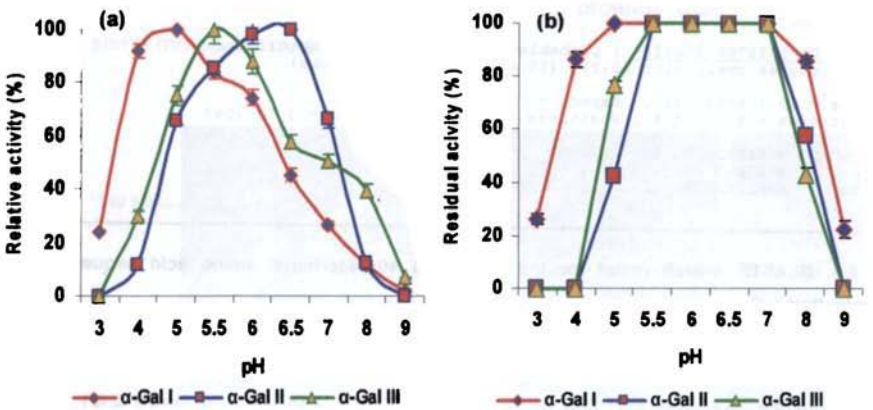


Fig. 5.6. (a) Effect of pH on activity of purified α-galactosidases. The enzyme assay was carried out at different pH (3.0-10.0) and the relative activity was determined at 55 °C. (b) Effect of pH on stability of purified α-galactosidases. The purified α-galactosidases were incubated at different pH (3.0-10.0) for 2 h and the residual activity was determined.

The optimum temperature for maximum activity of α-Gal I, α-Gal II and α-Gal III were 65, 50 and 55 °C respectively (Fig. 5.7a). Thermostability of α-galactosidases was determined by studying the time-dependent thermal inactivation of enzymes as shown in Fig 5.6b, c and d. α-Gal I was completely stable at 50-65 °C for 2 h. The half-life of

inactivation ($t_{1/2}$) of α-Gal I at 70 °C was 30 min. α-Gal II and α-Gal III behaved similarly with respect to thermal stability. Both α-galactosidases were completely stable upto 55 °C for 2 h and the $t_{1/2}$ at 60 °C was 30 min. *S. coelicolor* A3(2) family 36 α-galactosidase is reported to be most active at 40 °C and stable only upto 30 °C for 1 h (Kondoh et al. 2005).

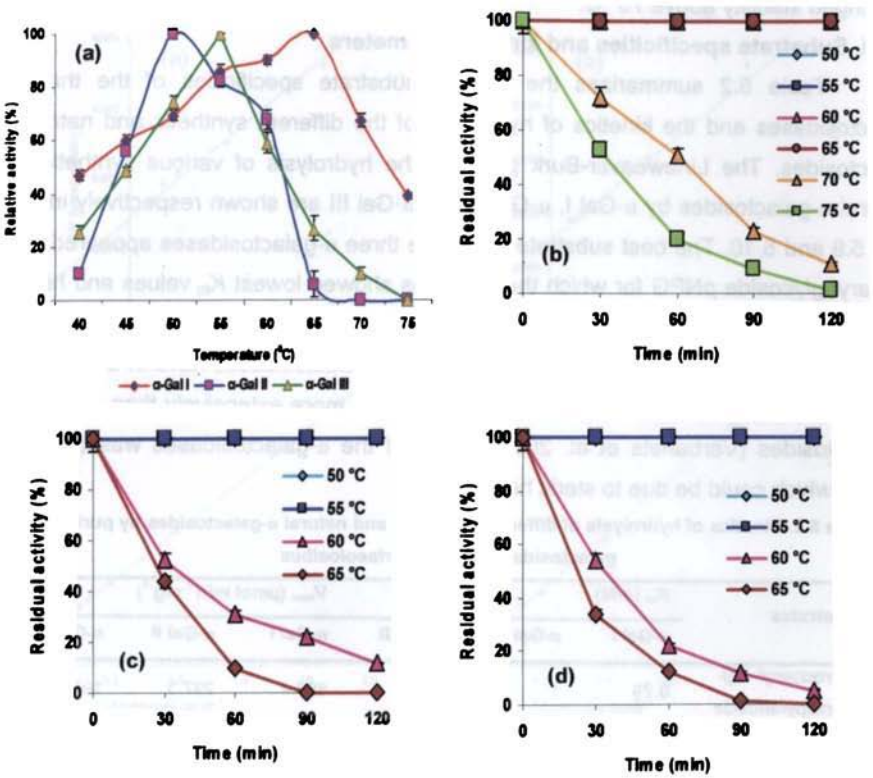


Fig. 5.7. (a) Effect of temperature on activity of purified α-galactosidases. The enzyme assay was carried out at different temperature (40-75 °C) and optimum pH and the relative activity was determined. **(b) Time-dependent thermal inactivation of α-Gal I at 50-75 °C, (c) α-Gal II and (d) α-Gal III at 50-65 °C.** The enzyme was incubated at the above mentioned temperatures and optimum pH for 2 h and the residual activity was determined at regular time intervals of 30 min.

The thermostability of the enzyme is advantageous for industrial application, since most of the industrial processes are carried out at high temperatures which lead to denaturation of thermolabile enzymes. α-Galactosidase of hyperthermophilic bacteria *Thermotoga maritima* ($t_{1/2}$ 6 h at 85 °C) (Miller et al. 2001) and *Thermotoga neapolitana* (75% activity after 4 h at 85 °C) (King et al. 1998) are reported to have activity and prolonged stability above 75 °C.

5.3.3. Substrate specificities and kinetic parameters

Table 5.2 summarizes the relative substrate specificities of the three α-galactosidases and the kinetics of hydrolysis of the different synthetic and natural α-galactosides. The Lineweaver-Burk plots for the hydrolysis of various synthetic and natural α-galactosides by α-Gal I, α-Gal II and α-Gal III are shown respectively in Figs. 5.8, 5.9 and 5.10. The best substrate for all the three α-galactosidases appeared to be the aryl glycoside *p*NPG for which the enzymes showed lowest K_m values and highest V_{max}. The K_m values are not absolute constants but depend on the temperature, substrate and source of enzyme. Most microbial α-galactosidases have in common the fact that they can hydrolyze the synthetic substrates more extensively than the natural α-galactosides (Varbanets et al. 2001). None of the α-galactosidases was active on *m*NPG which could be due to steric hindrance.

Table 5.2. Kinetics of hydrolysis of different synthetic and natural α-galactosides by purified α-galactosidases from *S. griseoloalbus*

Substrates	K_m (mM)			V_{max} (μmol min^{-1} mg^{-1})		
	α-Gal I	α-Gal II	α-Gal III	α-Gal I	α-Gal II	α-Gal III
p-nitrophenyl-α-D-galactopyranoside	0.79	1	1.3	693.4	297.3	195.3
o-nitrophenyl-α-D-galactopyranoside	0.96	1.9	1.6	129.7	125.9	54.7
Melibiose	3.2	2.6	2.9	101.1	228.6	108
Raffinose	5.8	3.7	5	163.6	280.4	121.9
Stachyose	6	2.5	2.5	139	228.9	109.9

The *S. griseoloalbus* α-galactosidases were also active on naturally occurring α-1-6 linked galactooligosaccharides like melibiose, raffinose and stachyose. The

specificities for the hydrolysis of these galactosides were in the order melibiose>raffinose>stachyose for α-Gal I (Table 5.2 and Figs. 5.8c, d, e). For α-Gal II (Table 5.2 and Figs. 5.9c, d, e) and α-Gal III (Table 5.2 and Figs. 5.10c, d, e) the substrate specificities were in the order stachyose>melibiose >raffinose.

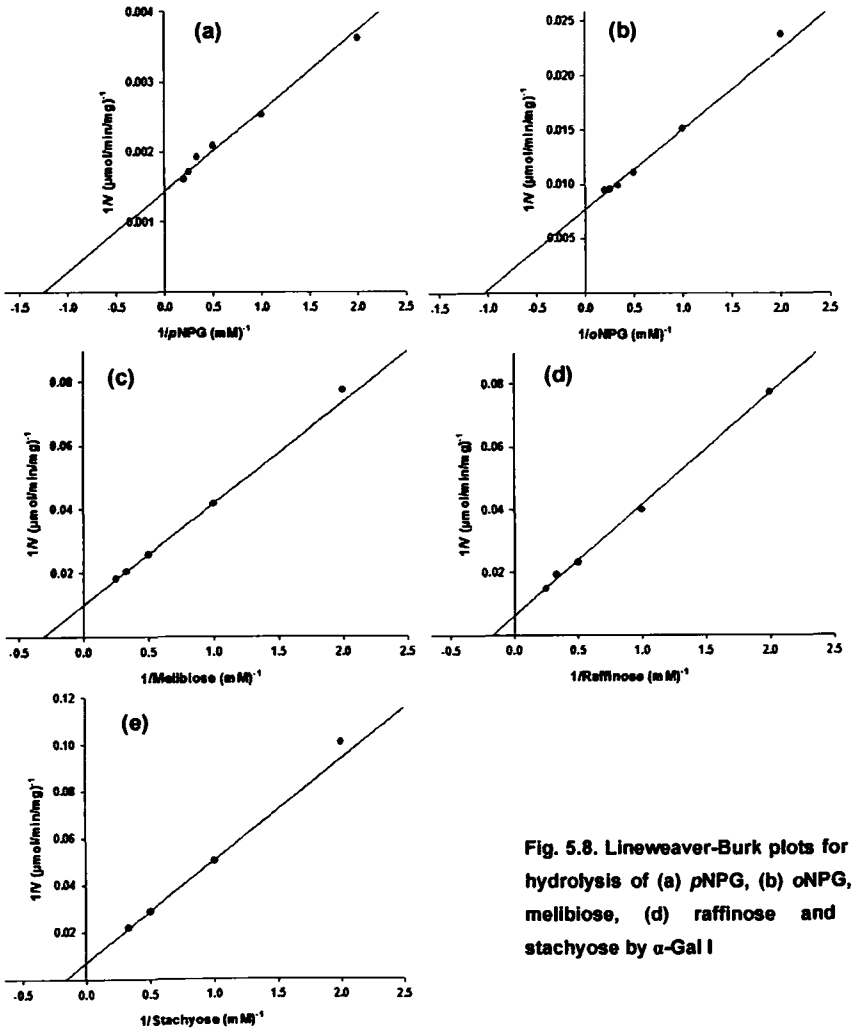


Fig. 5.8. Lineweaver-Burk plots for the hydrolysis of (a) *p*NPG, (b) oNPG, (c) melibiose, (d) raffinose and (e) stachyose by α-Gal I

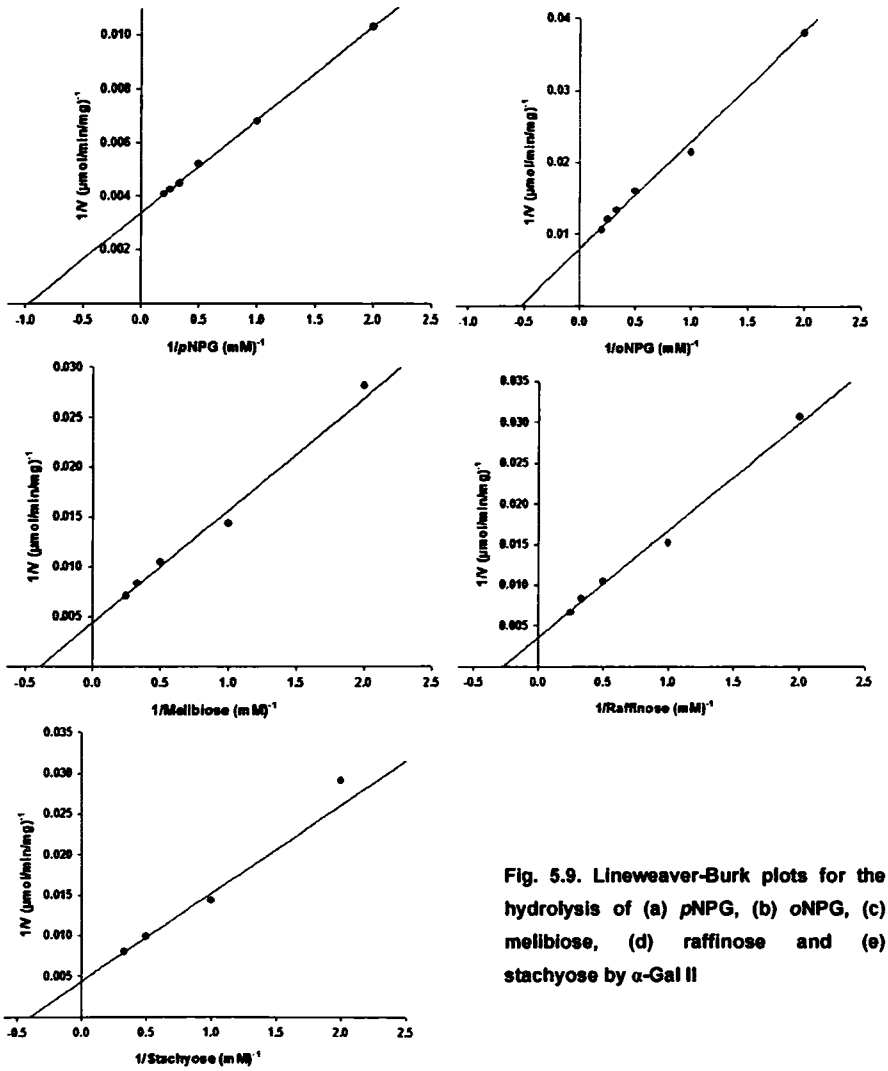


Fig. 5.9. Lineweaver-Burk plots for the hydrolysis of (a) *p*NPG, (b) oNPG, (c) melibiose, (d) raffinose and (e) stachyose by α-Gal II

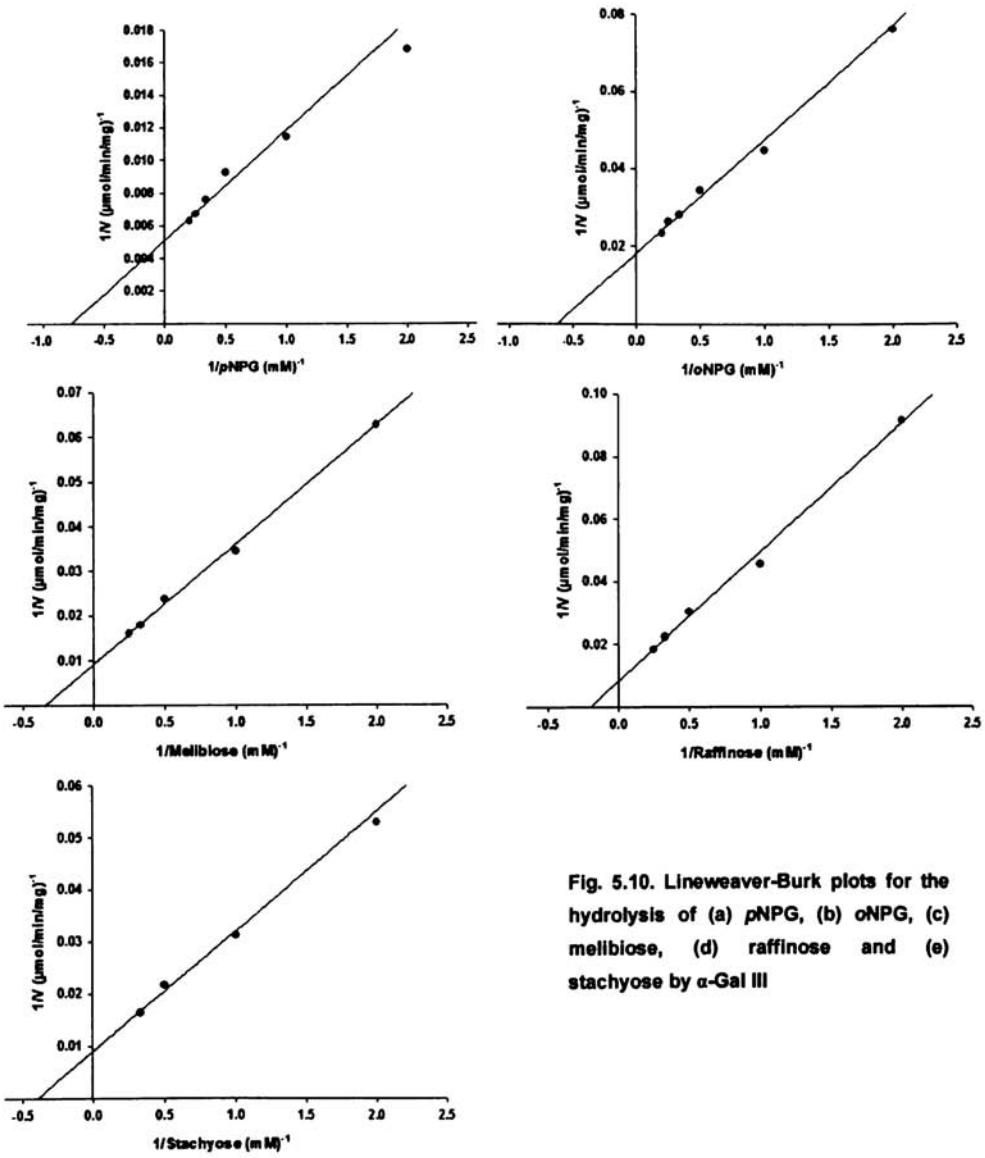


Fig. 5.10. Lineweaver-Burk plots for the hydrolysis of (a) *p*NPG, (b) oNPG, (c) melibiose, (d) raffinose and (e) stachyose by α-Gal III

S. griseoloalbus α-galactosidases showed remarkable differences from *S. coelicolor* A3(2) family 36 α-galactosidase with respect to substrate preference also. *S. coelicolor* A3(2) family 36 α-galactosidase is reported to hydrolyze raffinose and stachyose, but not melibiose (Kondoh et al. 2005). On the contrary, *S. griseoloalbus* α-galactosidases were also active on melibiose.

5.3.4. Hydrolysis of polymeric galactomannans

The hydrolysis of LBG as visually assayed by TLC (Fig. 5.11a) indicated that *S. griseoloalbus* α-Gal I was active on polymeric galactomannans. The other two α-galactosidases were not active on polymeric galactomannans (Fig. 5.11b).

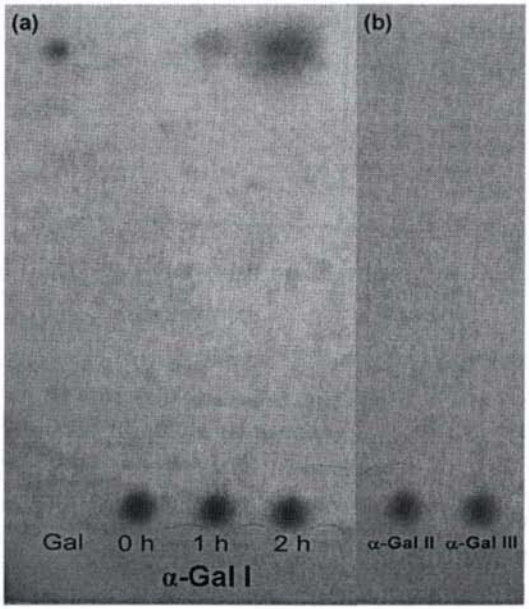


Fig. 5.11 (a) Thin layer chromatograph showing the galactose released from LBG by the hydrolytic action of *S. griseoloalbus* α-Gal I at regular time intervals of 1 h. Gal: Authentic galactose. (b) Thin layer chromatograph showing that α-Gal II and α-Gal III are not active on polymeric galactomannans, as there was no release of galactose.

α-Galactosidases can be classified into two groups on the basis of their hydrolytic properties (Dey & Pridham 1972) - one group specific for low molecular weight α-galactosides such as alkyl- and aryl galactosides, melibiose and the raffinose

family oligosaccharides, and the other type of enzymes specific for polymeric galactomannans, however they are also able to hydrolyze low molecular weight substrates to various extents. In general bacterial and fungal α-galactosidases of GH27 family are active on oligosaccharides and intact polymeric galactomannans, whereas α-galactosidases of GH36 family are more specific towards oligosaccharides and have little or no activity on polymeric substrates. The results obtained in the present investigation were confirmative of the fact that *S. griseoloalbus* α-Gal I belonged to GH27 family. Since the other two α-galactosidases, α-Gal II and α-Gal III, were not active on polymeric galactomannans, they could be members of GH36 family. There are reports available in the literature documenting the presence of both types of α-galactosidases from the same organism, for e.g. *Trichoderma reesei* (Margolles-Clark et al. 1996) and *A. niger* (Ademark et al. 2001).

5.3.5. Stereochemical analysis of hydrolysis products by ^{1}H NMR

The ^{1}H NMR spectrum of *p*NPG hydrolysis by α-Gal I, α-Gal II and α-Gal III (Fig. 5.12) clearly suggested a retentive, double displacement mechanism of hydrolysis for α-Gal I, α-Gal II and α-Gal III, though they belonged to two different families of glycosyl hydrolases. The α-anomer of D-galactose was formed at the initial stages of hydrolysis followed by its slow mutarotation to β-anomer at later stages. Recently Comfort et al. (2007) reported the mechanistic commonality of GH27 and GH36 α-galactosidases. The double displacement mechanism involves two catalytic residues, one responsible for the protonation of the glycosidic oxygen and the other stabilization of a carbocationic intermediate. The glycosyl-enzyme intermediate is decomposed by transfer of glycosyl moiety to an acceptor molecule, which in case of hydrolysis is water, but hydroxyl groups of sugar may also act as the glycosyl acceptor, leading to transglycosylation (Henrissat 1991).

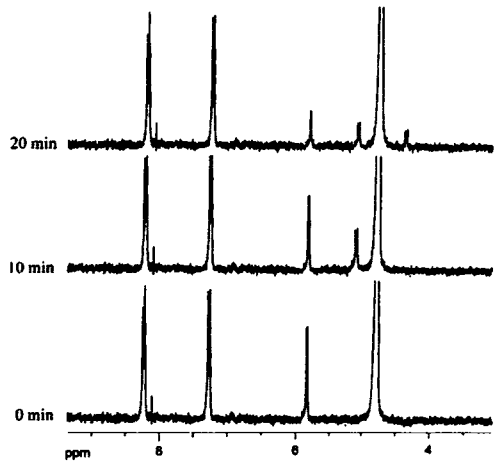


Fig. 5.12. Stereochemistry of hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside by *S. griseoloalbus* α-Gal I. The spectra show the proton resonances of free and bound 4-nitrophenol (7.2-8.2 ppm) and anomeric resonances of un-hydrolyzed substrate (5.8 ppm), released α-galactose (5.1 ppm) and β-galactose (4.4 ppm) at different periods of incubation. The anomeric resonances were determined relative to the chemical shift δ of HO^2H at 4.79 ppm. The α-Gal II and α-Gal III also had a similar retaining mechanism of action and the ^1H NMR spectra of their hydrolysis is not shown.

