Isolation, Purification and Characterization of Thermostable α-Galactosidase from *Bacillus stearothermophilus* (NCIM-5146).

A THESIS
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IN
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BY
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DEDICATED TO

MY MOTHER & LATE GRAND MOTHER

CONTENTS

National Chemical Laboratory, (Council of Scientific and Industrial Research, Pune –411 008 (INDIA)

DECLARATION

Certified that the work incorporated in the thesis entitled **"Isolation, Purification and Characterization of Thermostable α-Galactosidase from *Bacillus stearothermophilus* (NCIM-5146)."** submitted by **Mr. Manoj M. Gote** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. J. M. Khire
Research Guide

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ABSTRACT

Recently thermophiles have generated great interest as they appear to offer some major advantages to biotechnology and it is the theme for their extensive exploration. Thermophilic microorganisms are also known to be a source of thermostable hydrolytic enzymes including glycosidases. There is a considerable interest in enzymes that catalyzes hydrolysis of glycosidic bonds, due to their extensive industrial, therapeutic and biochemical applications.

α-Galactosidase (α-D-galactoside galactohydrolase E.C.3.2.1.22) is an exo-glycosidase that catalyzes hydrolysis of terminal α-1-6-linked galactosyl residues from a wide range of substrates including oligo-saccharides of raffinose family sugars; raffinose, stachyose, melibiose, verbascose and polysaccharides of galactomannans; locust bean gum and guar gum. More over it also hydrolyzes glycoconjugates; glycoproteins and glycosphingolipids. The use of these enzymes, which are specific in their action, is becoming more popular as tools for structural investigation and degradation of complex carbohydrate molecules. α-Galactosidases have many potential biotechnological and medicinal applications. It plays a crucial role in the enzymatic hydrolysis of raffinose from sugar beet syrup from sugar industry in order to facilitate the crystallization and consequently improve the yield of the sugar. The potential use of α-galactosidase for the processing of legume based food products has been demonstrated. Moreover, recently it has been used in the animal feed processing and in pulp and paper industry. Further more some of the α-galactosidase preparations are known to remove the terminal α-1-3-linked galactose residues from glycans and have potential medical use in transfusion therapy for the conversion of erythrocyte of blood group 'B' specificity to more universally transferable type 'O'. In view of these applications the present enzyme was isolated and purified from thermophilic microorganism *Bacillus stearothermophilus* (NCIM-5146). The purified enzyme has been extensively characterized for its biochemical and molecular properties and also for its substrate specificity towards the oligomeric and polymeric substrates. The structure function relationship of enzyme has also been investigated.

Chapter 1: General Introduction.

This chapter comprises of literature survey with reference to plant, animal and microbial α-galactosidases, their occurrence, properties and applications. The production of microbial α-galactosidases has been reviewed in detail.

Chapter 2 : Production of Thermostable α-Galactosidase by *Bacillus stearothermophilus* (NCIM-5146).

An extracellular α-galactosidase with wide pH and high temperature stability was produced by newly isolated strain of *Bacillus stearothermophilus*, (NCIM 5146), grown on agricultural residues, at 60 °C, under submerged fermentation. The maximum α-galactosidase activity (2.0 U/ml) was found when water extract of soybean meal (2.0 % w/v) was used as a carbon source along with yeast extract (0.3 % w/v) and ammonium sulfate (0.3 % w/v) as a nitrogen sources. The pH & temperature optimum of α-galactosidase was 6.5-7.0 and 65 °C, respectively. The enzyme was stable in the pH range 3-10. The half-life of the enzyme at 70 °C was 80 minutes. The enzyme α-galactosidase was active against synthetic as well as natural glycosides. The K_m values determined for *p*NPG, stachyose, raffinose and melibiose were 0.625, 3.33, 6.66 and 13.33 mM and the V_{max} values were 4.3 x 10^7, 18.03, 12.65, and 6.3 x 10^3 μmoles $min^{-1}mg^{-1}$ of protein, respectively for above substrates. The enzyme activity was strongly inhibited by Ag^+, Hg^{2+}, and Cu^{2+} metal ions. No significant inhibition was found with different sugars.

Chapter 3: Purification and Characterization of Thermostable α-Galactosidase from *Bacillus stearothermophilus* (NCIM-5146).