5.3.6. Effect of metal ions, sugars and inhibitor reagents

The effects of various metal ions, sugars and inhibitors on *S. griseoloalbus* α-galactosidases are summarized in Table 5.3. Among the metal ions tested Hg^{2+}, Cu^{2+} and Ag^{2+} completely inhibited all the three α-galactosidases. Similar results were reported previously for other thermostable α-galactosidases from *Thermus brockianus* (Fridjonsson et al. 1999) and *Baciullus stearothermophilus* (Gote et al. 2006). This inhibition usually suggests reaction with thiol groups and/or carboxyl, amino and imidazolium group of histidine in the active site (Dey & Pridham 1972).

Other metal cations like Na^+, K^+, Ca^{2+}, Co^{2+}, Mn^{2+} and Fe^{3+} did not influence the activity of *S. griseoloalbus* α-galactosidases. Mg^{2+} did significantly stimulate enzyme activity of α-Gal I. However, the metal chelating agent, EDTA did not inhibit the activity of *S. griseoloalbus* α-galactosidases, indicating that they are not metalloenzymes. These results suggested that metal cations are not involved in the catalytic site of the enzyme and activation by the specific cation Mg^{2+} could be due to its possible role in modulating enzyme activity according to environmental conditions. Mg^{2+} is known to

play a role in activating α-galactosidase from *Thermomyces lanuginosus* (Rezessy-Szabó et al. 2007).

Table 5.3. Effect of metal ions, sugars and inhibitor reagents on the activity of purified α-galactosidases from *S. griseoloalbus*

Cation/Sugar/Inhibitor[a]	Relative activity (%)[b]		
	α-Gal I	α-Gal II	α-Gal III
Na^+	100	100	100
K^+	100	97.1	100
Mg^{2+}	124	100	100
Mn^{2+}	98.2	95.7	100
Hg^{2+}	0	0	0
Ag^{2+}	0.19	0	0
Cu^{2+}	0.1	0	0
Ca^{2+}	97.8	99.5	100
Co^{2+}	99.9	98.6	100
Fe^{3+}	98.6	99.7	100
EDTA	99.4	98.2	100
SDS	0	0	0
β-mercaptoethanol	96.9	99.4	100
Urea	100	100	100
Galactose	100	74. 9	71.3
Melibiose	76.3	60.2	77.8

[a] The concentration of cations and β-mercaptoethanol were 1 mM, EDTA 10 mM, urea 1 M and sugars 10 mM.

[b] Relative activity was calculated as the percentage of activity obtained in the absence of cations/sugars/inhibitors.

Among the various sugars tested melibiose was found to be inhibitory to all the three α-galactosidases when *p*NPG was used as the assay substrate. α-Gal I was not inhibited by galactose where as the other two enzymes were inhibited by galactose.

5.3.7. Kinetics of enzyme inhibition

Melibiose and galactose (whenever inhibitory) acted as competitive inhibitors since the K_m for *p*NPG increased and V_{max} remained constant (Fig. 5.13 and 1.14). The

inhibition constant, K_i of galactose for α-Gal II and α-Gal III as determined from Dixon plot (Fig. 5.15) were 23.4 and 13.1 respectively. The K_i of melibiose (Fig. 5.16) were 13.3, 12.8 and 14.9 respectively for α-Gal I, α-Gal II and α-Gal III.

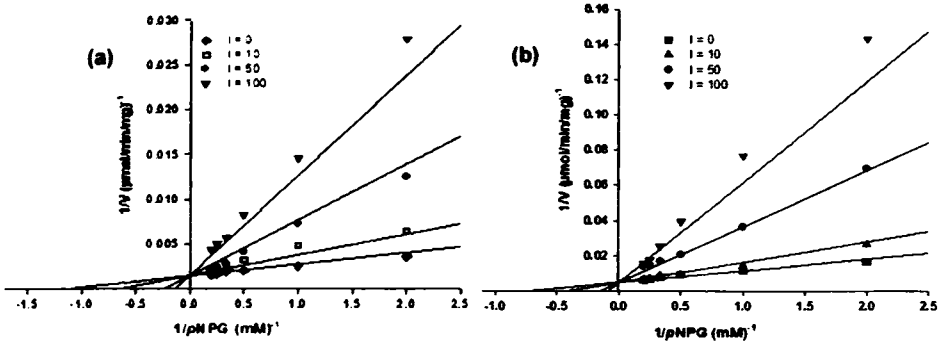


Fig. 5.13. Lineweaver-Burk plots showing competitive inhibition by galactose on (a) α-Gal II and (b) α-Gal III. To determine the type of inhibition, kinetic constants K_m and V_{max} of hydrolysis of *p*NPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations (10-100 mM) of galactose.

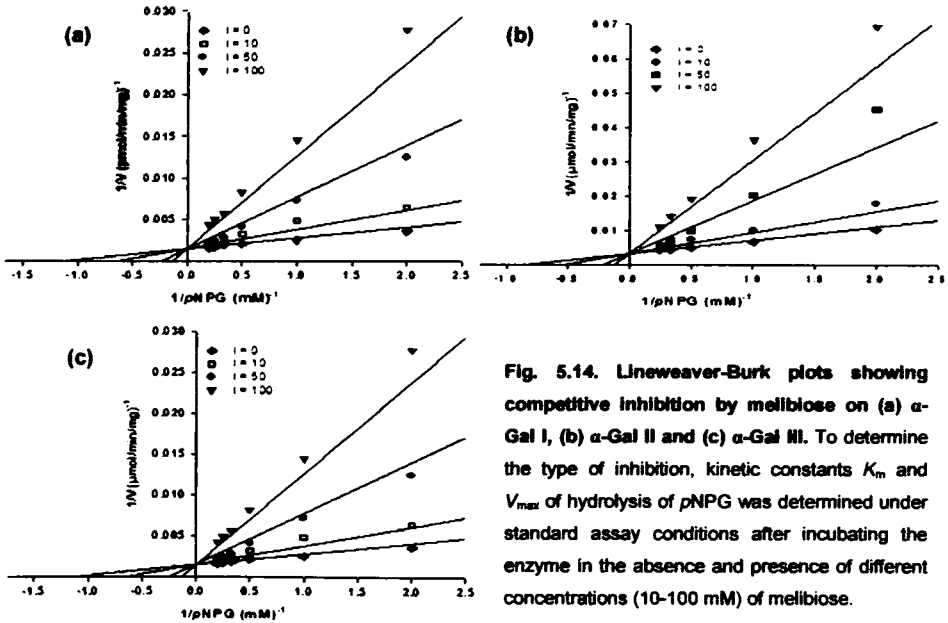


Fig. 5.14. Lineweaver-Burk plots showing competitive inhibition by melibiose on (a) α-Gal I, (b) α-Gal II and (c) α-Gal III. To determine the type of inhibition, kinetic constants K_m and V_{max} of hydrolysis of *p*NPG was determined under standard assay conditions after incubating the enzyme in the absence and presence of different concentrations (10-100 mM) of melibiose.

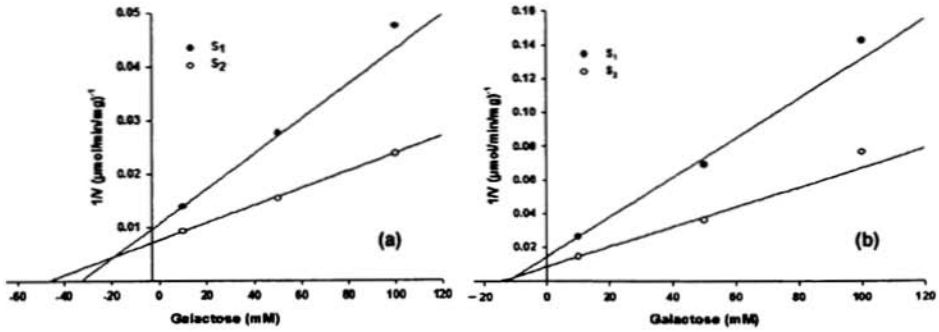


Fig. 5.15. Dixon plots for the inhibition of (a) α-Gal II and (b) α-Gal III by galactose. α-Galactosidase activity was determined under standard assay conditions at two different concentrations of *p*NPG after incubating the enzyme for 10 min with different concentrations of galactose (10-100 mM).

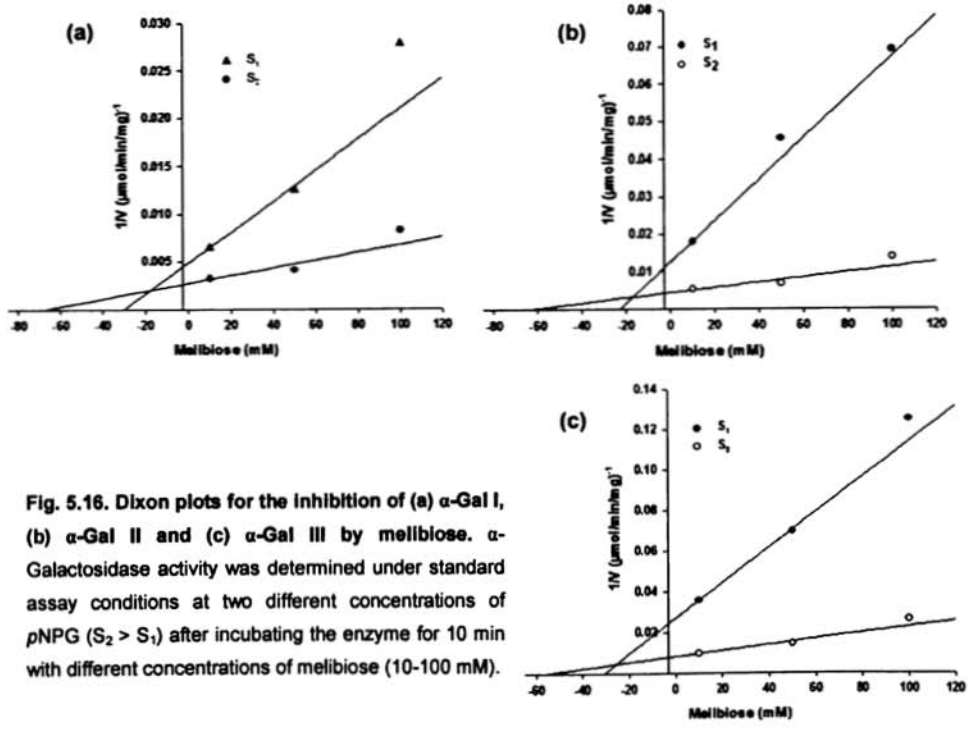


Fig. 5.16. Dixon plots for the inhibition of (a) α-Gal I, (b) α-Gal II and (c) α-Gal III by melibiose. α-Galactosidase activity was determined under standard assay conditions at two different concentrations of *p*NPG ($S_2 > S_1$) after incubating the enzyme for 10 min with different concentrations of melibiose (10-100 mM).

5.3.8. Galactose tolerance studies

It was interesting to find that α-Gal I was tolerant to even concentrations of galactose as high as 100 mM. The relative activity was 100% and the kinetic constants K_m (0.79 mM) and V_{max} (693.4 μmol min^{-1} mg^{-1}) of hydrolysis of *p*NPG remained constant despite the increase in galactose concentration. Galactose is reported to be a powerful competitive inhibitor of α-galactosidases from *Aspergillus niger* (Ademark et al. 2001), *Bacillus stearothermophilus* (Gote et al. 2006). But Suzuki et al. (1970) reported a mixed type of inhibition by D-galactose on α-galactosidase from *Mortierella vinacea* suggesting its competitive and non-competitive binding on the enzyme. Luonteri et al. (1998a) reported three α-galactosidases from *Penicillium simplicissimum* of which AGL II showed more resistance to product inhibition by galactose than the other two enzymes, AGL I and AGL III. α-Gal I from *S. griseoloalbus* is novel in this respect and is important from industrial point of view since galactose tolerance is an appreciable character which improves the efficiency of α-galactosidases in liberating galactose residues from oligomeric and polymeric α-galactosides. The exact reason behind this unique galactose tolerance is unclear and can be of interest for another detailed investigation.

5.3.9. Effect of amino acid group specific reagents

The role of amino acid functional groups on activity of the three α-galactosidases was studied using amino acid group specific reagents and the results are summarized in Table 5.4. NAI, TNBS, PMSF and 2,3-butanedione did not affect the activity of α-Gal I, α-Gal II and α-Gal III indicating that tyrosine, lysine, serine and arginine do not play any role in the catalytic action. Chemical modification of sulphydryl groups by NEM and DTNB did not influence the activity of *S. griseoloalbus* α-galactosidases, whereas PCMB almost inactivated the enzymes. This could be attributed to the non-competitive nature of inhibition by Hg^{2+} in PCMB rather than due to the modification of sulphydryl groups. Sulphydryl groups are reported to play an important role in maintaining the active conformation of the protein molecule rather than

taking part in catalysis (Malanchuk et al. 2001). α-Gal I, α-Gal II and α-Gal III were inhibited by DEPC, NBS, EDAC and WRK indicating the presence of histidine, tryptophan and carboxylate residues at or near the active site. Although DEPC is used for the specific chemical modification of histidine, it also reacts with cysteine, tyrosine and lysine residues. Since NEM, DTNB, NAI and TNBS did not inhibit enzyme activity in any way, the possible role of these amino acids could be excluded. Hence loss of enzyme activity with DEPC could be correlated to the chemical modification of histidine.

Table 5.4. Effect of amino acid group specific reagents on the activity of purified α-galactosidases from *S. griseoloalbus*

Amino acid group specific reagent	Possible amino acid modified	Concentration	Residual activity (%)		
			α-Gal I	α-Gal II	α-Gal III
NEM	Cys	10 mM	100	96.8	99.3
DTNB	Cys	1 mM	99.9	97.2	100
PCMB	Cys	1 mM	2.9	0.19	0
DEPC	His	2 mM	0	0	0
NAI	Tyr	1 mM	100	100	100
TNBS	Lys	1 mM	99.9	100	100
NBS	Trp	2 mM	0	0	0
PMSF	Ser	2 mM	100	100	100
2,3-Butanedione	Arg	10 mM	100	100	100
EDAC	Asx/Glx	10 mM	19.1	22.7	24.4
WRK	Asx/Glx	10 mM	17.9	12.3	14.7

Tryptophan is reported to play a significant role in the catalytic site as well as in the thermal stabilization of active site conformation of the enzyme molecule at high temperature (Gote et al. 2007). It is also known that hydrophobic interactions are often important contributors to the over all stability of proteins (Bund & Singhal 2002). The tryptophan residue at position 16 of coffee bean α-galactosidase has previously been shown to be essential for enzyme activity (Zhu et al. 1996). Involvement of carboxyl groups in the catalysis of α-galactosidases from *Humicola* sp. (Kotwal et al. 2000), *B. stearothermophilus* (NCIM 5146) (Gote et al. 2007) and coconut kernel (Mathew &

Balasubramaniam 1986) has already been reported. α-Galactosidase from *Vicia faba* also shows the presence of a single carboxylate and a histidine residue in catalysis (Dey & Pridham 1969a).

5.4. CONCLUSION

The multiple α-galactosidases- α-Gal I, α-Gal II and α-Gal III, produced by *S. griseoloalbus* were purified to homogeneity by a two-step chromatographic process. The purification protocol employed was rapid and provided a consistently pure source of α-galactosidases. The molecular masses and pI of the three enzymes were 72, 57 and 35 kDa, and 4.41, 5.6 and 6.13 respectively. α-Gal I showed *N*-terminal sequence homology to *S. coelicolor* A3(2) family 27 α-galactosidase. The optimum pH and temperature of the three α-galactosidases were 5.0, 6.5 and 5.5 and 65 °C, 50 °C and 55 °C respectively. α-Gal I was stable up to 65 °C and α-Gal II and α-Gal III up to 55 °C for 2 h. Based on the hydrolytic properties α-Gal I could be classified as a member of GH27 family and α-Gal II and α-Gal III as members of GH36 family with a retaining mechanism of hydrolysis. Metal cations like Hg^{2+}, Ag^{2+} and Cu^{2+} inhibited enzyme activity while Mg^{2+} enhanced the activity of α-Gal I. α-Gal I showed unusual tolerance to even higher concentrations of galactose, unlike the other two α-galactosidases which were competitively inhibited by galactose. Melibiose was a competitive inhibitor of all the three enzymes. Histidine, tryptophan and carboxylic residues were essential for catalytic action of the three α-galactosidases.

Chapter VI

APPLICATIONS OF *STREPTOMYCES GRISEOLOALBUS* α-GALACTOSDIASES

6.1. INTRODUCTION

What make an enzyme widely acceptable and attractive are its excellent industrial applications and also its characteristics that suit its field of application. The interest of scientific community in α-galactosidase research stems from its versatile biotechnological and medicinal applications in diverse fields. α-Ggalactosidases play a crucial role in improving the nutritional value of legume-based food. Legumes are widely grown throughout the world and their dietary and economic importance is globally appreciated and recognized. Dry legumes not only add variety to human diet, but also serve as an economical source of proteins, dietary fibers and a variety of micronutrients and phytochemicals (Messina 1999). Legumes also have the potential to lower cholesterol and serum glucose and quicken adjustment to high altitude induced stress (Mazur et al. 1998). Supplementing cereal-based diets with legumes improves overall nutritional status and is one of the best solutions to protein calorie malnutrition in the developing countries.

Soybean (*Glycine max*) is a legume, rich in proteins and having a well-balanced amino acid pattern (Smith & Circle 1972). It has received considerable attention for its potential role in the prevention and treatment of cancer and osteoporosis since it is a rich source of isoflavones (Messina 1999). Soymilk, the aqueous extract of soybeans is a nutritious beverage rich in high quality proteins and contains no cholesterol or lactose and only small quantities of saturated fatty acids. It is considered as a low-cost substitute for dairy milk in developing countries and as a nutritive supplement for the lactose-intolerant persons. Horse gram (*Dolichos biflorus*) and green gram or mung bean (*Vigna radiata* L.) are among the most important food legumes grown and consumed in India. In addition to proteins, horse gram is a rich source of iron and molybdenum. Green gram is an excellent source of carbohydrates,

proteins and minerals and its protein quality is similar to or better than other legumes such as chickpea, black gram, peas, pigeonpea, etc. (Jood et al. 1986). Horse gram and green gram are consumed as whole seeds or sprouts by a large population in rural areas of southern India. Despite these facts, the utilization of legume food crops is constrained due to the presence of large amount of anti-nutritive factors, mainly the raffinose-family oligosaccharides (RFO), which induce flatulence.

The production of flatulence is regarded as being due to the lack of ability of human intestinal tract to synthesize the enzyme α-galactosidase, which is necessary to hydrolyze oligosaccharides containing α-galactosidic linkages. The predominant RFO, raffinose and stachyose, are relatively large and are hence not resorbed by the intestinal wall. The intact oligosaccharides therefore enter the lower intestine where they are metabolized by the microflora into carbon dioxide, hydrogen and, to a lesser extent, methane by anaerobic fermentation. It is the production of these gases which leads to the characteristic features of flatulence, namely nausea, cramps, diarrhea, abdominal rumbling, and the social discomfort associated with the ejection of rectal gas (Cristofaro et al. 1974). RFOs make up a substantial part (40%) of the soluble sugars found in soybean seeds. It is highly desirable to decrease the oligosaccharide content of legumes if they are to be more effectively exploited as relatively cheap substitutes for good quality proteins.

Many methods are being practiced for the processing of legume seeds, such as soaking, cooking and sprouting (Mulimani & Devendra 1998; Viana et al. 2005), which may bring about changes in the levels of RFO. The newly released high-yielding cultivars may not only have different grain quality characteristics, but also may behave differently from existing cultivars after processing and cooking. Of all the techniques proposed, the enzymatic processing by α-galactosidase has proved most effective (Mansour & Khalil 1998; Kotwal et al. 1998; Scalabrini et al. 1998; Thippeswamy & Mulimani 2002; Viana et al. 2005).

Immobilization of enzymes is now a widely used approach for obtaining reusable derivatives of enzymes. It is generally carried out by adsorption, covalent coupling to solid matrices or entrapment in polymeric substances such as alginate, κ-

carragenan etc. Often, immobilization also results in improvement of enzyme stability under process conditions. In addition immobilization offers the advantage of reusability of enzymes. Thus lower capital/energy costs and better logistics are associated with a process using an immobilized system. α-Galactosidase has been extensively studied in immobilized forms. Immobilization of α-galactosidase in different gel matrices such as calcium alginate (Prasanth & Mulimani 2005), κ-carragenan (Girigowda & Mulimani 2006), gelatin (Naganagouda et al. 2007) etc. for treatment of soymilk has been well documented.

The transglycosylation property of α-galactosidases, in addition to the hydrolytic property, also makes them excellent candidates for industrial application. Until recently, oligosaccharides have been used in the food industry as a source of energy or as sweeteners. Nowadays knowledge of their biological function and their role in cell-surface interactions has opened a new field of glycotechnology (Gabius & Gabius 1997). Apart from their traditional use, oligosaccharides find new applications as immunostimulating agents or prebiotic compounds able to modulate the colonic microflora towards a healthy balance (Gibson & Roberfroid 1995; Murata & Usui 2006). α-Galactosidases have been used for the synthesis of α-galacto-oligosaccharides (Hashimoto et al. 1995; Weignerová et al. 2001; Tzortis et al. 2003; Yamashita et al. 2005) which are expected to have possible application as drug carriers in drug delivery systems (Kitahata et al. 1992; Hara et al. 1994; Koizumi et al. 1995). α-Galacto-oligosaccharides produced by transfer reaction of α-galactosidase can be used as a prebiotic in functional food (Rivero-Urgell & Santamaria-Orleans 2001).

This chapter describes the potential applications of *Streptomyces griseoloalbus* α-galactosidases in the processing of legumes as well as in galacto-oligosaccharide synthesis by transglycosylation reaction.

6.2. MATERIALS AND METHODS

6.2.1. Production and extraction of crude α-galactosidase

The production of α-galactosidase from *S. griseoloalbus* was done by solid-state fermentation and the crude enzyme was extracted as described in Chapter IV.

6.2.2. Partial purification of α-galactosidase

The proteins in the crude enzyme extract were concentrated by ammonium sulphate precipitation. The α-galactosidase active fractions (50-80% saturated fractions) were pooled and the precipitate obtained by centrifugation (10,000 rpm, 20 min) was dissolved in minimal amount of buffer (50 mM sodium phosphate buffer, pH 7.0), dialyzed against the same buffer overnight at 4 °C and used as partially purified enzyme preparation.

6.2.3. Characterization of crude and partially purified α-galactosidase

The optimum pH for α-galactosidase activity was determined in the assay mixture over a pH range of 4.0 to 9.0 and the relative activity was calculated. For pH stability determination, enzyme preparations were incubated at pH values in the range of 4.0 to 9.0 for 5 h at optimum temperature and residual activity was determined at regular time intervals of 30 min.

The optimum temperature for enzyme activity was determined by incubating the assay mixture at different temperatures ranging from 40 to 75 °C and the relative activity was calculated. For temperature stability determination enyme preparations were incubated at temperatures in the range of 50 to 75 °C for 5 h at optimum pH and residual activity was determined as described previously.

6.2.4. α-Galactosidase assay

α-Galactosidase assay was carried out using the chromogenic substrate *p*-nitrophenyl α-D-galactopyranoside as described in Section 2.2.4 of Chapter II.

6.2.5. Processing of soymilk

6.2.5.1. Preparation of soymilk

Soybeans (*Glycine max*) (Fig. 6.1) purchased from the local market were used for the preparation of soymilk (Fig. 6.2) according to the method described by Thippeswamy & Mulimani (2002).

Fig. 6.1. Soybean seeds used for treatment with α-galactosidase

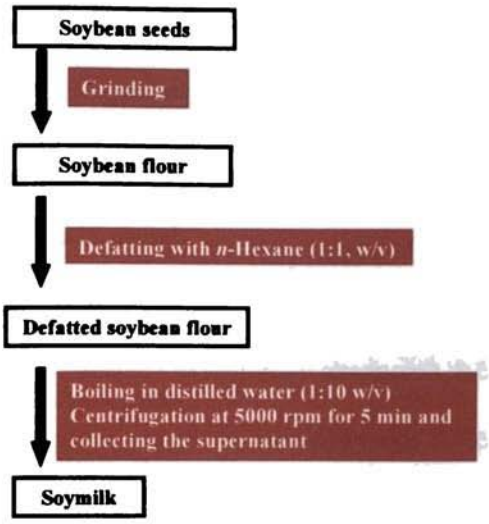


Fig. 6.2. Scheme for preparation of soymilk

6.2.5.2. Immobilization of α-galactosidase in gelatin-alginate blended beads

Partially purified α-galactosidase was entrapped in gelatin-alginate blended beads according to the method of Tanriseven & Dogan (2002). The partially purified α-galactosidase preparation (80 U) was mixed thoroughly with a solution containing 0.5 g alginate, 1 g gelatin, 4 mL glycerol and 2 mL acetate buffer (0.1 M, pH 5.0). The mixture obtained was then extruded drop-wise through a syringe fitted with a luer-lock needle into 50 mL of 0.2 M $CaCl_2$ prepared in 0.1 M acetate buffer (pH 5.0) containing 5% (v/v) glutaraldehyde. The beads were allowed to harden for 2 h, washed with acetate buffer and then with double-distilled water. The beads were stored in the same buffer until use. Since citrate and phosphate are reported to have a dissolution effect on alginate matrices, the immobilization experiments were carried out using 0.1 M acetate buffer (pH 5.0) instead of the McIlvaine buffer (0.1 M, pH 5.0) used in all other experiments.

The activity yield (%) of α-galactosidase immobilized in gelatin-alginate beads was calculated as the ratio of enzyme activity of the immobilized enzyme to the total units of soluble enzyme used for immobilization. The activity of immobilized enzyme was calculated as the difference between the total amount of enzyme used for immobilization and the enzyme activity left in the curing solution and wash outs.

$$\text{Activity yield} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100 \qquad \text{Eq. (1)}$$

6.2.5.3. Enzymatic treatment of soymilk

6.2.5.3.1. Batch experiments with free and immobilized α-galactosidase

Fifty milliliters each of soymilk was incubated separately with 50 U of free and immobilized α-galactosidase at 65 °C for 3 h. Two mL aliquots were removed at specific time intervals and kept in boiling water bath for 5 min to terminate the reaction. The proteins were precipitated by adding 0.2 mL of 0.3 M barium hydroxide and 0.2 mL of 0.18 M zinc sulphate. The sample was centrifuged to remove the precipitated proteins and the galactose liberated by enzymatic treatment was determined as reducing sugar by the method of Nelson (1944).

The effectiveness factor of the immobilized system was defined as the ratio of galactose liberated by the immobilized α-galactosidase to that of the free α-galactosidase.

$$\text{Effectiveness factor} = G_{imm}/G_{free} \qquad \text{Eq. (2)}$$

where G_{imm} is the amount of galactose liberated by immobilized enzyme and G_{free} is the amount of galactose liberated by free α-galactosidase.

6.2.5.3.2. Repeated batch experiments with immobilized α-galactosidase

Repeated batch experiments were carried out to study the operational efficiency of immobilized enzyme system. Fifty milliliters each of soymilk was treated

with 50 U of immobilized α-galactosidase in a rotary shaker at 50 and 65 °C for 3 h. After 3 h the increase in the amount of galactose liberated was estimated and the immobilized enzyme beads from the spent soymilk samples were transferred to fresh soymilk samples after washing with sterile distilled water and the catalytic cycle was continued for 3 h. Six successive catalytic cycles were performed and the operational efficiency of the immobilized enzyme system at each operating temperature (50 and 65 °C) was determined by the following equation:

$$\text{Operational efficiency (\%)} = 100 \times (G_x/G_1) \qquad \text{Eq. (3)}$$

where G_1 is the amount of galactose liberated in the 1st catalytic cycle and G_x is the amount of galactose liberated in the xth catalytic cycle.

6.2.5.4. Thin layer chromatographic analysis of soymilk

For the preparation of samples for thin layer chromatography (TLC), 50 mL of soymilk was incubated with 50 U of free enzyme at 65 °C for 3 h with agitation. Twenty five mL aliquots were removed at specific time intervals and the reaction was terminated by boiling the reaction mixture for 20 min in a boiling water bath. The boiled reaction mixture was poured into 60 mL absolute ethanol to partially precipitate the soy proteins. Complete precipitation of the proteins was effected by adding 0.25 mL of 0.3 M barium hydroxide and 0.25 mL of 0.18 M zinc sulphate. The precipitated proteins were removed by filtration through Whatman No. 1 filter paper. The filtrate was then extracted with 40 mL of chloroform. The aqueous phase was concentrated to 2.5 mL under vacuum and analyzed using TLC.

TLC was performed on silica gel G plates (10 x 20 cm) (Merck Co. Ltd.) using *n*-propanol: ethyl acetate: water (6:1:3, v/v) as the solvent system (Tanaka et al. 1975). The sugars spots were located by keeping the plates in an oven at 140 °C for 5 min after spraying with 1% (w/v) α-naphthol in absolute ethanol containing 10% of *ortho*-phosphoric acid (Albon & Gross 1950).

6.2.6. Processing of legume seed flour

Dry whole seeds of horse gram (*Dolichos biflorus*) and green gram or mung bean (*Vigna radiata* L.) (Fig. 6.3) purchased from the local market were used for the study.

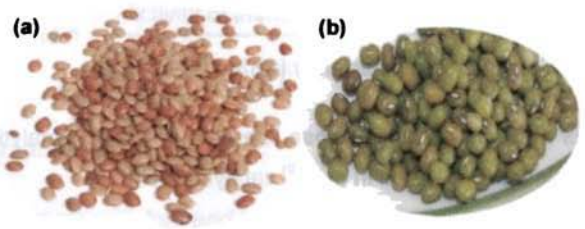


Fig. 6.3. (a) Horse gram and (b) green gram used for treatment with α-galactosidase

6.2.6.1. Conventional methods of legume processing

The conventional methods of legume processing such as soaking and cooking were done with dry whole seeds of horse gram and green gram. The seeds were soaked in distilled water (1:10, w/v) for 12 h at room temperature. After 12 h, the water was drained off and the soaked seeds were washed three times with distilled water. In cooking treatment the seeds were cooked in distilled water (1: 10, w/v) on a hot plate for 60 min and rinsed three times with distilled water. The seeds were then dried and powdered before estimation of the oligosaccharide content.

6.2.6.2. α-Galactosidase treatment of legume seed flour

Five grams of horse gram and green gram seed flour, which passes through a 500 μm sieve, was treated with 40 U of α-galactosidase diluted in 50 mL of 0.1 M McIlvaine buffer (pH 5.0), in a rotary shaker at 120 rpm and 65 °C for 2 h. After incubation, the treated seed flour samples were filtered through a Whatman No.1 filter paper, dried and the oligosaccharide content was determined. For control, the volume of enzyme was replaced with equal volume of buffer.

6.2.6.3. Determination of oligosaccharide content

The raffinose oligosaccharides were extracted by treating 5 g of seed flour sample with 50 mL of 70 % ethanol (v/v) in a rotary shaker at 120 rpm for 12 h. The alcoholic extract obtained after filtration through Whatman No.1 filter paper was concentrated under vacuum at 40 °C in a rotary evaporator. The concentrated sugar syrup was made up to 10 mL with distilled water. Ten microliters each of the sugar extract was applied to silica gel G plates (20 x 20 cm) (Merck Co. Ltd.) and developed by ascending thin layer chromatography and the sugar spots were visualized according to the method described above in Section 6.2.5.4. For quantitative determination, the area (2 x 2 cm) corresponding to each oligosaccharide spot was scraped from unsprayed duplicate plates and eluted with 3 mL distilled water for 12 h. The mixture was centrifuged to remove silica gel and 1 mL of the supernatant was used for the estimation of oligosaccharides by the method of Tanaka et al. (1975). One millilitre of the supernatant was treated with 1mL of 0.2 M thiobarbituric acid followed by 1 mL of concentrated HCl and boiled in a water bath for exactly 6 min. After cooling, the oligosaccharide contents were quantified spectrophotometrically at 432 nm. The oligosaccharide content of the samples was expressed on dry weight basis.

6.2.6.4. Determination of total soluble sugars and reducing sugars

Total soluble sugars in the concentrated sugar syrup were estimated by phenol-sulphuric acid method (Dubois et al. 1956). The reducing sugars were estimated by the method of Nelson (1944).

6.2.7. α-Galacto-oligosaccharide synthesis (Transglycosylation)

Transglycosylation reactions were carried out separately with the three purified α-galactosidases - α-Gal I, α-Gal II and α-Gal III - obtained by the purification steps described in Chapter V. Transgalactosylation reactions were carried out in a reaction mixture containing 50 µL of suitably diluted enzyme (1 U), 50 µL of galactosyl donor, 50 µL of acceptor sugar and 50 µL of McIlvaine buffer (0.1 M, pH 5.0). Reactions were carried out separately for 2 h at the temperature optimum for each α-galactosidase (α-

Gal I, 65 °C; α-Gal II 50 °C and α-Gal III 55 °C). Aliquots were removed at suitable time intervals and heated in a boiling water bath for 5 min to terminate the reaction. Galactosyl donors used were stachyose, raffinose and melibiose, at final concentrations of 100 mM in the reaction mixture. Acceptor sugars used were galactose and glucose at a final concentration of 500 mM. Transfer products were detected by TLC on silica gel G plates (10 x 20 cm) (Merck Co. Ltd.) using *n*-propanol: acetic acid: water (1:1:0.1, v/v/v) as the solvent system. The sugar spots were visualized as described above in Section 6.2.5.4.

6.2.8. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the average of triplicate determinations and the standard deviation for all the values were <± 5%.

6.3. RESULTS AND DISCUSSION

6.3.1. Characterization of crude and partially purified α-galactosidase

Generally crude or partially purified α-galactosidase preparations are used for the processing of legume-based food. Hence characterization of α-galactosidase in its crude and partially purified state is essential to identify the operational conditions suitable for the enzyme. Characterization studies of the crude and partially purified enzyme preparation indicated that the optimum pH for highest α-galactosidase activity was 5.0 (Fig. 6.4). The enzyme was active over a wide range of pH (4.0-9.0) with more activity towards the acidic range than towards alkaline range. At pH 4.0 the crude and partially purified α-galactosidase showed respectively 90% and 85% of the activity obtained at pH 5.0. Only a negligible amount of activity was obtained at pH 9.0. The α-galactosidase activity of both crude and partially purified enzyme preparations remained stable for 5 h over a wide pH range (5.0 to 7.0) (Fig. 6.4). This is an appreciable character for application in the treatment of legume-based food. The pH of legume slurries meant for enzymatic treatment varies considerably. An enzyme with activity over a wide range of pH is advantageous, because it will eliminate the necessity

for pH adjustment, which may cause precipitation of proteins leaving a sour taste to such food (Gote et al. 2004). Mansour & Khalil (1998) have reported α-galactosidase activity in *Aspergillus oryzae* and *Aspergillus niger* that is stable in the pH range of 4.0 to 7.0 and *Cladosporium cladosporides* α-galactosidase is stable in the pH range of 5.0 to 7.0.

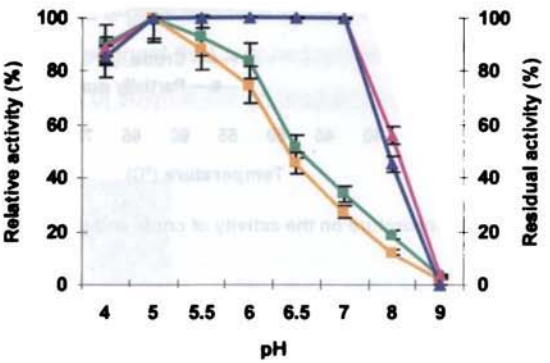


Fig. 6.4. Effect of pH on activity and stability of crude (■, ▲) and partially purified (■, ▲) α-galactosidase

The optimum temperatures of the crude and partially purified α-galactosidase preparations were determined at pH 5.0. The crude and partially purified α-galactosidase preparations were most active at 65 °C and exhibited 83% and 74% activity respectively at 70 °C (Fig. 6.5). Thermostability studies showed that both crude and partially purified enzyme preparations were completely stable up to 65 and 55 °C respectively for 5 h (Fig. 6.6). The half-life ($t_{1/2}$) of inactivation of partially purified α-galactosidase at 65 °C was 3.5 h. At 70 and 75 °C the enzyme activity decreased rapidly. The high thermostability of the enzyme is advantageous for industrial application, especially when removing RFO from soymilk and other soy-based products, as high temperature (usually 65–70 °C) used during the pasteurization step following the soybean processing leads to the denaturation of thermolabile enzymes.

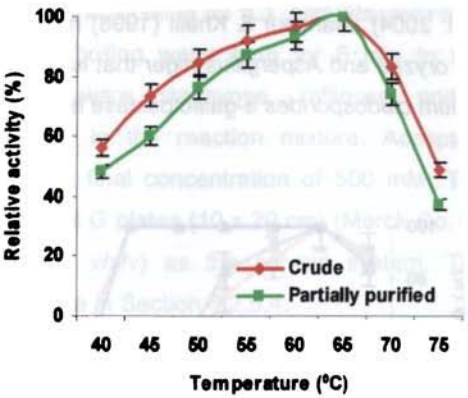


Fig. 6.5. Effect of temperature on the activity of crude and partially purified α-galactosidase

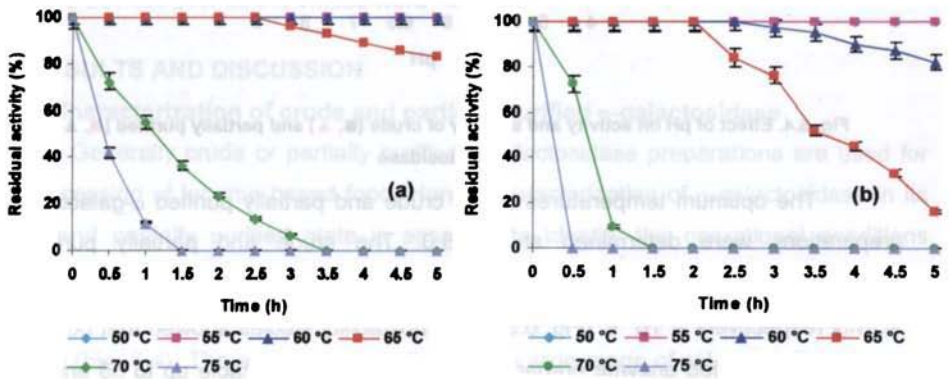


Fig. 6.6. Effect of temperature on the stability of (a) crude and (b) partially purified α-galactosidase

6.3.2. Enzymatic treatment of soymilk

Analysis of enzyme treated soymilk by TLC (Fig. 6.7) indicated the complete disappearance of stachyose from the soymilk samples within 30 min of incubation and raffinose within 1 h of incubation. Unlike the reports of Gote et al. (2004) on the hydrolysis of soymilk by *Bacillus stearothermophilus* α-galactosidase, no melibiose was

found to accumulate in the soymilk samples after 1 h of incubation, which indicated the complete hydrolysis of the soy oligosaccharides to the respective end products. Sucrose, the hydrolysis product of raffinose, was not found to appear after enzyme treatment of soymilk indicating the presence of invertase activity in the culture supernatant of *S. griseoloalbus*. These results could be advantageous as the treatment of soymilk with *S. griseoloalbus* α-galactosidase would render the soymilk free of all the oligosaccharides. The results suggest that α-galactosidase from *S. griseoloalbus* has a great potential in development of soymilk-based food products.

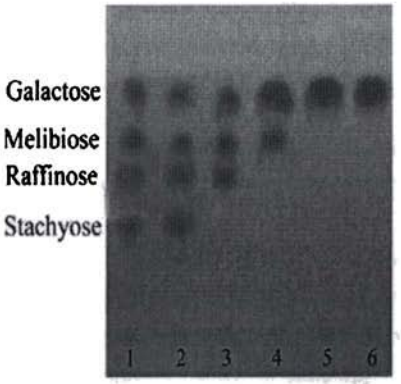


Fig. 6.7. Thin layer chromatogram of soymilk samples. Lanes: (1) Standard sugars, (2) Soymilk before treatment, (3) Soymilk after 30 min, (4) 1 h, (5) 2 h and (6) 3 h incubation with free α-galactosidase.

6.3.2.1. Comparison of free and immobilized α-galactosidase

A time course of the hydrolysis of oligosaccharides in soymilk by free α-galactosidase and α-galactosidase immobilized in gelatin-alginate blended beads showed an increase in the amount of reducing sugars liberated with increase in incubation time (Fig. 6.8). However, free α-galactosidase liberated more galactose compared to immobilized α-galactosidase. This could be due to the diffusional limitations in the immobilized system which causes resistance to the substrate to diffuse into the immobilized matrix and to the products to diffuse out (Abdel-Naby et al. 1999; Erginer et al. 2000). On the contrary, free α-galactosidase has easy access to the RFO in soymilk.

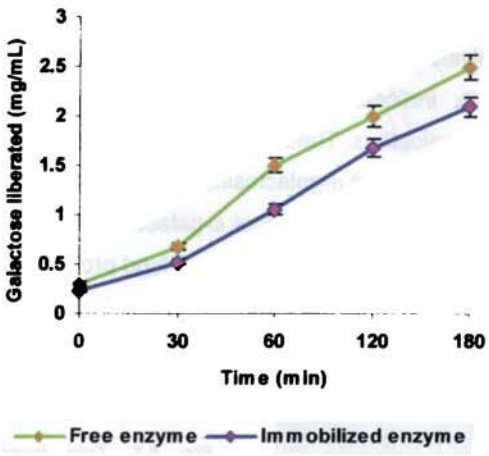


Fig. 6.8. Time course of the hydrolysis of oligosaccharides in soymilk by free and immobilized α-galactosidase at 65 °C.

The partially purified α-galactosidase was physically entrapped in gelatin-alginate blended beads (Fig. 6.9). The activity yield obtained after immobilization was 69.7%. Generally calcium alginate beads used for the immobilization of enzymes have the disadvantage of large pore sizes leading to enzyme leakage. Immobilization of enzymes in gelatin requires additional processes such as freezing and thawing which denatures the enzyme and lowers the immobilization yield. In the present study the advantages of gelatin and alginate are combined. Gelatin is a protein used for the immobilization of cells and enzymes using glutaraldehyde as a cross-linking agent. Gelatin is cross-linked with glutaraldehyde under mild conditions and the reaction between gelatin and glutaradehyde, accompanied by a colour change due to the formation of the aldimine linkage, involves only the lysine residue of the protein (Tanriseven & Dogan 2002). To improve the immobilization system, sodium alginate and gelatin were used along with glycerol. The polyol or glycerol imparts a stronger resistance and confers better characteristics with respect to smoothing and rehydration

effects (Casas et al. 1990), probably because these polyols provides more hydroxyl groups and these polyhydroxyllic additives have a positive effect on enzyme stability. Similar results were reported by Naganagouda & Mulimani (2006) for immobilization of α-galactosidase and Tanriseven & Dogan (2002) for immobilization of β-galactosidase in alginate-gelatin fibers. An inherent problem in enzyme immobilization is the leakage of enzyme from beads, even at higher concentrations, which gradually leads to loss of enzyme activity. In order to avoid leakage 5% glutaraldehyde solution was used as a hardener. Ates & Mehmetoglu (1997) have reported that on treatment with glutaraldehyde solution, the activity of β-galactosidase in the alginate beads was stable. Glutaraldehyde cross-links enzyme and gelatin forming an insoluble structure. Glutaraldehyde treatment also stabilizes the alginate gel, helping in the prevention of enzyme leakage (Aetes & Mehmetoglu 1997; Tanriseven & Dogan 2002).

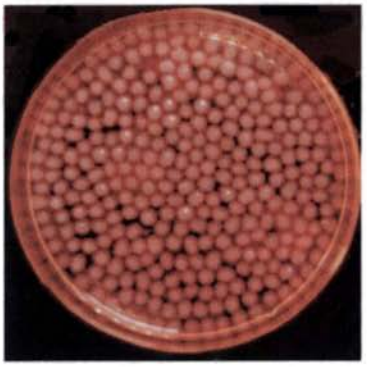


Fig. 6.9. α-Galactosidase physically entrapped in gelatin-alginate blended beads

6.3.2.2. Operational efficiency of immobilized α-galactosidase

The operational stability of the immobilized α-galactosidase was evaluated in a repeated batch process at 50 and 65 °C. The results indicated that the immobilized enzyme system was 100% efficient up to 4 catalytic cycles at 50 °C and up to 2 catalytic cycles at 65 °C. Even after six cycles of usage the immobilized enzyme system could maintain 77% operational efficiency at 50 °C. At 65 °C the operational stability of immobilized enzyme dropped considerably after two cycles of usage and

after six cycles the operational efficiency was only 36%. Though the α-galactosidase is thermostable, the immobilization matrix used in the present study could not withstand the operating temperature as high as 65 °C as a result of which enzyme leakage increased correspondingly. This could be the reason for the rapid decrease in operational efficiency at 65 °C. The immobilized enzyme system was operationally more efficient at 50 °C. Naganagouda & Mulimani (2006) and Tanriseven & Dogan (2002) also reported 50 °C as the most suitable temperature for α-galactosidase and β-galactosidase immobilized in alginate-gelatin fibers. In the present study the immobilized enzyme system working at 55 °C could liberate only less amount of galactose from soymilk as compared to the free α-galactosidase working at 65 °C, which is the optimum temperature. The lower hydrolysis efficiency of the immobilized enzyme can, however, be compensated by re-using the system, whereas the free enzyme is lost after single use. The glutaraldehyde treatment rendered high stability to the gelatin-alginate blended beads and prevented the leakage of enzyme. Naganagouda & Mulimani (2006) reported the use of α-galactosidase immobilized in alginate-gelatin fibers for the removal of RFO from soymilk, where the immobilized fibers maintained 60% operational efficiency after five runs. Ates & Mehmotoglu (1997) found that after treatment with glutaraldehyde Co-alginate immobilized β-galactosidase could be used eight times with high activity.