An extracellular thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM-5146) has been purified to homogeneity by single chromatographic step, using Phenyl Sepharose CL4B. The molecular mass of the enzyme as determined by SDS-PAGE and gel filtration column were 79.9 and 165.9 kDa, respectively, suggested that enzyme consists of two identical subunits. The purified α-galactosidase is a non-glycosylated protein with a p*I* value of 4.9. The enzyme is rich in acidic and hydrophobic amino acids and partial N-terminal sequence exhibited remarkable homology with the earlier reported α-galactosidase from *Bacillus stearothermophilus* (NUB 3621) of family 36 of glycosyl hydrolases. The secondary structure of the enzyme determined by CD spectroscopy and analyzed by prediction method, exhibited α/β class of protein. The pH and temperature optima for the purified enzyme are 6.5-7.0 and 65 ºC, respectively. The α-galactosidase was stable over a broad pH range (3-9) and its half-life of inactivation ($t_{1/2}$) at 70 ºC was 30 minutes. The enzyme exhibited strict anomer specificity towards the substrates and hydrolyzes only the α-galactosidic linkages and not the β-galactosidic linkages. The relative substrate specificity of α-galactosidase towards the various synthetic and natural galactosides is in the order of *p*NPG > *o*NPG > melibiose > stachyose > raffinose > methyl-α-D-galactopyranoside > locust bean gum and guar gum. The enzyme also hydrolyzes α-1-3 and α-1-4 galactosidic linkages from disaccharide

sugars. The kinetics of hydrolysis of number of natural and synthetic substrates showed that *p*NPG and *o*NPG has lower K_m (higher affinity) and higher K_{cat} (higher catalytic efficiency). However natural substrates, melibiose, raffinose, and stachyose showed higher K_m (lower affinity) and lower K_{cat} (lower catalytic efficiency). Among the natural substrates, a disaccharide, melibiose (K_m = 5.0 mM) was hydrolyzed at higher rate than the trisaccharide, raffinose (K_m = 5.0 mM), although, both the substrates has similar K_m (similar affinity). However by contrast, although, trisaccharide, raffinose and tetrasaccharide, stachyose (K_m = 2.5 mM) showed similar K_{cat} (similar rate of hydrolysis), both the substrates has different K_m values (different affinities). Thermodynamic parameters calculated from the temperature dependence studies of binding and hydrolysis of different glycosides indicated that binding might be enthalpy as well as entropy driven process. Hence, it suggests that hydrophobic interactions as well as hydrogen bonds play a predominant role in substrate binding. The high catalytic efficiency of *p*NPG and melibiose is due to the highest transition state stabilization and ground state destabilization, suggested that these substrates fit properly in the active site cleft. By contrast high entropy loss in binding and activation of raffinose and stachyose indicated strained substrates and hence hydrolyzes at lower rate due to lower transition state stabilization and ground state destabilization. Purified α-galactosidase showed biphasic Arrhenius plots with break point at 55 ºC for *p*NPG and 50 ºC for melibiose, raffinose and stachyose, suggested temperature dependent conformational states. Besides the hydrolytic activity, present α-galactosidase also exhibited weak transferase activity and produces two new sugars, tentatively identified as melibiose and stachyose. The enzyme was completely inhibited by Ag^+, Hg^{2+} and Cu^{2+} metal ions, nature of inhibition was non competitive. Galactose, melibiose (K_i = 16.25), stachyose (K_i = 33.0) inhibited α-galactosidase activity competitively, whereas lactose (K_i = 14.5) inhibition was uncompetitive.

Chapter 4 : Active Site Directed Chemical Modification of α-Galactosidase from *Bacillus stearothermophilus* (NCIM -5146).

The catalytic amino acid residues of α-galactosidase from *Bacillus stearothermophilus* (NCIM -5146) was investigated by the pH dependence of the reaction kinetic parameters and active site directed chemical modification studies. The pH dependence curves gave apparent pK_a value of 6.1 and 8.2 for the free enzyme, while pK_a value of 7.84 was obtained for the enzyme substrate complex using *p*-nitrophenyl-α-D-galactopyranoside as a substrate. These results suggested that an ionized and a protonated group, presumably perturbed carboxylate and a lysine residue took part in catalysis and only lysine residues were essential for substrate binding. Chemical modification studies by amino acid group specific reagents also showed that a carboxylate, tryptophan and