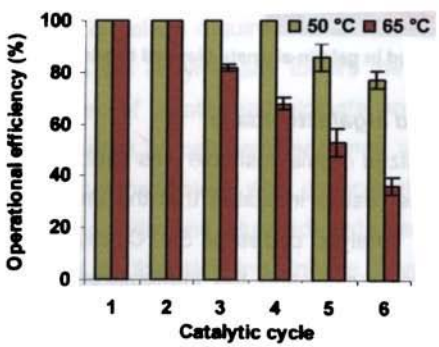


Fig. 6.10. Operational efficiency of gelatin-alginate-entrapped α-galactosidase in liberating galactose from soymilk at 50 and 65 °C over repeated catalytic cycles. The duration of a single catalytic cycle was 3 h.

6.3.3. Treatment of legume seed flour

The levels of RFO in raw horse gram and green gram flour samples are presented in Table 6.1. The results showed that green gram contained more RFO than horse gram and the concentration of stachyose was highest in both horse gram and green gram. The relative levels of raffinose and stachyose obtained in our study were in confirmation with those presented by other workers (Rathbone 1980; Adsule et al. 1986).

Table 6.1. Oligosaccharide content of raw horse gram and green gram

Seed flour sample	Total soluble sugars (g/kg DM[a])	Raffinose (g/kg DM[a])	Stachyose (g/kg DM[a])
Horse gram	28.9	6.8	19.4
Green gram	59.2	16.5	27.5

The data are mean of three independent samples with triplicate determinations

[a] Dry matter

6.3.3.1. Effect of soaking

The reduction of RFO in dry whole seeds of horse gram and green gram by various treatments is given in Fig. 6.11a and 6.11b respectively. Soaking of dry whole seeds of horse gram in distilled water for 12 h resulted in a mean reduction of raffinose content by 23.8 % and stachyose content by 12.3 %. For green gram flour samples, the reduction of raffinose content was by 19% and that of stachyose was by 10%. The reduction of raffinose content was higher compared to that of stachyose content in both the cases. Mulimani et al. (1997) have reported that soaking of whole soybean seeds led to a mean decrease of 80.3% for raffinose and 44.8% for stachyose. Reduction in raffinose and stachyose content of red gram flour by 54.6% and 55.4% respectively was reported by Mulimani & Devendra (1998). Reduction of RFO in cow pea seeds by soaking is reported by Somiari & Balogh (1993). Leaching could be one of the reasons for the reduction of raffinose family of sugars during soaking (Price et al. 1988). Upadhyay & Garcia (1988) have demonstrated that the differential solubility of individual sugars and their diffusion rates are the two factors that influence the sugar

losses during soaking. The extent of reduction in level of oligosaccharides can be enhanced by increasing the soaking time and employing different soaking media (Pugalenthi et al. 2006). But the presence of off-odour in flours obtained from the legume seeds after soaking would affect the acceptability of such products (Somiari & Balogh 1993).

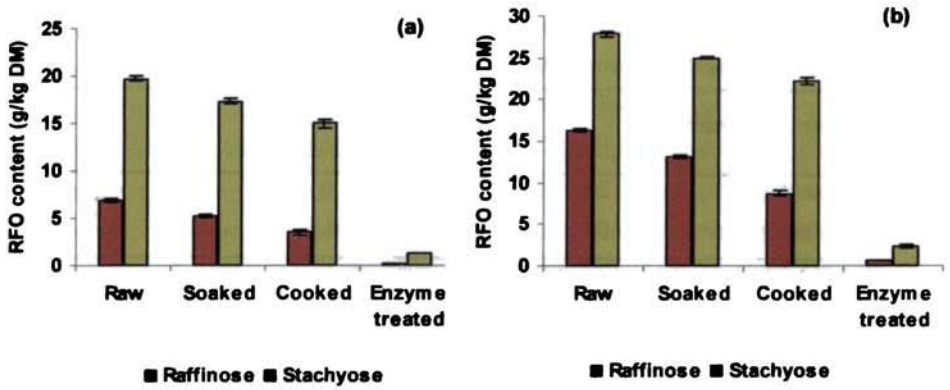


Fig. 6.11. Raffinose and stachyose contents of raw, soaked, cooked and α-galactosidase treated (a) horse gram flour and (b) green gram flour

6.3.3.2. Effect of cooking

Cooking brought about a greater reduction in raffinose family sugars than soaking (Fig. 6.11a and 6.11b). Cooking of horse gram and green gram seeds for 60 min resulted in a mean decrease of 49.6% and 46.3% respectively for raffinose and 24.3% and 20.1% respectively for stachyose. Mulimani et al. (1997) reported 52.3% removal of raffinose and 20.7% removal of stachyose from soybean seeds after cooking. Somiari & Balogh (1993) reported that cooking of cow pea for 50 min reduced the raffinose content by 44% and stachyose by 28.6%. Onigbinde & Akinyele (1983) have proposed that decrease in the levels of raffinose and stachyose during cooking might be attributed to heat hydrolysis to disaccharides and monosaccharides or due to the formation of other compounds. In contrast, Rao & Belavady (1978) reported an increase in the level of oligosaccharides after cooking of pulses.

Though cooking resulted in a decrease in the level of RFO, it affected the colour, texture and aroma of the seed flours. It is also reported that legumes such as horse gram require prolonged cooking to obtain products of acceptable nature (Kadam & Salunkhe 1985). Price et al. (1988) have reported that treatments such as soaking and cooking could change the physicochemical properties of legumes. Moreover, soaking and cooking alone will not be sufficient to bring about any significant reduction in the flatulence-inducing activity of legumes (Price et al. 1988).

6.3.3.3. Effect of crude α-galactosidase treatment

Horse gram flour when treated with α-galactosidase resulted in a reduction of raffinose content by 97.5% and stachyose content by 93.2% (Fig. 6.11a and 6.11b). The enzyme treatment of green gram samples resulted in 96.3% and 91.8% reduction of raffinose and stachyose respectively. On the other hand, no reduction of RFO was observed in the control. The reduction in RFO by crude α-galactosidase treatment was due to the conversion of oligosaccharides into di- and monosaccharides by the hydrolysis of α-galactosidic linkages between the sugar molecules. The crude α-galactosidase extracts from *S. griseoloalbus* remarkably reduced the levels of raffinose and stachyose in horse gram and green gram flours.

There are several reports available in the literature of the use of α-galactosidase from fungal and plant sources for the removal of the RFO from soymilk and legume flours. Somiari & Balogh (1993) have used crude preparations of α-galactosidase from *Aspergillus niger* for the removal of raffinose and stachyose present in cowpea flours. Mansour & Khalil (1998) have reported 100% reduction of raffinose oligosaccharide content in chickpea flours by crude fungal α-galactosidase treatment. Mulimani et al. (1997) have used crude α-galactosidase from germinating guar seeds for the hydrolysis of galactooligosaccharides in soybean flour and have reported 90.4% reduction of raffinose and 91.9% reduction of stachyose.

6.3.4. α-Galacto-oligosaccharide synthesis by transglycosylation

α-Galactosidases have been generally known to catalyze hydrolytic reactions, however some of the α-galactosidases also found to have galactosyl transfer activity

(Hara et al. 1994; Hashimoto et al. 1995a, b; Eneyskaya et al. 1998; van Laere et al. 1999; Spangenberg et al. 2000; Yamashita et al. 2005) especially when incubated at very high substrate concentration. In the present study the three α-galactosidases- α-Gal I, α-Gal II and α-Gal III- purified from the crude enzyme extract of *S. griseoloalbus* were used to catalyze transglycosylation reactions. The results showed that among the three α-galactosidases, only α-Gal II possessed translycosylation property which can be exploited for the synthesis of novel α-galacto-oligosaccharides. When raffinose and galactose were incubated with α-Gal II, a transfer product with chromatographic mobility similar to that of stachyose was observed by TLC analysis (Fig.6.12). Two transfer products with chromatographic mobility similar to melibiose and stachyose were obtained when raffinose and glucose were incubated with α-Gal II. Incubation of stachyose and galactose with α-Gal II resulted in a new transfer product which was not similar to the chromatographic mobility of any of the standard sugars tested. When stachyose and glucose were incubated with α-Gal II two transfer products were obtained, one of which showed similarity to melibiose in chromatographic mobility and the other product was a new product observed as in the case of incubation with galactose.

Generally, α-galactosidases have been known to preferentially transfer galactosyl residues to the primary alcoholic groups of acceptor sugars (Dey & Pridham 1972; Mitsutomi & Ohtakara 1988). Since transfer of galactose to stachyose can yield a sugar which is structurally similar to the tetrasaccharide verbascose of the RFO, the new product obtained in the present investigation by incubation with stachyose and galactose/glucose was tentatively identified as verbascose. *Pycnoporus cinnabarinus* (Mitsutomi & Ohtakara 1988) α-galactosidase also produced mainly RFO consisting of stachyose, verbascose and ajugose by transglycosylation reaction. Tzortis et al. (2003) reported the yield of a trisaccharide from melibiose and a tetrasaccharide from raffinose with *Lactobacillus reuteri* α-galactosidase. Melibiose was not a good acceptor for the transglycosylation reaction of α-Gal II, since only the hydrolytic products were obtained when melibiose and galactose/glucose was incubated with α-Gal II (Fig. 6.12, Lanes 2

and 3). Among the various sugars tested, raffinose was the only saccharide which acted both as a galactosyl donor as well as acceptor.

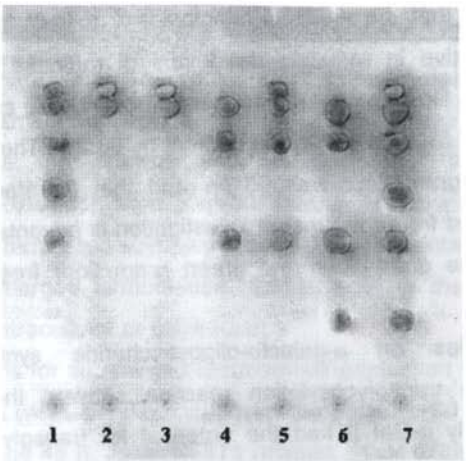


Fig. 6.12. Thin layer chromatogram showing the transfer products produced by transglycosylation reaction of α-Gal II from *S. griseoloalbus*. Lanes: (1) Standard sugars, (2) Melibiose + Galactose, (3) Melibiose + Glucose, (4) Raffinose + Galactose, (5) Raffinose + Glucose, (6) Stachyose + Galactose, (7) Stachyose + Glucose

6.4. CONCLUSION

The studies on the potential applications of thermostable α-galactosidases from *S. griseoloalbus* indicated the scope for their utilization in the processing of legume food. The crude and partially purified α-galactosidase preparations showed highest activity at pH 5.0 and 65 °C and were stable at pH 5.0 to 7.0 and up to 65 and 55 °C respectively for 5 h. The $t_{1/2}$ of the partially purified α-galactosidase at 65 °C was 3.5 h. Usually legume food processing requires a high operating temperature which necessitates the involvement of thermostable α-galactosidases.

The hydrolysis of flatulence causing oligosaccharides in soymilk by the free and immobilized α-galactosidase was investigated. Thin layer chromatographic analysis of enzyme treated soymilk samples showed the complete hydrolysis of soy oligosaccharides liberating galactose, the final product. Though the immobilized system was less efficient compared to the free α-galactosidase, it had the advantage of

reusability. The immobilized enzyme system was 77% operationally efficient at 50 °C even after six successive cycles of reuse. The investigations on legume seed flour treatment showed that the crude α-galactosidase extract from *S. griseoloalbus* was clearly most effective in reducing the levels of raffinose and stachyose than the traditional methods like soaking and cooking. The raffinose content in horse gram flour was reduced by 97.6% and stachyose content by 93.2%. The reduction in the raffinose content of green gram flour was 96.3% and that for stachyose was 91.8%. The information obtained from the present investigation is advantageous for the large-scale production of horse gram flour and green gram flour free from flatulence-causing oligosaccharides.

The studies on α-galacto-oligosaccharide synthesis by purified α-galactosidases *via* transglycosylation reaction showed that among the three α-galactosidases, only α-Gal II had the potential for transglycosylation. Incubation of raffinose/stachyose and galactose/glucose with α-Gal II resulted in the formation of transfer products. Raffinose and stachyose were good galactosyl donors, where as melibiose was not. Incubation of melibiose and galactose/glucose with α-Gal II yielded only the hydrolytic products. Among the sugars tested, raffinose acted both as galactosyl donor and acceptor. The transglycosylation potential of α-Gal II can be made use of in synthesizing α-galacto-oligosaccharides which are having excellent probiotic as well as medicinal applications.

Chapter VII

SUMMARY AND CONCLUSION

The results obtained during the course of this study clearly unraveled the potential of a novel filamentous acinobacterium *Streptomyces griseoloalbus* in producing α-galactosidase, an enzyme with excellent and versatile industrial applications. Since α-galactosidase from *S. griseoloalbus* is extracellular, it could be readily extracted from the fermented soybean in solid-state fermentation (SSF) or from the culture filtrate in submerged fermentation (SmF). Many of the α-galactosidases reported so far are either intracellular or cell-bound.

The optimum conditions for submerged fermentative production of α-galactosidase were locust bean gum 1% (w/v), pH 6.0, incubation temperature 30 °C, inoculum size 1.5 x 10^6 CFU, inoculum age 48 h, agitation 175 rev/min, yeast extract 4 g/L, $FeSO_4$ 2 x10^{-5} g/L $MgSO_4$ 0.25 g/L, salinity 24.9 g/L and incubation period 96 h. The optimization of α-galactosidase production in SmF increased the enzyme yield from 17 to 50 U/mL.

Cell immobilization technique was employed for enhancing the α-galactosidase yield in SmF. *Streptomyces griseoloalbus* was immobilized in calcium alginate gel and the optimal immobilization parameters for the enhanced production of α-galactosidase were determined. The alginate-entrapped cells were advantageous because there was a two-fold increase in the enzyme yield compared to the free cells. The optimization experiments with free cells showed salinity as the most statistically significant factor, since the organism under study was isolated from mangrove sediment soil sample. But using sea water for enzyme production in large scale fermenters is not appreciable as it may have some corroding effect. By taking advantage of whole cell immobilization, the sea water could be excluded from the fermentation medium without any loss of enzyme yield. Moreover, with immobilized cells the maximum yield was reached after 72 h of incubation in batch fermentation under optimal conditions, whereas in the case of free cells the maximum enzyme yield was obtained only after 96 h of incubation. The alginate beads had good stability and also retained 75% ability of enzyme production

even after 8 cycles of repeated batch fermentation. It is significant that this is the first report on whole cell immobilization for α-galactosidase production. Given the potential applications of α-galactosidase and the need for development of economical methods for improved enzyme production with an overall aim of reducing the cost of the industrial process, the use of whole-cell immobilization can serve as an excellent alternative for increasing enzyme yields in submerged fermentation.

SSF proved to be the best fermentation method for α-galactosidase production by *S. griseoloalbus*. Among the various solid-substrates screened soybean flour in the particle size range of 500-1000 μm was identified as the best in terms of supporting good growth and enzyme yield. Galactose was found to have an inducing effect, whereas no additional supplementation of nitrogen was required since soybean itself was enough to support the nitrogen demand of the culture. The other fermentation conditions beneficial for highest α-galactosidase yield were initial moisture content 40%, pH 7.0, temperature 30 °C, inoculum size 1.9 x 10^6 CFU/g initial dry substrate. The highest α-galactosidase yield obtained at flask level was 125 U/gds. A packed bed column bioreactor was designed and the effect of aeration was studied which enhanced the α-galactosidase yield still further to 197 U/gds. Since the overall yield in packed bed bioreactor was better than in flask fermentation, the former was considered as a possible option for the scale-up.

The scanning electron microscopic studies of the filaments of *S. griseoloalbus* grown on soybean flour showed that they are slender than the filaments of fungi like *Aspergillus niger*. It is advantageous because slender cells have faster reaction rates without having diffusion problems. It is very significant that this information is relevant for future studies of modeling the diffusion and consumption of oxygen across the network of filamentous microbial growth.

The electrophoretic analysis of the crude enzyme extract of *S. griseoloalbus* revealed the presence of three proteins with α-galactosidase activity - α-Gal I, α-Gal II and α-Gal III- which were then purified to electrophoretic homogeneity by a two-step chromatographic process with considerable recovery of purified enzyme. The biochemical and hydrolytic properties of the multiple α-galactosidases were

investigated. The molecular masses and pI of the three enzymes were 72, 57 and 45 kDa, and 4.41, 5.6 and 6.13 respectively. α-Gal I showed *N*-terminal sequence homology to *S. coelicolor* A3(2) family 27 α-galactosidase. The optimum pH and temperature of α-Gal I, α-Gal II and α-Gal III were 5.0, 6.5 and 5.5 and 65 °C, 50 °C and 55 °C respectively. The α-Gal I had a wider range of pH and thermal stability than α-Gal II and α-Gal III. α-Gal I was stable up to 65 °C and α-Gal II and α-Gal III up to 55 °C for 2 h. The α-Gal I was active on oligomeric α-galactosides as well as polymeric locust bean gum, whereas α-Gal II and α-Gal III were active only on oligomers. α-Gal I could be classified as a member of GH 27 family and α-Gal II and α-Gal III as members of GH 36 family based on the hydrolytic properties. All the three α-galactosidases had a retaining mechanism of hydrolysis. The metal cations like Hg^{2+}, Ag^{2+} and Cu^{2+} inhibited enzyme activity while Mg^{2+} enhanced the activity of α-Gal I. Melibiose was a competitive inhibitor of all the three enzymes. Histidine, tryptophan and carboxylic residues were essential for catalytic action of the three α-galactosidases.

Interestingly α-Gal I showed unusual tolerance to even higher concentrations of galactose, whereas the other two α-galactosidases were competitively inhibited by galactose. α-Gal I from *S. griseoloalbus* is novel in this respect and is important from industrial point of view since galactose tolerance is an appreciable character which improves the efficiency of α-galactosidases in liberating galactose residues from oligomeric and polymeric α-galactosides. Moreover, galactose is reported to be a powerful inhibitor of most α-galactosidases. The exact reason behind this unique galactose tolerance is unclear and can be of interest for another detailed investigation. This unusual galactose-tolerance gives α-Gal I an outstanding position among microbial α-galactosidases. The high resistance to galactose inhibition is surely what makes the newly purified *S. griseoloalbus* α-Gal I of great interest for biotechnological applications.

The versatility of *S. griseoloalbus* α-galactosidases was also revealed in their potentials for industrial applications. The characterization of crude and partially purified α-galactosidase preparations prior to application studies showed that *S. griseoloalbus* α-galactosidase has a relatively wide range of pH and temperature for activity and stability. The $t_{1/2}$ of the partially purified α-galactosidase at 65 °C was 3.5 h. Usually

legume food processing requires a high operating temperature which necessitates the involvement of thermostable α-galactosidases. The relatively wide range of pH and temperature for activity and stability exhibited by the α-galactosidase preparation from *S. griseoloalbus* is interesting and deserves attraction for food industrial applications.

The hydrolysis of flatulence causing oligosaccharides in soymilk by the free and immobilized α-galactosidase was investigated. Thin layer chromatographic analysis of enzyme treated soymilk samples showed the complete hydrolysis of soy oligosaccharides. The relatively low hydrolytic efficiency of immobilized α-galactosidase as compared to the free enzyme could be compensated by the reusability of immobilized enzyme system whereas the free enzyme is lost after single use. Alginate and gelatin used in this study were non-toxic, biocompatible, biodegradable and cheap. The immobilization of *S. griseoloalbus* α-galactosidase in gelatin-algiante beads is simple and its subsequent use is comparatively safe and cheap with durable enzyme activity. The high efficiency and operation conditions of immobilized α-galactosidase point towards the possibility of developing more viable process technologies, with the added advantages of reusability and cost-effectiveness.

The investigations on legume seed flour treatment showed that the crude α-galactosidase extract from *S. griseoloalbus* was clearly most effective in reducing the levels of raffinose and stachyose than the traditional methods like soaking and cooking. The raffinose content in horse gram flour was reduced by 97.6% and stachyose content by 93.2%. The reduction in the raffinose content of green gram flour was 96.3% and that for stachyose was 91.8%. The information obtained from the present investigation is advantageous for the large-scale production of horse gram flour and green gram flour free from flatulence-causing oligosaccharides. Although the results suggest that α-galactosidase from *S. griseoloalbus* has a great potential in the treatment of legume food, safety, palatability, functionality and storage properties of enzyme-treated flours have to be determined before they can be commercialized. Adoption of effective processing methods may further enhance the utilization of legumes as potential sources of proteins especially among the economically weaker section of people in developing countries.

The studies on α-galacto-oligosaccharide synthesis by purified α-galactosidases *via* transglycosylation reaction showed that among the three α-galactosidases, only α-Gal II had the potential for transglycosylation. Incubation of raffinose/stachyose and galactose/glucose with α-Gal II resulted in the formation of transfer products. The transglycosylation potential of α-Gal II can be made use of in synthesizing α-galacto-oligosaccharides which are having excellent probiotic as well as medicinal applications.

Since *S. griseoloalbus* α-Gal I is active on polymeric substrates it can also be employed for the processing of galactomannans which can find possible applications in pharmaceuticals, cosmetics, paper products, paints and plastics.

BIBLIOGRAPHY

Ademark P, Larsson M, Tjerneld F, Stalbrand H (2001) Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificity. Enzyme Microb. Technol. 29:441-448.

Adsule RN, Kadam SS, Salunkhe DK (1986) Chemistry and technology of green gram (*Vigna radiata* L.) Wilezek. Crit. Rev. Food Sci.Nutr., 25:73-106.

Aduse-Opoku J, Tao L, Ferretti JJ, Russell RB (1991) Biochemical and genetic analysis of Streptococcus mutans a-galactosidase. J. Gen. Microbiol. 137: 757–764.

Adya S, Elbein D (1977) Glycoprotein enzymes secreted by *Aspergillus niger*: Purification and properties of α-galactosidase. J. Bacteriol. 129:850-856.

Aetes S, Mehmetoglu U (1997) A new method for immobilization of β-galactosidase and its utilization in a plug flow reactor. Process Biochem. 32: 433-436.

Ajisaka K, Fujimoto H (1989) Regioselective synthesis of trisaccharides by use of a reversed hydrolysis of α and β-D-galactosidase. Carbohydr. Res.185: 139-146.

Akiba T, Horikoshi K (1976) Identification and growth characteristics of α-galactosidase-producing microorganisms. Agr. Biol. Chem. 40:1845-1849.

Albon N, Gross D (1950) The chromatographic determination of raffinose in raw sugars. Analyst 75:454-457.

Annunziato ME, Mahoney RR, Mudgett RE (1986) Production of α-galactosidase from *Aspergillus oryzae* grown in solid state culture. J. Food Sci. 51:1370-1371.

Archana A, Satyanarayana T (1997) Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. Enzyme Microb. Technol. 21:12-17.

Armstrong GD, Rowe PC, Goodyer P, Orrbine E, Klassen TP, Wells G, MacKenzie A, Lior H, Blanchard C, Auclair F, Thompson B, Rafter DJ, McLaine PN (1995) A phase I study of chemically synthesized verotoxin (Shiga-like toxin) Pk-trisaccharide receptors attached to chromosorb for preventing haemolytic-uremic syndrome. J. Infect. Dis. 171:1042-1045.

Arnaud N, Bush D, Horisberger M (1976) Study of an intracellular α-galactosidase from the thermophilic fungus *Penicillium duponti*. Biotech. Bioeng. 18: 581.

Aslanidis C, Schimid K, Schmitt R (1989) Nucleotide sequences of operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in Escherichia coli. J. Bacteriol. 171:6753–6763.

Aspinall GO (1980) Chemistry of cell wall polysaccharides. In: Preiss J (Ed.) The Biochemistry of Plants. Vol 3. New York: Academic press. pp. 473-500.

Azimzadeh A, Wolf P, Thibaudeau K, Cinqualbre J, Soulillou JP, Anegon I (1997) Comparative study of target antigens for primate xenoreactive natural antibodies in pig and rat endothelial cells. Transplantation 64: 1166-74.

Bahl OP, Agarwal KML (1969) Glycosidases of *Aspergillus niger*. J. Biol. Chem. 244: 2970–2978.

Bajpai P (1999) Application of enzymes in the pulp and paper industry. Biotechnol. Prog. 15:147–157.

Baker JE (1991) Purification and partial characterisation of amylase allozymes from the lesser grain borer *Rhyzopertha dominica*. Insect Biochem. 21:303-311.

Bakunina IY, Sova VV, Nedashkovskaya OI, Kuhlmann RA, Likhosherstov LM, Martynova MD, Mihailov VV, Elyakova LA (1998) α-galactosidase of the marine bacterium *Pseudoalteromonas* sp. KMM 701. Biochemistry (Mosco); 63:1209-1215.

Bakunina YI, Nedashkovskaya OI, Zvyagintseva TN, Shchipunov YA (2006) Immobilization of α-galactosidase inside hybrid silica nanocomposites containing polysaccharides. Russian J. Appl. Chem. 79:827-832.

Balasubramaniam K, Mathew D (1986) Purification of α-galactosidase from coconut. Phytochem. 25:1817.

Barham D, Dey PM, Griffiths D, Pridham JB (1971) Studies on the distribution of α-galactosidases in seeds. Phytochem. 10:1759 – 1763.

Barry V, McCleary RA, Robert W, Hans N (1981) Effect of galactose content on the solution and interaction properties of guar and carob galactomannans. Carbohydr. Res. 92:269-285.

Bau A (1895) Chem. Ztg. Chem. App. 19:1873. [Cited in Adv. Carbohydr. Chem.,16, pp. 290. (1961)].

Beecher J, Grogan G, Roberts S, Willetts A (1996) Enantioselective oxidations by the diketocamphane monooxygenase isozymes from *Pseudomonas putida*. *Biotechnol. Lett.* 18: 571–576.

Beg QK, Bhushan B, Kapoor M, Hoondal GS (2000) Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. Enzyme Microb. Technol. 27:459-466.

Bei-Zhong H, Yong M, Frans MR, Robert NMJ (2003) Effects of temperature and relative humidity on growth and enzyme production by *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation. Food Chem. 81: 27-34.

Berg JO, Lindqvist L, Nord CE (1980) Purification of glycoside hydrolases from *Bacteroides fragilis*. Appl. Environ. Microbiol. 40: 40-47.

Beutler E, Grabowski GA (2001) Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (Eds.) The Metabolic and Molecular Bases of Inherited Disease, 8th edit., McGraw-Hill, New York.

Bierry H (1913) Diastatic cleavage of glucosides and galactosides. Compt. Rend. Soc. Biol. 156:265.

Bishop DF, Desnick RJ (1981) Affinity purification of α-galactosidase A from human spleen, placenta, and plasma with elimination of pyrogen contamination. Properties of the purified splenic enzyme compared to other forms. J. Biol. Chem. 256:1307-1316.

Boucher I, Parrot M, Gaudreau H, Champagne CP, Vadeboncoeur C, Moineau S (2002) Novel Food-Grade Plasmid Vector Based on Melibiose Fermentation for the Genetic Engineering of *Lactococcus lactis*. Appl. Environ. Microbiol. 68:6152–6161.

Box GEP, Behnken DW (1960) Some new three level designs for the study of quantitative variables. Technometrics 2:455-475.

Box GEP, Wilson KB (1951) On the experimental attainment of optimum conditions. J. Roy. Statist. Soc. Ser. B Metho. 13:1-45.

Brady RO, Schiffmann R (2000) Clinical features of and recent advances in therapy for Fabry disease. JAMA 284:2771–2775.

Breunig F, Knoll A, Wanner C (2003b) Enzyme replacement therapy in Fabry disease: clinical implications. Curr. Opin. Nephrol. Hypertens. 12:491-495.

Breunig F, Weidemann F, Beer M, Eggert A, Krane V, Spindler M, Sandstede J, Strotmann J, Wanner C (2003a) Fabry disease: diagnosis and treatment. Kidney Int. Suppl. 84: S181-185.

Brodelius P, Vandamme EJ (1987) Immobilized cell systems. In: Rehm HJ, Reed G (Eds.) Biotechnology. Weinheim: VCH Verlagsgesellschaft, pp. 405-464.

Brouns SJJ, Smits N, Wu H, Ambrosius PL, Snijders, Wright PC, de Vos WM, van der Oost J (2006) Identification of a Novel α-Galactosidase from the Hyperthermophilic Archaeon *Sulfolobus solfataricus*. J. Bacteriol. 188: 2392–2399

Bruhlmann F, Leupin M, Erismann KH, Fiechter A (2000) Enzymatic degumming of ramie bast fibers. J. Biotechnol. 76:43–50.

Brumer H, Sims PFG, Sinnott ML (1999) Lignocellulose degradation by *Phanerochaete chrysosporium*: purification and characterization of the main α-galactosidase. Biochem. J. 339:43-53.

Buckeridge MS, Panegassi VR, Rocha DC, Dietrich SMC, (1995) Seed galactomannan in the classification and evolution of the leguminosae. Phytochem. 38: 871-875..

Bulpin PV, Gidley MJ, Jeffcoat R, Underwood DR (1990) Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase. Carbohydr. Poly. 12: 155-168.

Bund RK, Singhal RS (2002) An alkali stable cellulase by chemical modification using maleic anhydride. Carbohydr. Poly. 47:137–141.

Burstein C, Kepes A (1971) The α-galactosidase from *Escherichia coli* K12. Biochim. Biophys. Acta. 230: 52-63.

Cairns T, Hammelmann D, Gray K, Welsh K, Larson G (1994) Enzymatic removal from various tissues of the galactose α-1,3-galactose target antigens of human antispecies antibodies. Transplant. Proc. 26:1279-1280.

Cao Y, Yang P, Shi P, Wang Y, Luo H, Meng K, Zhang Z, Wu N, Yao B, Fan Y (2007) Purification and characterization of a novel protease-resistant α-galactosidase from *Rhizopus* sp. F78 ACCC 30795, Enzyme Microb. Technol. 41: 835–841.

Carmi N, Zhang G, Petreikov M, Gao Z, Eyal Y, Granot D, Schaffer AA (2003) Cloning and functional expression of alkaline α-galactosidase from melon fruit: similarity to plant SIP proteins uncovers a novel family of plant glycosyl hydrolases. Plant J. 33:97–106.

Casas LT, Fernandodomingnez, Brito E (1990) Characterization and optimization of a new immobilized system of κ-carrageenan through interaction with carob bean gum and polyols. J. Ferment. Bioeng. 2:98–101.

Cavazzoni V, Adami A, Craveri R (1987) α-Galactosidase from the yeast *Candida javanica*. Appl. Microbiol. Biotechnol. 26: 555-559.

Cechowska-Pasko M, Ma‡gorzata, Wola ńska, Pa‡‡ka J (2002), Glycosaminoglycan-degrading enzymes in the skin of fasted rats, Comparative Biochem. Physiol. Part B 131: 551–557.

Centeno MSJ, Ponte PIP, Ribeiro T, Prates JAM, Ferreira LMA, Soares MC, Gilbert HJ, Fontes CMGA (2006) Galactanases and mannanases improve the nutritive value of maize and soybean meal based diets for broiler chicks. J. Poultry Sci. 43:344-350.

Chandrasekaran M (1997) Industrial enzymes from marine microorganisms: The Indian scenario, J. Marine Biotechnol. 5: 86-89.

Chen X, Andreana PR, Wang PG (1999) Carbohydrates in transplantation. Curr. Opin. Chem. Biol. 3: 650-658.

Chiba Y, Sakuraba H, Kotani M, Kase R, Kobayashi K, Takeuchi M, Ogasawara S, Maruyama Y, Nakajima T, Takaoka Y, Jigami Y (2002) Production in yeast of α-galactosidase A, a lysosomal enzyme applicable to enzyme replacement therapy for Fabry disease. Glycobiol. 12: 821-828.

Chinen I, Nakamura T, Fukuda N (1981) Purification and properties of α-galactosidase from immature stalks of *Saccharum officinarum* (sugar cane). J. Biochem. 90:1453-1461.

Chrost B, Schmitz K (1997) Changes in soluble sugar and activity of α-galactosidases and acid invertase during muskmelon (*Cucumis melo* L.) fruit development. J. Plant Physiol. 151:41–45.

Church FC, Meyers SP, Srinivasan VR (1980) Isolation and characterization of α-galactosidase from *Pichia guilliermondii*. Dev. Ind. Microbiol. 21:339-348.

Civas A, Eberhard R, Dizet PL, Petek F (1984a) Glycosidases induced in *Aspergillus tamarii*: Mycelial α-D-galactosidases. Biochem. J. 219:849-855.

Civas A, Eberhard R, Le Dizet P, Petek F (1984b) Glycosidases induced in *Aspergillus tamarii*: Secreted α-D-galactosidase and β-D-mannanase. Biochem. J. 219:857-863.

Clancy MJ, Whelan WJ (1967) Enzymatic polymerization of monosaccharides. Arch. Biochem. Biophys. 118:730-735.

Clarke JH, Davidson K, Rixon JE, Halstead JR, Fransen MP, Gilbert HJ, Hazlewood GP (2000) A comparison of enzyme-aided bleaching of softwood pulp using a combination of xylanase, mannanase and α-alactosidase. Appl. Microbiol. Biotechnol. 53:661–667.

Clarke JH, Rixon JE, Ciruela A, Gilbert HJ, Hazlewood GP (1997) Family-10 and Family-11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. Appl. Microbiol. Biotechnol. 48:177-183.

Comfort DA, Bobrov KS, Ivanen DR, Shabalin KA, Harris JM, Kulminskaya AA, Brumer H, and Kelly RM (2007) Biochemical analysis of *Thermotoga maritima* GH36 α-Galactosidase (*Tm*GalA) confirms the Mechanistic Commonality of Clan GH-D Glycoside Hydrolases. Biochemistry 46: 3319-3330.

Coombs J, Brenchley JE (2001) Characterization of two new glycosyl hydrolases from the lactic acid bacterium *Carnobacterium piscicola* strain BA. Appl. Environ. Microbiol. 67:5094-5099.

Coutinho PM, Henrissat B (1999a) Carbohydrate-active enzymes: an integrated database approach. In: Gilbert HJ, Davies G, Henrissat B, Svensson B (Eds.) Recent advances in carbohydrate bioengineering. Cambridge, UK: The Royal Society of Chemistry. pp 3–12.

Coutinho PM, Henrissat B (1999b) The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In: Ohmiya K, Hayashi K, Sakka K, Kobayashi Y, Karita S, Kimura T (Eds.) Genetics, biochemistry and ecology of cellulose degradation. Tokyo: Uni Publishers Co. pp 15–23.

Cristofaro E, Mattu F, Wuhrmann JJ (1974) Involvement of the raffinose family of oligosaccharide in flatulence. In: Sipple HL, McNatt KW (Eds.) *Sugars in Nutrition*, Academic Press, New York.

Cronin CE, Giannouli P, McCleary BV, Brooks M, Morris ER (2002) Formation of strong gels by enzymic debranching of guar gum in the presence of ordered xanthan. Special Publication – Royal Society of Chemistry, (Gums and Stabilisers for the Food Industry 11) 278: 289-296.

Cross T (1981) Aquatic actinomycetes: a critical survey of the occurrence, growth and role of actinomycetes in aquatic habitats. J. Appl. Bacteriol. 50:397–423.

Cruz R, Batistella JC, Wosiacki G (1981) Microbial α-galactosidase for soymilk processing. J. Food Sci. 46:1196-1200.

Cruz R, Park YK (1982) Production of fungal α-galactosidase and its application to the hydrolysis of galactooligosaccharides in soybean milk. J. Food Sci. 47:1973-1975.

Davies G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3:853-859.

de Rezende TS, Guimaraes VM, Rodrigues MC, Felix CR (2005) Purification and characterization of an α-galactosidase from *Aspergillus fumigatus*. Braz. Arch. Biol. Technol. 48:195-202.

de Vries RP, van den Broeck HC, Dekkers E, Manzanares P, de Graaff LH, Visser J (1999) Differential expression of three α-galactosidase genes and a single β-galactosidase gene from *Aspergillus niger*. Appl. Environ. Microbiol. 65:2453–2460.

Dea ICM, Morrison A (1975) Chemistry and interaction of seed galactomannan. Adv. Carbohydr. Chem. Biochem. 31:241-245.

Dean KJ, Sweeley CC (1979) Studies on human liver α-galactosidases. III. Partial characterization of carbohydrate-binding specificities. J. Biol. Chem. 254: 10006-10010.

Delente J, Johnson JH, Kuo MJ, O'Connor RJ, Weeks LE (1974) Production of a new thermostable neutral α-galactosidase from a strain of *Bacillus stearothermophilus*. Biotechnol. Bioeng. 16:1227-1243.

Deloach J.R., Spates GE (1984) Glycosidase activity from midgut region of *Stomoxys calcitrans* (Diptera: Muscidae). Insect Biochem. 14:169–173.

den Herder IF, Rosell AMM, van Zuilen CM, Punt PJ, van den Hondel CAMJJ (1992) Cloning and expression of a member of the *Aspergillus niger* gene family encoding α-galactosidase. Mol. Gen. Genet. 233:404–410.

Desnick RJ, Ioannou YA, Eng CM (2001) α-Galactosidase A deficiency: Fabry disease. In: The Metabolic and Molecular Bases of Inherited Disease. New York, NY: McGraw Hill, 3733-3774.

Dey G, Mitra A, Banerjee R, Maiti BR (2001) Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. Biochem. Eng. J. 7: 227-231.

Dey PM (1978) Biochemistry of plant Galactomannan. Adv. Carbohydr. Chem. Biochem. 35:341-376.

Dey PM (1979) Transgalactosylation activity of sweet almond α-galactosidase: synthesis of saccharides. Phytochem.; 18:35.

Dey PM (1980) Biochemistry of α-galactosidic linkages in the plant kingdom. Adv. Carbohydr. Chem. Biochem. 37:283-371.

Dey PM (1984) Characteristic features of an α-galactosidase from mung beans (*Vigna radiata*). Eur. J. Biochem. 140:385-90.

Dey PM, Malhotra OP (1969) Kinetic behavior of sweet almond α-galactosidase. Biochim. Biophys. Acta. 185: 402-408.

Dey PM, Patel S, Brownleader MD (1993) Induction of alpha-galactosidase in *Penicillium ochrochloron* by guar (*Cyamopsis tetragonoloba*) gum. Biotechnol. Appl. Biochem. 17:361-371.

Dey PM, Pridham JB (1969a) Purification and properties of α-galactosidases from *Vicia faba* seeds. Biochem. J. 113:49 – 55.

Dey PM, Pridham JB (1969b) Substrate specificity and kinetic properties of α-galactosidase from *Vicia faba*. Biochem. J. 115: 47P.

Dey PM, Pridham JB (1972) Biochemistry of α-D-galactosidase. In: Meister A, (Ed.) Advances in Enzymology, Vol. 36, Academic, New York, pp. 91-130.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for the determination of sugars and related substances. Anal. Chem. 28:350-356.

Duffaud GD, McCutchen CM, Leduc P, Parker KN, Kelly RM (1997) Purification and characterization of extremely thermostable β-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. Appl. Environ. Microbiol. 63: 169-177.

Elgavish S, Shaanan B (1997) Lectin-carbohydrate interactions: different folds, common recognition principles. Trends Biochem. Sci. 22:462–467.

Elgavish S, Shaanan B (1998) Structures of the *Erythrina corallodendron* lectin and of its complexes with mono- and disaccharides. J. Mol. Biol. 277:917–932.

Ellaiah P, Prabhakar T, Ramakrishna B, Taleb AT, Adinarayana K (2004) Production of lipase by immobilized cells of *Aspergillus niger*. Process Biochem. 39:525–528.

Elshafei AM, Foda MS, Aboul-Enein A, Afify AS, Ali NH (1993) Purification and enzymic properties of α-galactosidase from *Penicillium janthinellum*. Acta Biotechnol.13 : 351-359.

Elshafei AM, Foda, MS, Mobde EA, Ali NH (2001) Optimization of alpha-galactosidase production in *Streptomyces erythrus*. Acta. Microbiol. Pol. 50: 53-63.

Eneyskaya EV, Golubev AM, Kachurin AM, Savel'ev AN, Neustroev KN (1998) Transglycosylation activity of α-D-galactosidase from *Trichoderma reesei*. An investigation of the active site. Carbohydr. Res. 205: 83-91.

Eng CM, Guffon N, Wilcox WR, Germain DP, Lee P, Waldek S (2001) Safety and efficacy of recombinant human α-galactosidase A—replacement therapy in Fabry's disease. N. Engl. J. Med. 345:9–16.

Evans RW, Orians CE, Ascher NL (1992) The potential supply of organ donors: an assessment of the efficiency of organ procurement efforts in the United States. JAMA 267: 239.

FAO (1990) Protein quality evaluation. Report of a Joint FAO/WHO Expert Consultation. Rome: Bethesda M.D.D. FAO/WHO.

Ferguson MAJ, Homans SW, Dwek RA, Rademacher TW (1988) Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science 239: 753-759.

Ferreira C, Marana SR, Silva C, Terra WR (1999) Properties of digestive glycosidases and peptidases and the permeability of the peritrophic membranes of *Abracris flavolineata* (Orthoptera: Acrididae). *Comp. Biochem. Physiol.* 123B:241–250.

Fischer E, Lindner P (1895) "Uber die Enzyme einigen Hefen," Chem. Ber. 28:3034.

Flórez IG, Laro PS, Ochoa AG, Gascón S (1981) The specificity of induction of α-galactosidase from *Saccharomyces carlsbergenensis*. Biochim. Biophys. Acta 674:71-77.

Foda MS, Elshafei AM, Aboul-Enein A, Afify AS, Ali NH (1995) Physiological studies on the formation of α-galactosidase by fungi. Chem. Mikrobiol. Technol. Lebensm. 17:25-32.

Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, Szakacs G, Pandey A (2003) Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. Biochem. Eng. J. *15* 107-115.

Fridjonsson O, Mattes R (2001) Production of recombinant α-galactosidases in *Thermus thermophilus* Appl. Env. Microbiol. 67: 4192-4198.

Fridjonsson O, Watzlawick H, Gehweiler A, Mattes R (1999a) Thermostable α-galactosidase from *Bacillus stearothermophilus* NUB3621: cloning, sequencing and characterization. FEMS Microbiol. Lett. 176: 147–153.

Fridjonsson O, Watzlawick H, Gehweiler A, Rohrhirsch T, Mattes R (1999b) Cloning of the gene encoding a novel thermostable α-galactosidase from *Thermus brockianus* ITI360. Appl. Environ. Microbiol. 65: 3955–3963.

Fujimoto Z, Kaneko S, Momma M, Kobayashi H, and Mizuno H (2003) Crystal structure of rice α-galactosidase complexed with D-galactose. J. Biol. Chem. 278: 20313–20318.

Gabius HJ, Gabius S (Eds). Glycosciences- status and perspectives. Chapman and Hall, Weinheim, 1997.

Galas E, Miszkiewicz H (1996) Purification and some properties of α-galactosidase from *Mortierella vinacea* IBT-3. Acta Microbiol. Pol. 45:143-154.

Ganter C, Bock A, Buckel P, Mattes R (1988) Production of thermostable recombinant α-galactosidase suitable for raffinose elimination from sugar beet syrup. J. Biotechnol. 8: 301-310.

Gao Z, Schaffer AA (1999) A novel alkaline α-galactosidase from melon fruit with a substrate preference for raffinose. Plant Physiol. 119:979–987.

Garman SC (2006) Structural studies on α-GAL and α-NAGAL: The atomic basis of Fabry and Schindler diseases. Biocat. Biotrans. 24:129-136.

Garman SC, Garboczi DN (2002) Structural basis of Fabry disease. Mol. Genet. Metab. 77: 3-11.

Garman SC, Garboczi DN (2004) The Molecular Defect Leading to Fabry Disease: Structure of Human α-Galactosidase. J. Mol. Biol. 337: 319–335.

Garman SC, Hannick L, Zhu A, Garboczi DN (2002) The 1.9 Å structure of α-*N*-acetylgalactosaminidase: Molecular basis of glycosidase deficiency diseases. Structure 10:425-434.

Garro MS, de Valdez GF, Oliver G, de Giori GS (1996) Purification of α-galactosidase from *Lactobacillus fermentum*. J. Biotechnol. 45: 103-109.

Gaudreault PR, Webb JA (1986) Alkaline α-galactosidase activity and galactose metabolism in the family cucurbitaceae. Plant Sci. 45: 71 – 75.

Gdala J, Johansen HN, Bach KE, Knap IH, Wagner P, Jorgensen OR (1997) The digestibility of carbohydrates, protein and fat in the small and large intestine of piglets fed non-supplemented and enzyme supplemented diets. Ani. Feed Sci. Technol. 65:15-33.