amino group of lysine are present at the active site. Further, Carbodiimide modification of enzyme 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 by acetic and citraconic anhydride and sodium borohydride suggested that the protonated lysine residues carrying positive charge on its ε-amino group and exist in solution as NH_3^+ species provides the positive charge density for binding of the substrate and in the stabilization of transition state. We suppose that binding could take place through **H-bonding** between electrostatic hydroxyl oxygen of galactose and protonated amino group of lysine. The kinetic analysis of lysine modified enzyme showed partial increase in the K_m and significant decrease in the $K_{cat,}$ suggest a mechanism in which substrate and enzyme combine irrespective of the protonated state of the groups required for binding and catalysis but only the correctly protonated enzyme substrate complex is catalytically active. Kinetic study suggested that one of lysine residue of the four that are present at the active site must in some manner directly or indirectly implicated in the catalysis. Additionally four tryptophan residues also 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.

LIST OF ABBREVIATIONS

CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
DEP	Diethylpyrocarbonate
DTNB	2,2-Dithiobisnitrobenzoic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylene diamine tetraacetic acid
HEPES	2-(2-Hydroxyethyl) Piperazine-N-(4-Butanesulfonic acid).
HNBB	2- Hydroxy nitro benzyl bromide
MES	2-(N-Morpholinolethanesulfonic acid)
NAI	N-acetylimidazole
NBS	N-Bromosuccinimide
NCIM	National Collection of Industrial Microorganisms
NEM	N-ethylmaleimide
NTEE	3-nitro-L-tyrosine ethylester
PCMB	p-Chloromercurybenzoate
PG	Phenyl glyoxal
PMSF	Phenyl methyl sulfonyl fluoride
*p*NPG	*p*-nitrophenyl-α-D-Galactopyranoside.
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
TNBS	2,4,6-trinitrobenzenesulfonic acid
WRK	Woodward's Reagent K

CHAPTER-1

General Introduction

1.0 Thermophiles and thermostable enzymes :

Microorganisms are the most technologically practical sources of enzymes because they multiply at extremely high rate and synthesize biologically active products under conditions that can be controlled by humans. During the past decades, the interest in thermophilic microorganisms, which live and thrive at temperatures up to 100 °C, has been increasing. Thermophilic bacteria were first isolated in 1879 by Miquel [1], who found bacteria capable of growing at 72 °C. The discovery of these organisms as well as microorganisms from other extreme environments has extended our concept of the limits of life and has had a great impact on our view on the early history of the evolution. The study of the molecular biology and biochemistry of thermophiles has resulted in development of novel biotechnological tools of great importance and provided us with a broader understanding of biological structures and processes. Thermophiles have been isolated from a variety of hot environments such as terrestrial and submarine geothermal areas, hot oil-field production waters, deep subterranean cores, and from biologically self-heated materials. Important areas of thermophile research are: (1) factors contributing to thermostability of macromolecules, (2) application and screening of new thermophiles and thermostable proteins for use in novel or improved biotechnological processes, (3) phylogeny and evolution of thermophiles, and (4) metabolic pathways and mechanisms for regulation.

Thermophilic microorganisms are known to be a source of thermostable hydrolytic enzymes including glycosidases [2]. Enzymes from thermophilic microorganisms are generally thermostable and also stable in presence of denaturing agents and organic solvents. Many of these enzymes find wide industrial use in food and feed, paper and pulp, modification of complex polysaccharides and in organic biosynthesis [3,4,5,6].

1.1 Glycosidases :

O-Glycoside hydrolases (E.C.3.2.1.-) are a widespread group of enzymes, which hydrolyses α or β glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glycosyl hydrolases transfers the glycosyl bond to a water molecule; glycosyl transferases transfer this glycosyl bond to the -OH group of another glycosyl residue. Enzymes, which catalyze the hydrolysis of glycosidic

linkages are widely distributed in nature and include β-galactosidase, invertase, maltase, α-galactosidase, β-glucosidase, amylase etc. Glycosidases are also known as carbohydrolases. The existence of glycosidases has been known for more than 100 years and they were the very first biological catalysts investigated.