Gebler J, Gilkes M, Claeyssens D, Wilson P, Beguin W, Wakarchuk D, Kilburn R, Miller Jr, Warren R, Withers S (1992) Stereoselective hydrolysis catalyzed by related β-1,4-glucanases and β-1,4-xylanases. J. Biol. Chem. 267:12559-12561.

Gherardini F, Babock M, Salyers AA (1985) Purification and characerization of two α-galactosidases associated with catabolism of guar gum and other α-galactosides by *Bacteroides ovatus*. J. Bacteriol. 161: 500-506.

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota. Introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Gillard BK, Thurmon LT, Marcus DM (1993) Variable subcelluler localization of glycosphingolipids. Glycobiol. 3: 57-67.

Girigowda K, Mulimani VH (2006) Hydrolysis of galacto-oligosaccharides in soymilk by κ-carrageenan-entrapped α-galactosidase from *Aspergillus oryzae*. World J. Microbiol. Biotechnol. 22:437–442.

Giuseppin MLF, Almkerk JW, Heistek CJ, Verrips CT (1993) Comparative study on the production of guar α-galactosidase by *Saccharomyces cerevisiae* SU50B and *Hansenula polymorpha* 8/2 in continuous cultures. Appl. Environ. Microbiol. 59: 52-59.

Glaser V (1998) Carbohydrate-based drugs move closer to market: drugs in clinical pipeline validate glycobiology targets. Genet. Eng. News 18: 1-32.

Glicksman M (1969) Plant seed gums. In: Glicksman (Ed.) Gum Technology in the Food Industry. New York: Academic Press, New York.

Goldstein J (1989) Conversion of ABO blood groups. Transfus. Med. Rev. 3:206–212.

Goldstein J, Siviglia G, Hurst R (1982) Group B erythrocytes enzymatically converted to group O survive normally in A, B, and O individuals. Science 215:168-170.

Golubev AM, Nagem RAP, Neto JRB, Neustroev KN, Eneyskaya EV, Kulminskaya AA, Shabalin KA, Savelev AN, Polikarpov I (2004) Crystal structure of α-galactosidase from *Trichoderma reesei* and its complex with galactose: implications for catalytic mechanism. J. Mol. Biol. 339:413–422.

Golubev AM, Neustroev KN (1993) Crystallization of α-galactosidase from *Trichoderma reesei.* J. Mol. Biol. 231:933-934.

Gomes J, Gomes II, Terler K, Gubala N, Ditzelmuller G, Steiner W (2000) Optimization of culture medium and conditions for α-L-arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*. Enzyme Microb. Technol. 27:414-422.

Goriely A, Tabor M (2003) Biomechanical models of hyphal growth in actinomycetes. J. Theor. Biol. 222:211–218.

Gote M, Umalkar H, Khan I and Khire J (2004) Thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) and its application in the removal of flatulence causing factors from soymilk. Process Biochem. 39:1723-1729.

Gote MM, Khan MI, Gokhale DV, Bastawde KB, Khire JM (2006) Purification, characterization and substrate specificity of thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM-5146). Process Biochem. 41:1311–1317.

Gote MM, Khan MI, J.M. Khire (2007) Active site directed chemical modification of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146): Involvement of lysine, tryptophan and carboxylate residues in catalytic site. Enzyme Microb. Technol. 40:1312–1320.

Gowthaman MK, Krishna C, Moo-Young M (2001) Fungal solid state fermentation-An overview. In: Khachatourians GG, Arora DK (Eds.) Applied Mycology and Biotechnology, Vol.1. Agriculture and Food Productions, Elsevier Science, The Netherlands.

Grossmann GA, Terra WR (2001) α-Galactosidases from the larval midgut of *Tenebrio molitor* (Coleoptera) and *Spodoptera frugiperda* (Lepidoptera). Comp. Biochem. Physiol. Part B 128:109-122.

Guimarães VM, de Rezende ST, Moreira MA, de Barros EG, Felix CR (2001) Characterization of α-galactosidases from germinating soybean seed and their use for hydrolysis of oligosaccharides. Phytochem. 58: 67-73.

Gupta MN (1993) Thermostable enzymes. In: Gupta. M.N (Ed.) Thermostability of enzymes. Narosa Publishing House, New Delhi.

Haibath F, Hata J, Mitra M, Dhar M, Harmata M, Sun P, Smith D (1991) Purification and characterization of a *Coffea cabephora* α-D-galactosidase isozyme. Biochem. Biophys. Res. Commun. 181:1564 – 1571.

Hakomori S (2003) Structure, organization, and function of glycosphingolipids in membrane. Curr. Opin. Hematol. 10:16-24.

Halstead JR, Fransen MP, Eberhart RY, Park AJ, Gilbert HJ, Hazlewood GP (2000) α-Galactosidase A from *Pseudomonas fluorescens* subsp. cellulosa: cloning, high level expression and its role in galactomannan hydrolysis. FEMS Microbiol. Lett. 192:197-203.

Hara K, Fujita K, Kuwahara N, Tanimoto T, Hashimoto H, Koizumi K, Kitahara S (1994) Galactosylation of cylcodextrins and branched cyclodextrins by α-galactosidases. Biosci. Biotechnol. Bichem. 58: 652-659.

Harpaz N, Flowers HM, Sharon N (1975) Studies on B antigenic sites of human erythrocytes by use of Coffee bean α-galactosidase. Arch. Biochem. Biophys. 170:676–683.

Hart DO, He S, Chany CJ 2nd, Withers SG, Sims PF, Sinnott ML, Brumer H 3rd (2000) Identification of Asp-130 as the catalytic nucleophile in the main α-galactosidase from *Phanerochaete chrysosporium*, a family 27 glycosyl hydrolase. Biochemistry 39:9826-9836.

Hashimoto H, Katayama C, Goto M, Kitahata S (1993) Purification and some properties of α-galactosidase from *Candida guilliermondii* H-404, Biosci. Biotechnol. Biochem. 57:372-378.

Hashimoto H, Katayama C, Goto M, Okinaga T, Kitahata S (1995a) Enzymatic synthesis of α-linked galactooligosaccharides using the reverse reaction of a cell-bound α-galactosidase from *Candida guilliermondii* H-404, Biosci. Biotechnol. Biochem. 59: 179-183.

Hashimoto H, Katayama C, Goto M, Okinaga T, Kitahata S (1995b) Transglycosylation catalyzed by α-galactosidase from *Candida guilliermondii* H-404, Biosci. Biotech. Biochem., 59: 61-623.

Hazlewood GP, Gilbert HJ (1998) Structure and function analysis of *Pseudomonas* plant cell wall hydrolases. Prog. Nucleic Acid Res. Mol. Biol. 61: 211-241.

Helferich B, Appel H (1932) Emulsin-X., Z. Physiol. Chem. 237: 254.

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280:309–316.

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on aminoacid sequence similarities. Biochem. J. 293: 781-788.

Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. 316: 695-696.

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc. Natl. Acad. Sci. USA 92:7090-7094.

Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol. 7:637–644.

Herman EM, Shannon LM (1985) Accumulation and subcellular localization of α-galactosidase hemagglutinin in developing soybean cotyledons. Plant Physiol. 77:886–890.

Hernaiz MJ, Crout DHG (2000) Immobilization/stabilization on Eupergit C of the β-galactosidase from *B. circulans* and an α-galactosidase from *Aspergillus oryzae,* Enzyme Microb. Technol. 27: 26–32.

Holt S, Heading RC, Carter DC (1979) Effect of gel fiber on gastric emptying of glucose and paracetamol. Lancet 1: 636-639.

Hunter RL, Markert CL (1957) Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125: 1294–1295.

Imanaka T, Kaieda T, Sato K, Taguchi H (1972) Optimization of α-galactosidase production by mold. (I) α-Galactosidase production in batch and continuous culture and a kinetic model for enzyme production. J. Ferment. Technol. 50:633-646.

Irving DE, Hurst PL, Ragg JS (1997) Changes in carbohydrates and carbohydrate metabolism enzymes during the development, maturation and ripening of buttercup squash (*Cucurbita maxima* D. Delica). J. Am. Soc. Hortic. Sci. 122:310–314.

Ishiguro M, Kaneko S, Kuno A, Koyama Y, Yoshida S, Park GG, Sakakibara Y, Kusakabe I, Kobayashi H (2001) Purification and characterization of the recombinant *Thermus* sp. strain T2 α-galactosidase expressed in *Escherichia coli*. Appl. Environ. Microbiol. 67: 1601–1606.

Ivanova V, Stefanova M, Tonkova A, Dobreva E, Spassova D (1995) Screening of a growing cell immobilization procedure for the biosynthesis of thermostable α-amylases. Appl. Biochem. Biotechnol. 50:305–316.

Jamuna R, Ramakrishna SV (1992) Continuous synthesis of thermostable α-amylase by *Bacillus* cells immobilized in calcium alginate. Enzyme Microb. Technol. 14:36–41.

Jang HD, Chen KS (2003) Production and characterization of thermostable cellulases from *Streptomyces transformant* T3-1. World. J. Microbiol. Biotechnol. 19: 263–268.

Jesperson HM, MacGregor EA, Sierks MR, Svensson B (1991) Comparison of the domain-level organization of starch hydrolases and related enzymes. Biochem. J. 280:51–55.

Jin F, Li Y, Zhang C, Yu H (2001) Thermostable α-amylase and α-galactosidase production from the thermophilic and aerobic *Bacillus* sp. JF strain. Process Biochem. 36: 559-564.

Jing-Lian Y, Lu-Yang Y, Li-He G (2003) Human alpha galactosidase and alpha 1,2 fucosyltransferase concordantly inhibit xenoreactivity of NIH 3T3 cells with human serum, Acta Pharmacol. Sin. 24: 878-884.

Joersbo M, Marcussen J, Brunstedt J (2001) *In vivo* modification of the cell wall polysaccharide galactomannan of guar transformed with a α-galactosidase gene cloned from senna. Mol. Breed. 7: 211-219.

Jood S, Mehta U, Singh R (1986). Effect of processing on carbohydrates in legumes. J. Agri. Food Chem. 34: 417-420.

Kachurin AM, Golubev AM, Geisow MM, Veselkina OS, Isaeva-Ivanova LS, Neustroev KN (1995) Role of methionine in the active site of α-galactosidase from *Trichoderma reesei*. Biochem. J. 308: 955-964.

Kadam SS, Salunkhe DK (1985) Nutritional composition, processing, and utilization of horse gram and moth bean. Crit. Rev. Food Sci.Nutr. 22: 1-26.

Kaji A, Yoshihara O (1972) Purification and properties of α-galactosidase produced by *Corticium rolfsii*. Agric. Biol. Chem. 36:1335-1342.

Kalcheva EO, Faiziev MM, Shanskaya VO, Maluta SS (1994) Regulation of two aspartokinase isozymes in *Streptococcus bovis*. Can. J. Microbiol. 40: 224–227.

Kandler O, Hopf H (1980) Occurrence, metabolism and function of oligosaccharides, In: Stumpf PK, Conn EE (Eds.) The Biochemistry of Plants. A Comprehensive Treatise, Vol. 3, Academic, New York, pp. 221 – 270.

Kaneko R, Kusakabe I, Ida E, Murakami K (1991) Substrate specificity of α-galactosidase from *Aspergillus niger* 5-16. Agric. Biol. Chem. 55: 109–115.

Kang HC, Lee SH (2001) Characteristics of an α-galactosidase associated with grape flesh. Phytochem. 58:213 – 219.

Karel SF, Libicki SB, Robertson CR (1985) The immobilization of whole cells: engineering principles. Chem. Eng. Sci. 40:1321-1354.

Keller F, Pharr DM (1996) Metabolism of carbohydrates in sink and sources: galactosyl-sucrose oligosaccharides, In: Zamski E, Schaffer AA (Eds.) Photoassimilate distribution in plants and crops, Marcel Dekker, New York, pp. 157 – 183.

Kew OM, Douglas HC (1976) Genetic co-regulation of galactose and melibiose utilization in *Saccharomyces*, J. Bacteriol. 125: 33-41.

Kim SM, Baker DH (2003) Use of enzyme supplements in pig diets based on soyabean meal. Pig News Inform. 24: 91N-96N.

Kim WD, Kaneko S, Park GG, Tanaka H, Kusakabe I, Kobayashi H (2003) Purification and characterization of α-galactosidase from sunflower seeds. Biotechnol. Lett. 25:353-358.

Kim WD, Kobayashi O, Kaneko S, Sakakibara Y, Park GG, Kusakabe I, Tanaka H, Kobayashi H (2002) α-Galactosidase from cultured rice (*Oryza sativa* L. var. Nipponbare) cells. Phytochem. 61:621-630.

King MR, White BA, Blaschek HP, Chassy BM, Mackie RI, Cann IK (2002) Purification and characterization of a thermostable α-galactosidase from *Thermoanaerobacterium polysaccharolyticum*. J. Agric. Food Chem. 50: 5676-82.

King MR, Yernool DA, Eveleigh DE, Chassy BM (1998) Thermostable α-galactosidase from *Thermotoga neapolitana* cloning, sequencing and expression. FEMS Microbiol. Lett. 163: 37-42.

Kitahata S, Hara K, Fujita K, Kuwahara N, Koizumi K (1992) Synthesis of 6-O- α-D-galactosyl α-cyclodextrin by coffee bean α-galactosidase. Biosci. Biotech. Biochem. 56:1518-1519.

Kiyonobu K, Ohsaki T, Azuma A, Osawa G, Kanda Y, Kamoi K (1998) Effect of an oligosaccharide (raffinose) on immunocytes. *Shigaku* (in Japanese) 85: 551-558.

Knap IH, Hjort CM, Halkier T, Kofod LV (2001) α-Galactosidase enzyme. U. S Patent 6,197,566 (Assignee: Novo Nordisk A/S (Bagsvaerd, DK).

Kobayashi H, Suzuki H (1972) Studies on the decomposition of raffinose by α-galactosidase of mold. II. Formation of mold pellet and its enzyme activity. J. Ferment. Technol. 50: 625.

Kobayashi H, Suzuki H (1976) Kinetic studies of α-galactosidase-containing mold pellets on PNPG hydrolysis. Biotechnol. Bioeng. 18: 37-51.

Kocher A, Choct M, Porter MD, Broz J (2002) Effects of feed enzymes on nutritive value of soyabean meal fed to broilers. British Poultry Sci. 43: 54-63.

Kolatkar AR, Leung AK, Isecke R, Brossmer R, Drickamer K, Weis WI (1998) Mechanism of *N*-acetylgalactosamine binding to a C-type animal lectin carbohydrate-recognition domain. J. Biol. Chem. 273:19502–19508.

Kondoh K, Morisaki K, Kim WD, Park GG, Kaneko S, Kobayashi H (2005) Cloning and expression of the gene encoding *Streptomyces coelicolor* A3(2) α-galactosidase belonging to family 36. Biotechnol. Lett. 27: 641–647.

Konsoula Z, Kyriakides LM (2006) Thermostable α-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. Enzyme Microb. Technol. 39: 690–696.

Koshland DEJ (1953) Stereochemistry and the mechanism of enzymatic reactions. Biol. Rev.28: 416-436.

Kotwal SM, Gote MM, Khan MI, Khire JM (1999) Production, purification and characterization of constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp. J. Ind. Microbiol Biotechnol. 23: 661-667.

Kotwal SM, Gote MM, Sainkar SR, Khan MI, Khire JM (1998) Production of α-galactosidase by thermophilic fungus *Humicola* sp. in solid-state fermentation and its application in soya milk hydrolysis. Process Biochem. 33: 337-343.

Kotwal SM, Khan MI, Khire JM (1995) Production of thermostable α-galactosidase from thermophilic fungus *Humicola* sp, J. Ind. Microbiol. 15: 116-120.

Kotwal SM, Khire JM, Khan MI (2000) Chemical modification of α-galactosidase from the thermophilic fungus *Humicola* sp. J. Biochem. Mol. Biol. Biophys. 4:66–72.

Kourkoutas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA (2004) Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol. 21:377–397.

Kramer DM, Lehmann K, Pennewiss M, Plainer H (1975) Photo-beads and oxirane beads as solid supports for catalysis and biospecific adsorption. In: Peeters H (editor). Proteins of Biological Fluids 23th Colloquium. Oxford: Pergamon Press. pp. 505–511.

Kramer DM, Lehmann K, Pennewiss M, Plainer, H (1978) Oxirane-acrylic beads, preparation 2878-C. Enzyme Engineering 4. In: Broun GB, Manecke G, Wingard LB, editors. New York: Plenum Publishing,pp. 153–4.

Kremnicky L, Biely P (1997) β-Mannanolytic system of *Aureobasidium pullulans*. Arch. Microbiol. 165: 350-355.

Krishna C (2005) Solid-State Fermentation Systems—An Overview. Crit. Rev. Biotechnol. 25:1–30.

Kuhad RC, Kapoor M, Rustagi R (2004) Enhanced production of an alkaline pectinase from *Streptomyces* sp. TCK-SC by whole-cell immobilization and solid-state cultivation. World J. Microbiol. Biotechnol. 20:257-263.

Kunitz M (1947) Crystalline soybean trypsin inhibitor II. General properties. J. Gen. Physiol. 30:291-310.

Kuster E, Williams ST (1964) Selective media for the isolation of *Streptomycetes*. Nature 202: 928-929.

Labrie S, Bart C, Vadeboncoeur C, Moineau S (2005) Use of an α-Galactosidase gene as a food-grade selection marker for *Streptococcus thermophilus* J. Dairy Sci. 88: 2341-2347.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Laine A (1994) A calculation of all possible oligosaccharide isomers both branched and linear yields 1.05×10^{12} structures for a reducing hexasaccharide: the *Isomer Barrier* to development of single-method saccharide sequencing or synthesis systems. Glycobiol. 4:759–767.

Landsteiner K (1900) Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. Zbl Bakt, 27: 357–362.

Laskowski M (1955) Enzymes of protein metabolism: trypsinogen and trypsin. In: Colowick SP, Kaplan NO (Eds.) Methods in enzymology, vol. 2, New York Academic Press, pp 26-36.

Lazo PS, Ochoa AG, Gascon S (1978) α-Galactosidase (melibiase) from *Saccharomyces carlsbergensis*: structrual and kinetic properties. Arch. Biochem. Biophys. 191: 316-324.

LeBlanc JG, Silvestroni A, Connes C, Juillard V, de Giori GS, Piard JC, Sesma F (2004) Reduction of non-digestible oligosaccharides in soymilk: application of engineered lactic acid bacteria that produce α-galactosidase. Genet. Mol. Res. 3: 432-440 .

Lechevalier HA (1989) The Actinomycetes III, A Practical Guide to Generic Identification of Actinomycetes. Bergey' s Manual of Systematic Bacteriology. Williams & Wilkins Company, Baltimore. Vol. 4: 2344-2347,

LeClerc GM, Grahame DA (1996) Methylcobamide: coenzyme M methyltransferase isozymes from *Methanosarcina barkeri*. J. Biol. Chem. 271: 18725–18731.

Leder S, Hartmeier W, Marx SP (1999) α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083, Current Microbiol. 38: 101–106

LeDonne NC, Fairley JL, Sweeley CC (1983) Biosynthesis of α-galactosidase A in cultured chang liver cells. Arch. Biochem. Biophys. 224: 186-195.

Lee K, Jin X, Zhang K, Copertino L, Andrews L, Baker-Malcolm J (2003) A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. Glycobiol. 13: 305–313.

Lee RH, Lin MC, Chen SC (2004) A novel alkaline alphagalactosidase gene is involved in rice leaf senescence. Plant Mol. Biol. 55: 281–295.

Lerouge P, O'Neill MA, Darvill AG, Albersheim P, (1993) The purification of commercially available endo-a-L-arabinases and α-L-arabinosidase for use in the structural analysis of pectic polysaccharides. Carbohydr. Res. 243: 373-378.

Lesley SA, Kuhn P, Godzik A, Deacon AM, Mathews I, Kreusch A, Spraggon G, Klock HE, McMullan D, Shin T, Vincent J, Robb A, Brinen LS, Miller MD, McPhillips TM, Miller MA, Scheibe D, Canaves JM, Guda C, Jaroszewski L, Selby TL, Elsliger MA, Wooley J, Taylor SS, Hodgson KO, Wilson IA, Schultz PG, Stevens RC (2002) Structural genomics of the *Thermotoga maritima* proteome implemented in a high-throughput structure determination pipeline. Proc. Natl. Acad. Sci. U.S.A. 99: 11664-11669.

Li H, Liang WQ, Wang ZY, Luo N, Wu XY, Hu JM, Lu JQ, Zhang XY, Wu PC, Liu YH (2006) Enhanced production and partial characterization of thermostable α-galactosidase by thermotolerant *Absidia* sp.WL511 in solid-state fermentation using response surface methodology. World J. Microbiol. Biotechnol. 22:1–7.

Li J, Robertson DE, Short JM and Wang PG (1999) Chemical and enzymatic synthesis of glycoconjugates 5: one-pot regioselective synthesis of bioactive galactobiosides using a Clonenzyme™ thermophilic glycosidase library. Bioorg. Med. Chem. Lett. 9: 35-38.

Li X, Yang L, Yan P, Zuo F, Jin F, (1997) Factors regulating production of α-galactosidase from *Bacillus* sp. JF_2, Lett. Appl. Microbiol. 25: 1-4.

LI YT, and Shetlar MR (1964) Occurrence of α-galactosidase in higher fungi: Isolation of α-galactosidase from Calvatia cyathiformis. Arch. Biochem. Biophys. 108: 523.

LI YT, Li SC, Shetlar MR (1963) α-Galactosidase from *Diplococcus pneumoniae*. Arch. Biochem. Biophys. 103: 436-442.

Liebl W, Wagner B, Schellhase J (1998) Properties of an alpha-galactosidase, and structure of its gene galA, within an alpha- and betagalactoside utilization gene cluster of the hyperthermophilic bacterium *Thermotoga maritima*. Syst. Appl. Microbiol. 21:1–11.

Lin SS, Michael JH, Gonzalo VGS (2000) The role of anti-Gal α-1-3Gal antibodies in acute vascular rejection and accommodation of xenografts. Transplantation 70: 1667-1674.

Linden JC (1982) Immobilized α-D-galactosidase in the sugar beet industry. Enzyme Microb. Technol. 4: 130-136.

Liu C, Ruan H, Shen H, Chen Q, Zhou B, Li Y, and He G (2007a) Optimization of the fermentation medium for alpha-galactosidase production from *Aspergillus foetidus* ZU-G1 using response surface methodology, J. Food Sci. 72: 121-125.

Liu C-Q, Chen Q-H, Cheng Q-J, Wang J-L, He G-Q (2007b) Effect of cultivating conditions on α-galactosidase production by a novel *Aspergillus foetidus* ZU-G1 strain in solid-state fermentation, J. Zhejiang Univ. Science B 8(5): 371-376.

Liu C-Q, Chen Q-H, Tang B, Ruan H, He G-Q (2007c) Response surface methodology for optimizing the fermentation medium of alpha-galactosidase in solid-state fermentation, Lett. Appl. Microbiol. 45: 206–212.

Lowry OH, Rosebrough NJ, Farr AL, Randall RL (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265 – 275.

Luonteri E, Alatalo E, Siika-Aho M, Penttila M, Tenkanen M (1998a) α-Galactosidases of *Penicillium simplicissimum*: production, purification and characterization of the gene encoding AGLI. Biotechnol. Appl. Biochem. 28: 179-188.

Luonteri E, Tenkanen M, Viikari L, (1998b) Substrate specificities of *Pencillium simplicissimum* α-galactosidases. Enzyme Microb. Technol. 22: 192-198.

Ly HD, Howard S, Shum K, He S, Zhu A, Withers SG (2000) The synthesis, testing and use of 5-fluoro-alpha-D-galactosyl fluoride to trap an intermediate on green coffee bean alpha-galactosidase and identify the catalytic nucleophile. Carbohydr. Res. 329:539-547.

Ly HD, Withers SG (1999) Mutagenesis of glycosidases. Annu. Rev. Biochem. 68: 487-522.

Lyons AJ Jr, Pridham TG, Hesseltine CW (1969) Survey of some *Actinomycetales* for α-galactosidase activity. Appl. Micobiol. 18: 579-583.

Machaiah JP, Pednekar MD (2002) Carbohydrate composition of low dose radiation-processed legumes and reduction in flatulence factors. Food Chem. 79: 293–301.

Mamo G, Gessesse A (1997) Thermostable amylase production by immobilized thermophilic *Bacillus* sp. Biotechnol. Tech. 11: 447–450.

Mansour EH, Khalil AH (1998) Reduction of raffinose oligosaccharides in chickpea (*Cicer arietinum)* flour by crude extracellular fungal α-galactosidase. J. Sci. Food. Agric. 78: 175-181.

Manzanares P, de Graaff LH, Visser J (1998) Characterization of galactosidases from *Aspergillus niger*: purification of a novel α-galactosidase activity. Enzyme Microb. Technol. 22: 383-390.

Maranville E, Zhu A (2000) Assessment of amino-acid substitutions at tryptophan 16 in α-galactosidase. Eur. J. Biochem. 267: 1495-501.

Margolles-Clark E, Tenkanen M, Luonteri E, Penttilä M (1996) Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. Eur. J. Biochem. 240:104–111.

Mathew CD, Balasubramaniam K (1986) Chemical modification of α-galactosidase from coconut. Phytochem. 25: 2439-2443.

Matsuda M, Takeuchi S, Nagura T (1998) Influence of raffinose for atopic dermatitis. Allergy in Practice (in Japanese) 18: 1092-1095.

Mattiasson B, Larsson M, Hahn-Haegerdal B (1984) Metabolic behavior of immobilized cells-effects of some microenvironmental factors. Ann. N. Y. Acad. Sci.: Enzyme Eng. 434: 475–478.

Mazur WM, Duke JA, Wahala K, Rasku S, Adlerventz H (1998). Isoflavanoids and lignans in legumes: Nutritional and health aspects in humans. Nutritional Biochem. 9: 193–200.

McCarter JD, Withers SG (1994) Mechanisms of enzymatic glycoside hydrolysis. Curr. Opin. Struct. Biol. 4: 885-892.

McCleary BV (1983) Enzymic interactions in the hydrolysis of galactomannan in germinating guar: The role of exo-β-mannanase, Phytochem. 22: 649-658.

McCleary BV (1994) Enzymatic analysis of the fine structure of galactomannans. In: BeMiller JN, Manners DJ, Sturgeon RJ (Eds.) Methods in Carbohydrate Chemistry. Vol 10. John Wiley & Sons, Inc. pp 175-182.

McCleary BV, Matheson NK (1974) α-D-Galactosidase activity and galactomannan and galactosylsucrose oligosaccharide depletion in germinating legume seeds, Phytochem. 13: 1747 – 1757.

McCleary BV, Matheson NK (1986) Enzymatic analysis of polysaccharide structure. Adv. Carbohydr. Chem. Biochem. 44: 147-276.

McCutchen CM, Duffaud GD, Leduc P, Petersen ARH, Tayal A, Khan SA, Kelly RM (1996) Characterization of extremely thermostable enzymatic breakers (α-1,6-galactosidase and β-1,4-mannanase) from the hyperthermophilic bacterium *Thermotoga neapolitana* 5068 for hydrolysis of guar gum. Biotechnol. Bioeng., 52 : 332-339.

McKay AM (1991), Production of extracelluar β-glucosidase and α-galactosidase during fungal growth on polygalacturonate. J Food Sci. 56: 1749.

Messina MJ (1999) Legumes and Soybeans overview of their Nutritional Profiles and Health Effects. Am. J. Clin. Nutr. 70: 439S-450S.

Milagres AMF, Santos E, Piovan T, Roberto IC (2004) Production of xylanase by *Thermoascus aurantiacus* from sugar cane bagasse in an aerated growth fermentor. Process Biochem. 39: 1387–1391.

Miller ES, Kimberley JR, Parker N, Liebl W, Lam D, Callen W, Snead MA, Mathur EJ, Short JM, Kelly RM (2001) α-D-Galactosidases from *Thermotoga* species. In: Adams MW, Kelly RM (Eds.) Methods in Enzymology, Academic Press, New York.

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal Chem, 31 426-428.

Miranda PM, Horowitz DL (1978) High fiber and diets in the treatment of diabetes mellitus. Ann. Intern. Med. 88: 482-486.

Mitchell DA, Krieger N, Stuart DM, Pandey A (2000) New developments in solid-state fermentation II. Rational approaches to the design, operation and scale-up of bioreactors. Process Biochem. 35:1211–1225.

Mitchell DA, von Meien O F, Krieger N (2003) Recent developments in modeling of solid-state fermentation: heat and mass transfer in bioreactors. Biochem. Eng. J. 13:137–147.

Mitsutomi M, Ohtakara A (1988) Isolation and identification of oligo-saccharides produced from raffinose by transgalactosylation reaction of thermostable alpha-galactosidase from *Pycnoporus cinnabarinus*. Agri. Biol.Chem. 52: 2305-2311.

Mitsutomi M, Uchida Y, Ohtakara A (1985) J. Ferment. Technol. 63: 325-329.

Modi AT, McDonald MB, Streeter JG (2000) Soluble carbohydrates in soybean seeds during development and imbibition. Seed Sci. Technol. 28: 115–127.

Monis B, Wasserkrug H (1967) Histochemistry of glycosidases of Megakaryocytes and Platelets, A comparative study. Histochemie. 10: 363-368.

Monteil MD, Hernandez M, Rodriguez J, Arias ME (2002) Evaluation of an endo-β-mannanase produced by *Streptomyces ipomoea* CECT 3341 for the biobleaching of pine kraft pulp. Appl. Microbiol. Biotechnol. 58: 67–72

Moreira LRS, Filho EXF (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. 79:165–178.

Mulimani VH, Devendra S (1998) Effect of soaking, cooking and crude α-galactosidase treatment on the oligosaccharide content of red gram flour. Food Chem. 61: 475-479.

Mulimani VH, Ramlingam (1995) Enzymic hyrolysis of Raffinose and Stachyose in soymilk by α-galactosidase from *Gibberella fujikuroi*. Biochem. Mol. Biol. Intl., 36: 897-905.

Mulimani VH, Thippeswamy S, Ramalingam (1997). Enzymatic degradation of oligosaccharides in soybean flours. Food Chem. 59: 279-282.

Murata T, Usui T (2006) Enzymatic synthesis of oligosaccharides and neoglycoconjugates. Biosci. Biotechnol. Biochem. 70: 1049-1059.

Nadkarni MA, Nair CKK, Pandey VN, Pradhan DS (1992) Characterization of α-galactosidase from Corynebacterium murisepticum and mechanism of its induction. J. Gen. Appl. Microbiol. 38: 23-34.

Naessens M, Vandamme EJ (2003) Multiple forms of microbial enzymes, Biotechnol. Lett. 25: 1119–1124.

Naganagouda K, Mulimani VH (2006) Gelatin blends with alginate: Gel fibers for α-galactosidase immobilization and its application in reduction of non-digestible oligosaccharides in soymilk. Process Biochem. 41:1903–1907.

Naganagouda K, Prashanth SJ, Shankar SK, Dhananjay SK, Mulimani VH (2007) Immobilization of *Aspergillus oryzae* α-galactosidase in gelatin and its application in removal of flatulence-inducing sugars in soymilk. World J. Microbiol. Biotechnol. 23:1131–1137.

Nagura T, Hachimura S, Hashiguchi M, Ueda Y, Kanno T, Kikuchi H, Sayama K and Kaminogawa S (2002) Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity, Br. J. Nutr. 88: 421-426,

Nampoothiri KM, Pandey A (1998) Immobilization of *Brevibacterium* cells for the production of L-glutamic acid. Bioresour. Technol. 63: 101-106.

Narahara H, Koyama Y, Yoshida T, Pichangkura S, Ueda R, Taguchi H (1982) Growth and enzyme production in a solid-state culture of *Aspergillus oryzae*. J. Ferment. Technol. 60: 311-319.

Narita S, Naganishi H, Yokouchi A, Kagaya I (1976) Method for manufacture of α-galactosidase by microorganism. US Patent 3,957,578.

Naumoff DG (2001) Sequence analysis of glycosyl hydrolases: β-fructosidase and α-galactosidase superfamilies. Glycoconjugate J. 18: 109.

Naumoff DG (2002) Sequence analysis and classification of α-galactosidases. Int. Summer School "From Genome to Life: Structural, Functional and Evolutionary Approaches". Cargese, Corsica, France. P. 40. (http://www.archbac.u-psud.fr/Meetings/cargese2002/).

Naumoff DG (2004) Phylogenetic analysis of α-galactosidases of the GH27 family. Mol. Biol. 38: 388-399.

Naumoff DG (2005) GH97 is a new family of glycoside hydrolases, which is related to the α-galactosidase superfamily. BMC Genomics 6:112, 1-12.

Nelson N (1944) Photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-378.

Niladevi KN, Prema P (2005) Mangrove actinomycetes as the source of ligninolytic enzymes. Actinomycetologica. 19: 40-47.

Obendorf RL (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. Seed Sci. Res. 7: 63–74.

Oda Y, Tonomura K (1996) α-Galactosidase from the yeast *Torulaspora delbrueckii* IFO 1255. J. Appl. Bacteriol. 80: 203-208.

Ofek I, Sharon N (1990) Adhesins as lectins: specificity and role in infection. Curr. Top. Microbiol Immunol. 151: 91-113.

Oh JK, Lee JY, Park HK, Kho HS (2008) α-Galactosidase activity in human saliva. Arch. Oral Biol. (In Press).

Ohtakara A, Mitsutomi M (1987) Immobilization of thermostable α-galactosidase from *Pycnoporus cinnabarinus* on chitosan beads and its application to the hydrolysis of raffinose in beet sugar molasses. J. Ferment. Technol. 65: 493-498.

Ohtakara A, Mitsutomi M, Uchida Y (1984) Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus*. Agr. Bio. Chem. 48: 1319-1327.

Oishi K, Aida K (1971) Blood group substance degrading enzymes obtained from *Streptomyces* sp. Part-II. Purification and characterization of α-D-galactosidase from *Sterptomyces* 9917S2. Agr. Biol. Chem., 35: 1101-1108.

Olivieri R, Pansolli P, Fascetti E, Cuiffiolotti P (1984) Process for the production of α-galactosidase and uses of the enzyme thus obtained. US Patent 4,431,737

Onigbinde AO, Akinyele IO (1983) Oligosaccharide content of 20 varieties of cowpeas in Nigeria. J. Food Sci. 48: 1250-1254.

Onysko KA (1993) Biological bleaching of chemical pulps: a review. Biotechnol. Adv. 11: 179–198.

Pai VB, Khan SA (2002) Gelation and rheology of xanthan /enzyme modified guar blends. Carbohydr. Poly. 49: 207-216.

Pandey A, Selvakumar P, Ashakumary L (1996) Performance of a column bioreactor for glucoamylase synthesis by *Aspergillus niger* in SSF. Process Biochem. 31:43-46.

Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P (2001) Solid-state fermentation in biotechnology. Asiatech Publishers Inc. New Delhi.

Park J, Murray GJ, Limaye A, Quirk JM, Gelderman MP, Brady RO, Qasba P (2003). Long-term correction of globotriaosylceramide storage in Fabry mice by recombinant adeno-associated virus-mediated gene transfer. Proc. Natl Acad. Sci. USA 100: 3450-3454.

Pastores GM, Thadhani R (2002) Advances in the management ofAnderson-Fabry disease: Enzyme replacement therapy. Expert Opin. Biol. Ther. 2:1-9.

Pederson DM, Goodman RE (1980) Isozymes of α-galactosidase from *Bacillus stearothermophilus*. Can. J. Microbiol, 26: 978-84.

Peters FPJ, Vermeulen A, Kho TL (2001) Anderson-Fabry's disease: α-galactosidase deficiency. Lancet 357:138–140.

Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33: 305–325.

Porter JE, Ladisch MR (1991) Ion exchange and affinity chromatography in the scaleup of the purification of α-galactosidase from soybean seeds, Biotechnol. Bioeng. 37: 356-363.

Post DA, Luebke VE (2005) Purification, cloning and properties of a-galactosidase from *Saccharopolyspora erythraea* and its use as a reporter system. Appl. Microbiol. Biotechnol. 67: 91-96.

Poutanen K, Tenkanen M, Korte H, Puls J (1991) Accessory enzymes involved in the hydrolysis of xylans. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. (ACS Symposium Series 460) American Chemical Society, Washington, Dc, 426-435.

Prasanth SJ, Mulimani VH (2005) Soymilk oligosaccharide hydrolysis by *Aspergillus oryzae* α-galactosidase immobilized in calcium alginate. Process Biochem. 40: 1199-1205.

Price KR, Lewis J, Wyatt GM, Fenwick GR (1988). Flatulence – causes, relation to diet and remedies. Die Nahrung 32: 609 – 626.

Przybylska M, Wu I-H, Zhao H, Ziegler RJ, Tousignant JD, Desnick RJ, Scheule RK, Cheng SH, Yew NS (2004) Partial correction of the α-galactosidase A deficiency and reduction of glycolipid storage in Fabry mice using synthetic vectors. J. Gene Med. 6: 85–92.

Puchart V, Vrsanska M, Bhat MK, Biely P (2000) Purification and characterization of α-galactosidase from a thermophilic fungus Thermomyces lanuginosus. Biochim. Biophys. Acta 1524: 27-37.

Pugalenthi M, Siddhuraju P, Vadivel V (2006). Effect of soaking followed by cooking and the addition of α-galactosidase on oligosaccharides levels in different *Canavalia* accessions. J. Food Composition Anal. 19: 512-517.

Ragupathi G, Park TK, Zhang S, Kim IJ, Graber L, Adluri S, Lloyd KO, Danishefski SJ, Livingston PO (1997) Angew. Chem. Int. Ed. Engl. 36: 125.

Ramachandran S, Roopesh K, Nampoothiri KM, Szakacs G, Pandey A (2005). Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. Process Biochem. 40: 1749-1754.

Ramakrishna SV, Prakasham RS (1999) Microbial fermentations with immobilized cells. Curr. Sci. 77: 87–100.

Rao PV, Belavady B (1978) Oligosaccharides in pulses: varietal differences and effect of cooking and germination. J.Agric. Food Chem. 26: 316-319.

Rathbone EB (1980) Raffinose and Melezitose. In: Lee CK (Ed.) *Developments in food carbohydrate.* vol. 2 (pp 145-185) Applied Science Publishers Ltd, London, United Kingdom.

Ren Y-F, He J-Y, Wang X-F (2007) Changes in Activities of Three Enzymes Degrading Galactomannan During and Following Rice Seed Germination, Rice Sci. 14: 295-301.

Reynolds JH (1974) An immobilized α-galactosidase continuous flow reactor. Biotechnol. Bioeng. 16: 135-147.

Rezessy-Szabó JM, Nguyen QD, Bujna E, Takács K, Kovács M and Hoschke Á, (2003) *Thermomyces lanuginosus* CBS 395.62/b strain as rich source of α-galactosidase enzyme, Food Technol. Biotechnol. 41: 55-59.

Rezessy-Szabó JM, Nguyen QD, Hoschke Á, Braet C, Hajós G, Claeyssens M (2007) A novel thermostable α-galactosidase from the thermophilic fungus *Thermomyces lanuginosus* CBS 395.62/b: Purification and characterization. Biochim et Biophys Acta 1770: 55–62.

Rigden DJ (2002) Iterative database searches demonstrate that glycoside hydrolase families 27, 31, 36 and 66 share a common evolutionary origin with family 13, FEBS Lett. 523: 17-22.

Rios S, Pedregosa AM, Fernandez Monistrol I, Laborda F (1993) Purification and molecular properties of an α-galactosidase synthesized and secreted by Aspergillus nidulans. FEMS Microbiol. Lett. 112: 35-41.

Rivero-Urgell M, Santamaria-Orleans A (2001) Oligosaccharides: application in infant food, Early Hu. Dev., 65 (2) 43-52.

Rye CS, Withers SG (2000) Glycosidase mechanisms. Curr. Opin. Chem. Biol. 4: 573-580.

Sakai K, Tachiki T, Kumagai H, Tochikura T (1987) Hydrolysis of α-D-galactosidase of *Bifidobacterium breve* 203, Agric. Biol. Chem. 51: 315-322.

Sakurai Y, Lee TH, Shiota H (1977) On the convenient method of glucosamine estimation in koji. Agric. Biol. Chem. 41: 619–624.

Savelév AN, Eneyskaya EV, Isaeva-Ivanova LS, Shabalin KA, Golubev AM, Neustroev KN (1996) Enzymatic properties of α-galactosidase from *Trichoderma reesei*. Carbohyd. Res. 296: 261-273.

Scalabrini P, Rossi M, Spettoli P, Matteuzzi D (1998) Characterization of Bifidobacterium strains for use in soymilk fermentation. Int. J. Food Microbiol. 39: 213-219.

Schaffer AA, Pharr DM, Madore MA, Cucurbits (1996) In: Zamski E, Schaffer AA (Eds.) Photoassimilate distribution in plants and crops, Marcel Dekker, New York, pp. 729 – 757.

Schiffmann R, Floeter MK, Dambrosia JM, Gupta S, Moore DF, Sharabi Y, Khurana RK, Brady RO (2003) Enzyme replacement therapy improves peripheral nerve and sweat function in fabry disease, Muscle Nerve 28: 703–710.

Schiffmann R, Kopp JB, Austin HA III, Sabnis S, Moore DF, Weibel T. (2001). Enzyme replacement therapy in Fabry disease: a randomized controlled trial. JAMA 285: 2743–2749.

Schmitt R, Rotman B (1966) α-galactosidase activity in cell free extract of *E. Coli*. Biochem. Biophys. Res. Commun. 22: 473.

Schuler R (1985) Kinetic properties of α-galactosidase from *Lactobacillus fermentum*. Enzyme Microb. Technol. 7: 207-211.

Scigelova M, Crout DHG (2000) Purification of alpha-galactosidase from *Aspergillus niger* for application in the synthesis of complex oligo-saccharides. J. Mol. Catalys. B; Enzymatic. 8: 175-181.

Scrivener AM, Watanabe H, Noda H (1997) Diet and carbohydrate digestion in the yellow-spotted longicorn leetle *Psacothea hilaris*. J. Insect Physiol. 43: 1039–1052.

Shabalin KA, Kulminskaya AA, Savelev, Shishlyannikov SM, Neustroev KN (2002) Enzymatic properties of α-galactosidase from *Trichoderma reesei* in the hydrolysis of galactooligosaccharides. Enzyme Microb. Technol. 30: 231-239.

Shankar SK, Girigouda K, Mulimani VH (2006) Production of α-galactosidase by *Aspergillus oryzae* using solid state fermentation. Indian J. Microbiol. 46: 165-7.

Shankar SK, VH Mulimani (2007) α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresour. Technol. 98: 958–961.

Sharma A, Okabe P, McClellan SB, Martin MJ, Platt JL, Logan JS (1996) Reduction in the level of Gal α-(1,3)Gal in transgenic mice and pigs by the expression of an α-(1,2)fucosyltransferase. Proc Natl Acad Sci USA 93: 7190-7195.

Shaw CR (1969) Isozymes: classification, frequency, and significance. Int. Rev. Cytol. 25: 297–332.

Shchipunov YA, Karpenko TY, Bakunina IY, Burtseva YV, Zvyagintseva TN (2004) A new precursor for the immobilization of enzymes inside sol–gel-derived hybrid silica nanocomposites containing polysaccharides, J. Biochem. Biophys. Methods 58: 25–38.

Shen W, Zhengyu Jin, Jianwei Zhao, Li Deng, Chao Yuan, Dandan Li, Xuehong Li (2008) New source of α-D-galactosidase: germinating coffee beans *Food Chemistry, In Press, Accepted Manuscript, Available online 8 March 2008*

Shibuya H, Kobayashi H, Park GG, Komatsu Y, Sato T, Kaneko R, Nagasaki H, Yoshida S, Kasamo K, Kusakabe I (1995) Purification and some properties of α-galactosidase from *Penicillium purpurogenum*. Biosci. Biotechnol. Biochem. 59: 2333-2335.

Shibuya H, Kobayashi H, Sato T, Kim WS, Yoshida S, Kaneko S, Kasamo K, Kusakabe I (1997) Purification, characterization, and cDNA cloning of a novel α-galactosidase from *Mortierella vinacea*. Biosci. Biotechnol. Biochem. 61: 592-598.

Silman RW, Black LT, McGhee JE, Bagley EB (1980) Hydrolysis of raffinose in a hollow-fiber reactor using an unrefined mixture of a-galactosidase and invertase. Biotechnol. Bioeng. 22: 533-541.

Silva CP, Terra WR (1997) α-Galactosidase activity in ingested seeds and in the midgut of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Archs. Insect Biochem. Physiol. 34: 443–460.

Simerská P, Kuzma M, Monti D, Riva S, Macková M, Křen V (2006) Unique transglycosylation potential of extracellular α-D-galactosidase from *Talaromyces flavus*, J. Mol. Cat. B: Enzymatic 39: 128–134.

Simerská P, Monti D, Čechová I, Pelantová H, Macková M, Bezouška K, Riva S, Křen V (2007) Induction and characterization of an unusual α-D-galactosidase from *Talaromyces flavus*, J. Biotechnol. 128: 61–71.

Singh S, Madlala AM, Prior BA (2003) *Thermomyces lanuginosus*: properties of strains and their hemicelulases. FEMS Microbiol. Rev. 27: 3–16

Sinnott ML (1990) Catalytic mechanisms of enzymatic glycosyl transfer. Chem. Rev. 90: 1171-1202.