1.1.1 Specificity and mechanism :

Glycosidases are classified into **exo-glycosidases** and **endo-glycosidases** based on the nature of hydrolysis. The exo-glycosidases (e.g. galactosidases, glucosidases) acts on the glycosidic bond present at the non-reducing end of the saccharide chain whereas endo-glycosidases (e.g. amylases) acts on the glycosidic bond within the saccharide chain. Numerous glycosidases from different sources have been classified in to 91 families (as on Dec-2003, CAZy-Carbohydrate-Active Enzymes database) [7], which form different products and display a complete range of different bonds specificity. For example, an α-amylase hydrolyzes endo-α-(1-4) bonds in polysaccharide chains and produces linear α-(1-4) linked oligo-saccharides (so-called retaining enzyme) [8], whereas a β-amylase hydrolyses also α-(1-4) bonds, but cuts off only maltose from the non-reducing ends (exo) and forms a different anomeric configuration (-β-D-glucose; a so-called inverting enzyme) [9]. Some enzymes prefer to use substrates of a certain chain length, for instance cyclodextrin glycosyltransferases use predominantly the linear and single branched chain from amylopectin with a degree of polymerization of 13, 14 or 15 [10].

The reaction specificity of some of the glycosidases acting on different oligo and polysaccharides is shown in Table 1.

Table 1. Reaction specificity of few glycosidases :

Enzyme	Type of specificity			Ref
	Spec. bond	**Substrate**	**Cleaves off**	
α-Amylase	α r h e 4	oligo- & polysacch	oligosacch	(8)
β-Amylase	α i h x 4	oligo- & polysacch	G2	(9)
Cyclodextrin glycosyltransferase	α r t x 4→4	oligo- & polysacch	cyclodextrins	(10)
Pullulanase	α r h e 6	pullulan	G3	(11)
α-Glucosidase (Maltase)	α r h d/x4/6	oligo- & polysacch	G1	(12)
Dextran glucosidase	α r h x 6	dextran	G1	(13)
Isoamylase	α r h e 6	oligo- & polysacch	oligosacch	(14)
β-Glucosidase	β r h x 4	oligo- & polysacch	hexose	(15)
α-Galactosidase	α r h x 6	galactose-sacch.	α -galactose	(16)
β-Galactosidase	α r h d 4	lactose (β-galactosides)	β-galactose + G1	(17)

Cyclodextrinase	α r h e 4/6	cyclodextrins	G3, G2, G1	(18)
Lysozyme	β r h x 4	peptidoglycan	di-sacch.	(19)
Xylanase	β r h x 4	xylan	xylobiose/-triose	(20)
Cellobiohydrolase	β i/r h x 4/3	β-glucans/xylans/cellulose	G2, G3	(21)

The bond specificity is indicated as follows: α or β indicate the anomeric configuration, which is retained (r) or inverted (i) in the product. The enzyme is hydrolase (h) or transglycolase (t), acting as endo (e), exo (x) or as a disaccharidase (d). The bond specificity is indicated with 3,4 or 6; for (1-3), (1-4) or (1-6) bonds, respectively; 4/6 or 4/3 indicate dual bond specificity;→ indicate the transfer action. G_2, G_3 are glucose oligomer with a polymerization degree 2 (maltose), 3 etc.

Catalytic mechanism :

The enzymatic hydrolysis of the glycosidic bond takes place via general acid catalysis that requiring two critical residues: a proton donor and a nucleophile/base [22]. This hydrolysis occurs via two major mechanisms giving rise to either an overall retention, or an inversion, of anomeric configuration [23,24,25]. In the proposed mechanism for inverting enzymes the glycosidic oxygen is initially protonated by a general acid catalyst, followed by a nucleophilic attack of a water molecule on the C1 atom of the sugar, which is activated by a carboxylate base (Figure 1a), leading to inversion of the anomeric conformation. This mechanism is known as a single-displacement mechanism [23], bond breaking and bond making both proceed in a single concerted step. The reaction rate depends on the concentrations of both nucleophile and substrate.

The retaining reactions proceed via a double-displacement mechanism [26]; the first step involves a similar protonation of the glycosidic oxygen by a general acid as in the inverting mechanism, creating an intermediate, which is attacked by a water nucleophile assisted by the base form of the acid catalyst in a second step. Each step inverts the configuration of the anomeric carbon. The two displacement steps therefore create an overall retention of the configuration. For retaining enzymes the intermediate could either be an oxo-carbonium ion (Figure 1b) which is electrostatically stabilized by a carboxylate, or involves formation of a covalent bond (Figure 1c), in which one of the catalytic aspartates (in some cases a glutamate) is presumed to act as a nucleophile.

Transglycosylases (e.g. Cyclodextrin glycosyl transferases) employ a similar reaction mechanism as described for retaining hydrolases. For these enzymes, however, the second step of the reaction does not involve a water nucleophile, but the non-reducing end of a saccharide, possibly assisted by the base form of the acid catalyst [25].

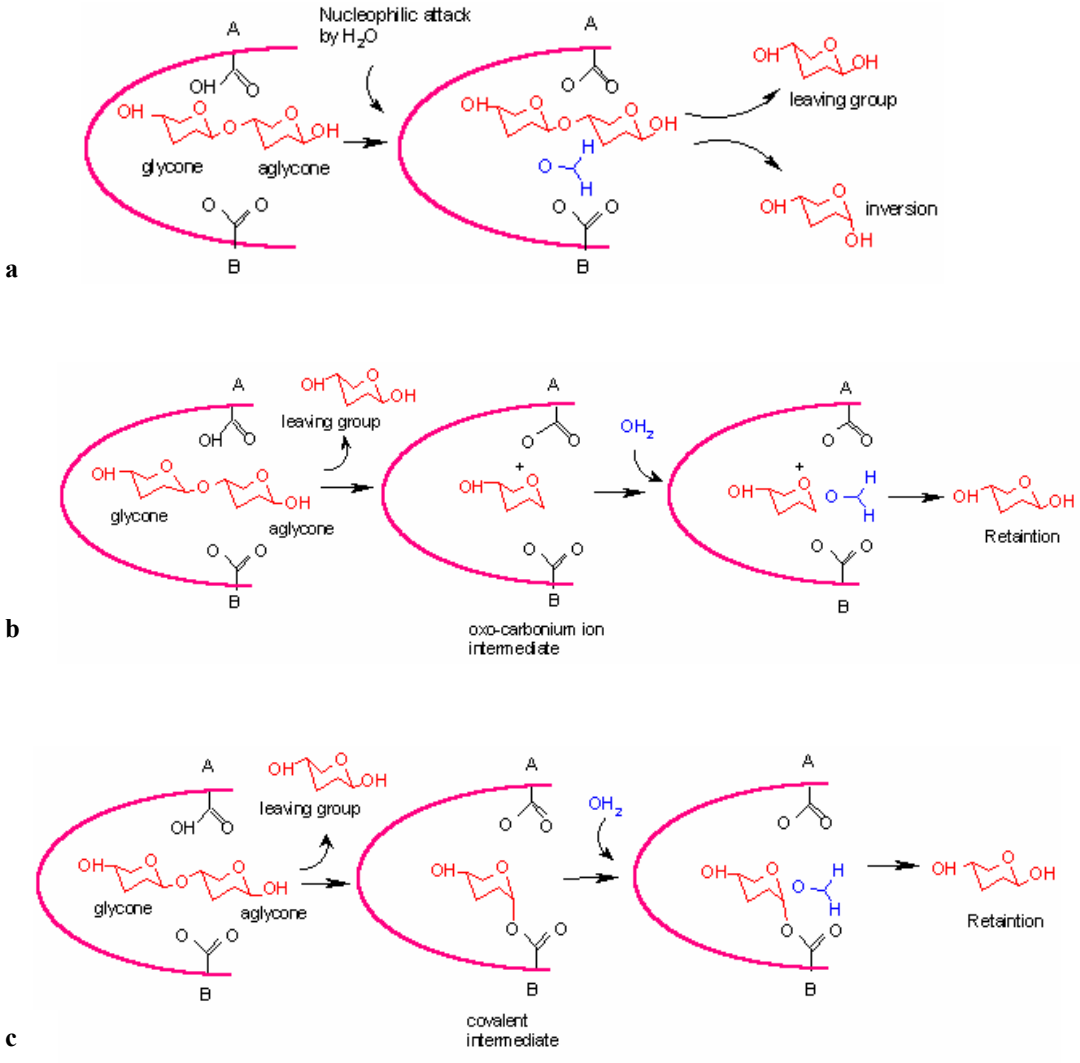


Figure 1. Catalytic mechanism of glycosidases; **a**) inverting enzyme **b**) retaining enzyme acting through oxo-carbonium or **c**) via covalent intermediate

1.1.2 Classification of glycosidases :

Classification of enzymes based on the type of reaction catalyzed and their substrate-specificity does not take into account evolutionary events or sequence (and structural) similarities. Henrissat in 1991 [27] proposed a classification of glycoside hydrolases in families based on amino acid sequence similarities (Table 2). Glycoside hydrolases from various sources, including bacteria, fungi, plants and animals were

classified into 91 families based on alignment of amino acid sequences [Database, Carbohydrate Active Enzymes; CAZy] [7,28,29]. Some of the families were found to be monospecific, e.g. α-amylases and cyclodextrin glycosyl transferases both belonging to family 13, have been found to share superimposable domains. On the other hand several enzymes were found to fall in to more than one family and are polyspecific, e.g. 10 families in the case of endoglucanase. Because there is a direct relationship between sequence and folding similarities, such a classification is expected to reflect: (i) the structural features of these enzymes better than their sole substrate specificity, (ii) help to reveal the evolutionary relationships between these enzymes, and (iii) provide a convenient tool to derive mechanistic information [27,28].