Sittikijyothin W, Torres D, Gonçalves MP (2005) Modelling the rheological behaviour of galactomannan aqueous solutions. Carbohydr. Polym. 59: 339–350

Smiley KL, Hensley DE, Gasdorf HJ (1976) Alpha-galactosidase production and use in a hollow-fiber reactor, Appl. Env. Microbiol. 32: 615-617.

Smith AK, Circle JJ (1972) Soybeans: Chemistry and Technology. Westpost, CT (USA): Westpost, CN Avi Publishing Co., Westport, CN.

Somiari RI, Balogh E (1992) Hydrolysis of raffinose and stachyose in cowpea (*Vigna unguiculata*) flour, using α-galactosidase from *Aspergillus niger*. World J. Microbiol. Biotechnol. 8: 564-566.

Somiari RI, Balogh E (1995) Properties of an extracellular glycosidase of *Aspergillus niger* suitable for removal of oligo-saccharides from cow pea meal. Enzyme Microb. Technol. 17: 311-316.

Sonia KG, Chadha BS, Saini HS (2005) Sorghum straw for xylanase hyper-production by *Thermomyces lanuginosus* (D2W3) under solid-state fermentation. Biores. Technol. 96: 1561–1569.

Sonnleitner B, Fiechter A (1983) Advantages of using thermophiles in biotechnological processes: expectations and reality. Trends Biotechnol. 1:74–80.

Spangenberg P, Adré C, Dion M, Rabiller C, Mattes R (2000) Comparative study of new α-galactosidases in transglycosylation reactions, Carbohydr. Res. 329: 65-73.

Spök A (2006) Safety Regulations of Food Enzymes. Food Technol. Biotechnol. 44:197–209.

Srinivas MRS, Chand N, Lonsane BK (1994) Use of Plackett-Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of α-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system. Bioprocess Eng. 10: 139-44.

Srinivas MRS, Padmanabhan S, Lonsane BK (1993) Growth kinetics, α-galactosidase biosynthesis, and concomitant invertase production by *Aspergillus niger* NCIM 839 in a solid-state fermentation system. Chem. Mikrobiol. Technol. Lebensm. 15: 41-46.

Sripuan T, Aoki K, Yamamoto K, Tongkao D, Kumagai H (2003) Purification and characterization of thermostable α-galactosidase from *Ganoderma lucidum*, Biosci. Biotechnol. Biochem. 67: 1485-1491.

Stromberg N, Marklund BI, Lund B, Ilver D, Hamers A, Gaastra W, Karlson KA and Normark S (1990) Host-specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to Galα1-4Gal-containing isoreceptors. EMBO J. 9: 2001-2010.

Sumner-Smith M, Bozzato RP, Skipper N, Davies RW, Hopper JE (1985) Analysis of the inducible MEL1 gene of *Saccharomyces carlsbergensis* and its secreted product, alpha galactosidase (melibiase). Gene 36: 333-340.

Suzuki H, Kobayashi H, Ozawa Y, Kamibayashi A(1974) Method for manufacture of α-galactosidase by microorganisms. U.S. Patent, 3,795,585;

Suzuki H, Li SC, Li YT (1970) α-Galactosidase from *Morteirella vinacea*: Crystallization and properties, J. Biol. Chem. 245: 781-786.

Suzuki H, Ozawa Y, Oota H, Yoshida H (1969) Stidies on the decomposition of raffinose by α-galactosidase of Mold. Part-I α-Galactosidase formation and hydrolysis of raffinose by the enzyme preparation. Agric. Biol. Chem. 33: 506.

Suzuki H, Ozawa Y, Tanabe O (1966) Studies on the decomposition of raffinose by actinomycetes. IV. Characteristics of α-galactosidase and estimation of raffinose by the enzyme preparation. Agric. Biol. Chem. 30: 1039-1046,

Suzuki H, Ozawa Y, Tanabe O (1972) Method of reducing raffinose content of beet molasses. US Patent, 3,647,625.

Szmigielski S (1966) An improved method for histochemical demonstration of β-glucuronidase and α-galactosidase activity in bone marrow and blood cells. J. Lab. Clin. Med. 67: 709

Takizawa M, Colwell RR, Hill RT (1993) Isolation and diversity of actinomycetes in the Chesapeake Bay. Appl. Environ. Microbiol. 59: 997–1002,

Talbot G, Sygusch J (1990) Purification and characterization of thermostable β-mannanse and α-galactosidase from *Bacillus stearothermophilus*, Appl. Environ. Microbiol. 56: 3505-3510.

Taniguchi M, Harada M, Kojo S, Nakayama T and Wakao H (2003) The regulatory role of Vα14NKT cells in innate and acquired immune response, Ann. Rev. Immunol. 21: 483-513.

Tanriseven A, Dogan S (2002) A novel method for the immobilization of bgalactosidase. Process Biochem. 38: 27–30.

Tenkanen M, Makkonen M, Perttula M, Viikari L, Teleman A (1997) Action of *Trichoderma reesei* mannose on galacto-glucomannan in pine kraft pulp. J. Biotechnol. 57: 191-204.

Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109B:1–62.

Terra WR, Ferreira C, Garcia ES (1988) Origin, distribution, properties, and functions of the major *Rhodnius prolixus* midgut hydrolases. Insect Biochem. 18: 423–434.

Teruel AML, Gontier E, Bienaime C, Nava Saucedo JE, Barbotin JN (1997) Response surface analysis of chlortetracycline and tetracycline production with κ-carragenan immobilized *Streptomyces aureofaciens*. Enzyme Microb. Technol. 21: 314-320.

Thananunkul D, Tanaka M, Chichester CO, Lee TC (1976) Degradation of raffinose and stachyose in soymilk by α-galactosidase from *Morteirella vinacea*. Entrapment of α-galactosidase within polyacrylamide gel. J. Food Sci. 41: 173-175.

Thippeswamy S, Mulimani VH (2002) Enzymatic degradation of raffinose family oligosaccharides in soymilk by immoblized α-galactosidase from *Gibberella fujikuroi*, Process Biochem. 38: 635-640.

Thomas B, Webb JA (1977) Multiple forms of α-galactosidase in mature leaves of *Cucurbita pepo*. Phytochem. 16, 203 – 206.

Thompson NS (1983) Hemicellulose as a biomass resource. In: Soltes E (Ed.) Wood and agricultural residues- research on use for feed, fuels and chemicals. Academic Press, London, pp 101-119.

Tomme P, Warren RA, Miller RC Jr, Kilburn DG, Gilkes NR (1995) Cellulose-binding domains: classification and properties. In: Saddler JN, Penner M (Eds.) Enzymatic Degradation of Insoluble Polysaccharides, American Chemical Society, Washington, DC. pp. 142–163.

Tzortis G, Jay AJ, Baillon MLA, Gibson GR, and Rastall RA (2003) Synthesis of α-galactooligosaccharides with α-galactosidase from *Lactobacillus reuteri* of canine origin. Appl. Microbiol. Biotechnol. 63: 286-292.

Ulezlo IV, Zaprometova OM (1982) Microbial alpha-galactosidase (a review). Prikl. Biokhim. Mikrobiol. 18: 3-15.

Uma Maheswari M, Chandra TS (2000) Production and potential applications of a xylanase from a new strain of *Streptomyces cuspidosporus*. World J. Microbiol. Biotechnol. 16: 257-263,

Upadhyay JK, Garcia VV (1988). Effect of soaking and cooking on reduction of oligosaccharides of cowpea (*Vigna unguiculata* (L) Walp.). Philip, J.Food Sci. Technol.12: 21-28.

Urashima T, Saito T, Nakamura T, Messer M (2001) Oligosccharides of milk and colostrums in non-human mammals. Glycoconj. J. 18: 357-371.

Urbano G, Jurado ML, Porres JM, Frejnagel S, Villalva EG., Frias J, Valverde CV, Aranda P (2007) Effect of treatment with α-galactosidase, tannase or a cell-wall-degrading enzyme complex on the nutritive utilisation of protein and carbohydrates from pea (*Pisum sativum* L.) flour. J. Sci. Food Agric. 87: 1356-1363.

Valera HR, Gomes J, Lakshmi S, Gururaja R, Suryanarayan S, Kumar D (2005) Lovastatin production by solid state fermentation using *Aspergillus flavipes*. Enzyme Microb. Technol. 37: 521–526.

van den Broek LAM, Ton J, Verdoes JC, van Laere KMJ, Voragen AGJ, Beldman G (1999) Synthesis of α-galacto-oligosaccharides by a cloned α-galactosidase from *Bifidobacterium adolescentis*. Biotechnol. Lett. 21: 441-445.

van Laere KMJ, Hartemink R, Beldman G, Pitson S, Dijkema C, Schols HA, Voragen AGJ (1999) Transglycosidase activity of *Bifidobacterium adolescentis* DSM 20083 α-galactosidase. Appl. Microbiol. Biotechnol. 52: 681-688.

Van Rantwijk F, Woudenberg-van Oosterom M, Sheldon RA (1999) Glycosidase-catalyzed synthesis of alkyl glycosides. J. Mol. Catal. B: Enzym. 6: 511–532.

Varbanets DL, Malanchuk VM, Buglova TT, Kuhlmann RA (2001) *Penicillium* sp. 23 α-galactosidase: purification and substrate specificity. Carbohydr. Poly. 44: 357-363.

Vasella A, Davies GJ, Bo¨hm M (2002) Glycosidase mechanisms. Curr. Opin. Chem. Biol. 6: 619-629.

Vernon CA (1967) The mechanisms of hydrolysis of glycosides and their relevance to enzyme-catalyzed reactions. Proc. R Soc. Ser B. 167: 389-401.

Viana PA, de Rezende ST, Falkoski DL, de Almeida Leite T, José IC, Moreira MA, Guimarães VM (2007) Hydrolysis of oligosaccharides in soybean products by *Debaryomyces hansenii* UFV-1 α-galactosidases, Food Chem. 103: 331-337.

Viana SF, Guimarães VM, Josè IC, Oliveira MGA, Costa NMB, Barros EG, et al. (2005). Hydrolysis of oligosaccharides in soybean flour by soybean α-galactosidase. Food Chem. 93: 665-670.

Viikari L, Kantelinen A, Sundquist J, Linko M (1994) Xylanases in bleaching: from an idea to the industry. FEMS Microbiol. Rev. 13: 335-350.

Viikari L, Tenkanen M, Suurnäkki A (2001) Biotechnology in the pulp and paper industry. In: Rehm HJ (Ed.) Biotechnology, 2nd edn, vol 10. VCH-Wiley, pp 523–546.

Viniegra-González G, Favela-Torres E (2006) Why solid-state fermentation seems to be resistant to catabolite repression?. Food Technol. Biotechnol., 44: 397-406.

Vosnidou NC, Johnson SA, Mitra MM, Wells DC, Li CQ, Evans ML, Harmata MA, Walker JC, Smith DS (1998) Seroconversion of type B to O erythrocytes using recombinant *Glycine max* a-D-galactosidase. Biochem. Mol. Biol. Int. 46: 175-186.

Vyas MN, Vyas NK, Quiocho FA (1994) Crystallographic analysis of the epimeric and anomeric specificity of the periplasmic transport/chemosensory protein receptor for D-glucose and D-galactose. Biochem. 33: 4762–4768.

Waldroup PW, Fritts CA, Keen CA, Yan F (2005) The effect of alpha-galactosidase enzyme with and without avizyme 1502 on performance of broilers fed diets based on corn and soybean meal. International J. Poultry Sci. 4: 920-937.

Wang AM, Bishop DF, Desnick RJ (1990). Human a-N-acetylgalactosaminidase-molecular cloning, nucleotide sequence, and expression of a full length cDNA. Homology with human α-galactosidase A suggests evolution from a common ancestral gene. J. Biol. Chem. 265: 21859–21866.

Wang AM, Ioannou YA, Zeidner KM, Desnick RJ (1998). Murine α-N-acetylgalactosaminidase: isolation and expression of a full-length cDNA and genomic organization: further evidence of an α-galactosidase gene family. Mol. Genet. Metab. 65: 165–173.

Wang CC, Lee JC, Luo SY, Fan HF, Pai CL, Yang WC, Lu LD and Hung SC (2002) Synthesis of biologically potent α-1→2-linked disaccharide derivatives *via* regioselective one-pot protection-glycosylation, Angew. Chem. Int. Ed. 41: 2360-2362.

Wang CL, Li DF, Lu WQ, Wang YH Lai CH (2004) Influence of cultivating conditions on the α-galactosidase biosynthesis from a novel strain of *Penicillium* sp. in solid-state fermentation, Lett. Appl. Microbiol. 39: 369-375.

Wang Q, Graham RW, Trimbur D, Warren RAJ, Withers SG (1994) Changing enyzmatic reaction mechanisms by mutagenesis: Conversion of a retaining glucosidase to an inverting enzyme. J. Am. Chem. Soc. 116:11594-11595.

Watanabe H, Sonoyama K, Watanabe J, Yamaguchi N, Kikuchi H, Nagura T, Aritsuka T, Fukumoto K and Kasai T (2004) Reduction of allergic airway eosinophilia by dietary raffinose in Brown Norway rats, Br. J. Nutr. 92: 247-255.

Watier H, Guillaumin JM, Piller F, Lacord M, Thibault G, Lebranchu Y (1996) Removal of terminal alpha galactosyl residues from xenogeneic porcine endothelial cells. Decrease in complement-mediated cytotoxicity by persistence of IgG1-mediated antibody-dependent cell-mediated cytotoxicity. Transplantation 62: 105-113.

Weignerová L, Huňková Z, Kuzma M, Křen V (2001) Enzymatic synthesis of three *p*NP-a-galactobiopyranosides: application of the library of fungal α-galactosidases, Journal of Molecular Catalysis B: Enzymatic 11: 219–224.

Weis WI, Drickamer K (1996). Structural basis of lectin-carbohydrate recognition. Annu. Rev. Biochem. 65, 441–473.

Weyland H (1986) Actinomycetes of the bottom sediments of various seas GERBAM — Deuxième Colloque International de Bactériologie marine — CNRS, Brest, 1-5 octobre 1984 IFREMER, Actes de Colloques. 3:73-79

Whistler RL, Bushway AA, Singh PP, Naxahara W, Tokuzen R (1975) Non cytotoxic, antitumor polysaccharides. Adv. Carbohydr. Chem. Biochem. 32: 235-275.

Williams ST, Cross T (1971) Actinomycetes, In: Methods in Microbiology, vol 4, Norris JR, Robbins DW (Eds.) (Academic Press, New York). pp 295- 334.

Wiseman A, (1985) Handbook of enzyme biotechnology, Ellis Horwood Ltd. New York, pp 274-379.

Withers SG (2001) Mechanisms of glycosyl transferases and hydrolases. Carbohydr. Poly. 44: 325-337.

Wolfenden R, Lu X, Young G. (1998) Spontaneous hydrolysis of glycosides. J. Am. Chem. Soc. 120: 6814-6815.

Wong H, Hu C, Yeh H, Su W, Lu H, Lin C (1986) Production, Purification and Characterization of α-galactosidase from Monascus pilosus. Appl. Environ. Microbiol. 52: 1147-1152.

Wong-Leung YL, Fong WF, Lam WL (1993) Production of α-galactosidase by *Monascus* grown on soybean and sugarcane wastes. World J. Microbiol. Biotechnol. 9: 529-533.

Xiao M, Tanaka K, Qian XM, Yamamoto K, Kumagai H (2000) High-yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose. Biotechnol. Lett. 22: 747-751.

Yamane T (1971) Decomposition of raffinose by -galactosidase. An enzymatic reaction applied in the factory-process in Japanese beet sugar factories. Sucr. Belge/Sugar Ind. Abstr. 90: 345-348.

Yamashita A, Hashimoto H, Fujita K, Okada M, Mori S, Kitahata S (2005) Reverse reaction of *Aspergillus niger* APC-9319 α-galactosidase in a supersaturated substrate solution: production of α-linked galactooligosaccharides (α-GOS) Biosci. Biotechnol. Biochem. 69: 1381-1388.

Yip VLY, Withers SG (2006) Family 4 glycoside hydrolases are special: The first β-elimination mechanism amongst glycoside hydrolases, Biocat. Biotransform. 24: 167-176.

Zapater IG, Ullah AHJ, Wodzinski RJ (1990) Extracellular α-galactosidase from *Aspergillus ficuum* NRRL3135 purification and characterization. Preparative Biochem. 20: 263-296.

Zaprometova OM, Ulezlo IV (1981) Studies on the optimal cultivation conditions of Cephalosporium sp., a producer of α-galactosidase., Mikrobiol. Promst. 4: 20.

Zaprometova OM, Ulezlo IV (1988) Isolation and purification of a mold α-galactosidase. Biotechnol. Appl. Biochem. 10: 232-241.

Zaprometova OM, Ulezlo IV, Lakhtin VM (1990) Structure and properties of a *Cephalosporium acremonium* α-galactosidase. Glycoconj. J. 7: 287-300.

Zechel DL, Withers SG (2000) Glycosidase mechanisms: Anatomy of a finely tuned catalyst. Acc. Chem. Res. 33:11-18.

Zechel DL, Withers SG. 2001. Dissection of nucleophilic and acid-base catalysis in glycosidases. Curr. Opin. Chem. Biol. 5: 643-649.

Zeilinger S, Kristufek D, Arisan-Atac I, Hodits R, Kubicek CP (1993) Conditions of formation, purification and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. Appl. Environ. Microbiol. 59: 1347-1353.

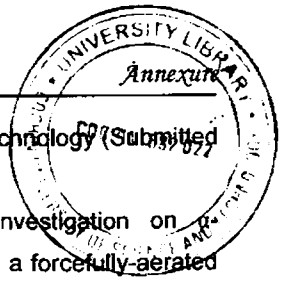
Zhang W, Kim JH, Franco CMM, Middelberg APJ (2000) Characterization of shrinkage of calcium alginate gel membrane with immobilized *Lactobacillus rhamnosus*. Appl. Microbiol. Biotechnol. 54: 28-32.

Zhu A, Monahan C, Wang Z (1996) Trp-16 is essential for the activity of α-galactosidase and α-*N*-acetylgalactosiminidase. Biochim. Biophys. Acta 1297: 99-104.

ANNEXURE

List of Publications

1. **G.S. Anisha**, P. Prema, (2008) Reduction of non-digestible oligosaccharides in horse gram and green gram flours using crude α-galactosidase from *Streptomyces griseoloalbus*, Food Chemistry, 106, 1175-1179.
2. **G.S. Anisha**, P. Prema, (2008) Cell immobilization technique for the enhanced production of α-galactosidase by *Streptomyces griseoloalbus*, Bioresource Technology, 99, 3325-3330.
3. **G.S. Anisha**, Rajeev K. Sukumaran, P. Prema, (2008) Evaluation of α-galactosidase biosynthesis by *Streptomyces griseoloalbus* in solid-state fermentation using response surface methodology, Letters in Applied Microbiology, 46, 338-343.
4. **G.S. Anisha**, P.J. Rojan, J. Nicemol, K.N. Niladevi, P. Prema, (2008) Production and characterization of partially purified thermostable α-galactosidases from *Streptomyces griseoloalbus* for food industrial applications, Food Chemistry, (doi:10.1016/j.foodchem.2008.04.029).
5. **G.S. Anisha**, R.K. Sukumaran, P. Prema, (2008) Statistical optimization of α-galactosidase production by *Streptomyces griseoloalbus* using response surface methodology, Food Technology and Biotechnology, (In Press).
6. **G.S. Anisha**, P. Prema, (2007) Production of α-galactosidase by a novel actinomycete *Streptomyces griseoloalbus* and its application in soymilk hydrolysis, World Journal of Microbiology and Biotechnology, 23, 859-864.
7. **G.S. Anisha**, P. Prema, (2006) Selection of optimal growth medium for the synthesis of α-galactosidase from mangrove actinomycetes, Indian Journal of Biotechnology, 5, 373-379.
8. **G.S. Anisha**, P.J. Rojan, P. Prema, Biochemical and hydrolytic properties of α-galactosidases from *Streptomyces griseoloalbus*: obvious existence of a novel



galactose-tolerant enzyme, Applied Microbiology and Biotechnology (Submitted revised manuscript).

9. **G.S. Anisha**, P.J. Rojan, P. Prema, A. Pandey, Investigation on α-galactosidase production by *Streptomyces griseoloalbus* in a forcefully-aerated packed bed bioreactor operating in solid-state fermentation condition Applied Biochemistry and Biotechnology (Accepted)
10. Nicemol Jacob, K.N. Niladevi, **G.S. Anisha** and P. Prema, Hydrolysis of pectin: An enzymatic approach and its application in banana fiber processing, *Microbiological Research*, 2006 (*Published online*)
11. K.N. Niladevi, Rajeev K. Sukumaran, Nicemol Jacob, **G.S. Anisha** and P. Prema, Optimization of laccase production from a novel strain-*Streptomyces psammoticus* using response surface methodology, *Microbiological Research*, 2007 (*Published online*)

Papers/posters presented in National/International Conferences

1. **G.S. Anisha** and P.Prema. Isolation and screening of halophilic actinomycetes producing α-galactosidase. Proceedings of the National Symposium on "Developments in Biotechnology – Emerging Trends and Challenges" and first BRSI convention, November 25-27, 2004, North Maharashtra University, Jalgaon.
2. **G.S. Anisha** and P.Prema. Production of α-galactosidase by *Streptomyces griseoloalbus*. Proceeding of the National conference on "Path to Health-Biotechnology Revolutions in India & Second BRSI Convention", 24-26 November 2005, Chennai, India.
3. **G.S. Anisha** and P. Prema, Statistical optimization of α-galactosidase production by *Streptomyces griseoloalbus* using response surface methodology, Proceedings of the International Conference on Bioprocess and Food Engineering, University of Patras, Greece, 2006, June.

4. **G.S. Anisha** and P.Prema, Production of α-galactosidase by *Streptomyces griseoloalbus* under solid-state fermentation, Proceedings of the International Conference on "Exploring Horizons in Biotechnology: A Global Venture" and 3rd BRSI convention, November, 2-4, 2006, Sardar Patel University, Vallabh Vidyanagar.
5. **G.S. Anisha** and P.Prema. Application of α-galactosidase from *Streptomyces griseoloalbus* for the reduction of flatulence-causing oligosaccharides in legumes, Proceedings of the International Conference on New Horizons in Biotechnology and 4th BRSI convention, November, 26-29, 2007, NIIST, Trivandrum.

Training programmes

1. A 12-day course on "Microbial Technology- Emerging Trends in Bio-analytical Techniques" organized by Thapar Institute of Engineering and Technology, Patiala, Punjab, during November 20th to December 1st, 2003.
2. Training programme on "Technological Entreprenuership" conducted by the Faculty of Indian Institute of Management, Bangalore from February 20th to March 22nd, 2006 at Central Leather Research Institute, Chennai.