Since the folding pattern of proteins is better conserved than their sequences, some of the families are grouped into 'clans'.

A detailed comparative analysis of the primary structures of glycoside hydrolases may also serve to locate the potential active site residues, which are strongly conserved among the similar family enzymes [28].

Table 2. Classification of glycosidases based on sequence similarity :

Enzyme	Abbreviation	E.C. number	Family
α-Amylase	AAMY	3.2.1.1	13
α-Galactosidase	AGAL	3.2.1.22	4,27,36,u
α-Fucosidase	AFUC	3.2.1.5	29
Agarase	AGAR	3.2.1.81	16,u
α-Glucosidase	AGLU	3.2.1.20	13,31
α-L-Iduronase	AIDU	3.2.1.76	39
α-Mannosidase	AMAN	3.2.1.24/114	38,u
Amyloglucosidase	MG	3.2.1.3	15,31
Amylase/pullulanase	APU	3.2.1.1/41	13
α-Arabinofuranosidase	ARAF	3.2.1.55	43,u
β-Amylase	BAMY	3.2.1.2	14
β-Galactosidase	BGAL	3.1.2.23	1,2,35,42
β-Glucuronidase	BGLR	3.2.1.31	2
β-Glucosidase	BGLU	3.2.1.21	1,3,40,41
β-Mannanase	BMAN	3.2.1.78	5,26,44
β-Xylosidase	BXYL	3.2.1.37	39,43,u
Cellobiohydrolase	CBH	3.2.1.91	6,7,5,10
Cyclodextrinase	CDX	3.2.1.54	13
Cellodextrinase	CED	3.2.1.74	3,5
Cyclodextrin Glycosyltransferase	CDGT	2.4.1.19	13

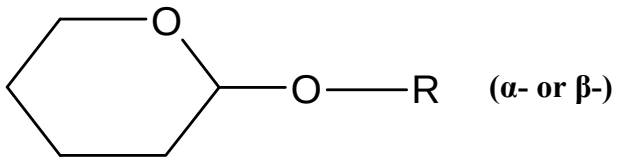
Chitinase	CHI	3.2.1.14	18,19
Chitosanase	CHITO	3.2.1. -	u
Dextranase	DEX	3.2.1.11	u
Evolved- β-galactosidase	EBGAL	3.2.1.23	2
Endoglucanase	EG	3.2.1.4	5,6,7,8,9,10, 12,44,45,48
Endo-N-acetyl-β-glucosaminidase endo	NAG	3.2.1.96	18,26,44,45,u
Exo-1,3-β-glucanase	EXG	3.2.1.58	5
Exo-β-fructosidase	FRU	3.2.1.80	32
Malto-tetraohydrolase	G4-AMY	3.2.1.60	13
Malto-pentaohydrolase	G5-AMY	3.2.1.-	13
Malto-hexaohydrolase	G6-AMY	3.2.1.98	13
Glucodextrinase	GDX	3.2.1.70	13
Glucosylceramidase	GLRB	3.2.1.45	30
Hyaluronidase	HYAL	3.2.1.36	u
Isoamylase	IAMY	3.2.1.68	13
Inulinase	INU	3.2.1.7	32
Invertase	INV	3.2.1.26	32
Laminarinase	LAM	3.2.1.39	16,17,u
Levanase	LEV	3.2.1.65	32,
Lichenase	LIC	3.2.1.73	8,16,17
Lactase/phlorizin hydrolase	LPH	3.2.1.62/108	1
Levansucrase	LVS	2.4.1.10	32
Lysozyme	LYS	3.2.1.52/17	21,22,23,24,25, u
N-Acetyl-α-galactosaminidase	NAAGAL	3.2.1.49	27
N-Acetyl-β-glucosaminidase	NABGLU	3.2.1.30/52	20
Neuraminidase	NEUR	3.2.1.18	33, 34
Neopullulanase	NPUL	3.2.1.-	13
Oligo-1,6-glucosidase	OGLU	3.2.1.10	13
6-Phospho-β-galactosidase	PBGAL	3.2.1.85	1
6-Phospho-β-glucosidase	PBGLU	3.2.1.86	1,4
Polygalacturonase	PGLR	3.2.1.15	28
Pullulanase	PUL	3.2.1.41	13
Spore-germinating spec. protein	SGSP	-	9
Sucrase/isomaltase	SI	3.2.1.48/10	31
Toxin-α-chain	-	3.2.1.14	18
Trehalase	TREH	3.2.1.28	37,u
Exo-laminarinase	XLAM	3.2.1.58	17
Exo-polygalacturonase	XPGLR	3.2.1.82	28

Xylanase	XYN	3.2.1.8	10
Open reading frame	ORF	-	0,11

Data from (Henrissat, 1991) [27] and (Henrissat and Bairoch, 1993) [28], for updated list of enzymes and their respective families, see the Database Carbohydrate Active Enzymes (CAZy) (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). u-indicates, some of the enzymes showing similar action are unclassified.

1.1.3 Glycosides :

Glycosides are compounds containing a carbohydrate and a non-carbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetal linkage at carbon atom 1 to a non-carbohydrate residue or aglycone. The nonsugar component is known as the aglycone and sugar component is called the glycone. If the carbohydrate portion is galactose, the resulting compound is a galactoside. The aglycone may be alcohol, glycerol, a sterol, a phenol, etc.



1.1.3.1 α-Galactosides :

α-Galactosides are glycosides containing α-linked D-galactosyl residues attached to the aglycone molecule by an acetal linkage. Here the non-reducing terminal monosaccharide (α-D-galactose) is called the glycone, because it contributes the anomeric carbon to the glycosidic center in the acetal bond. The adjoining monosaccharide and remainder of the oligosaccharide substrate is termed the aglycone, and is effectively the leaving group in the catalytic mechanism. The D-galactosyl groups are ubiquitous in the higher plants, mostly occurring in combined form and found in variety of oligo-saccharides, polysaccharides, and few non-sugars such as glycerol, inositol, and certain lipids. Such α-galactosides are mainly the substrate for enzyme α-galactosidase. The principal members of these galactosides, their structure, occurrence, distribution and physiological functions are given in Table 3.

Table 3. Occurrence, distribution and physiological functions of the α-galactosides :

Glycosides	Structure	Occurrence/Distribution	Physiological function
α-D-Galacto-oligosaccharides:-			
Planteose	O-α-D-Gal*p*-(1-6)-β-D-Fru*f* - (1-2)-α-D-Glu*p*	*Plantago major*/seeds *P. ovata* /seeds Fraxinus excelsior /seeds	Storage oligosaccharide
Umbeliferose	O-α-D-Gal*p*-(1-2)-α-D-Glu*p* - (1-2)-β-D-Fru*f*	Umbellifereae/ roots	Reserve carbohydrate.
Raffinose	O-α-D-Gal*p*-(1-6)-α-D-Glu*p* - (1-2)-β-D-Fru*f*	Family Leguminoceae/ seeds, roots, leaves, stems	Reserve carbohydrate, protection against frost and drought.
Isolychnose	O-α-D-Gal*p*-(1-6)-α-D-Glu*p* -3-O-α-D-Gal*p*--(1-2)-β-D-Fru*f*	*Lychnis dioica*/ roots	---
Lychnose	O-α-D-Gal*p*-(1-6)-α-D-Glu*p* -1-O-α-D-Gal*p*--(1-2)-β-D-Fru*f*	*Lychnis dioica*/ roots *Sesamum indicum*/ seeds	Storage oligosaccharide
Sesamose	O-α-D-Gal*p*-(1-6)-O-α-D- Gal*p*-(1-6)-β-D-Fru*f*-(1-2)-α-D-Glu*p*	*Sesamum indicus*/ seeds	Storage oligosaccharide
Stachyose	O-α-D-Gal*p*-(1-6)-O-α-D-Gal*p*-(1-6)-α-D-Glu*p*-(1-2)-β-D-Fru*f*	*Stachys tuberifera*, and family Leguminoceae/ Rhizomes, seeds	Frost resistant to plant
α-D-galactosides of polyols :			
Isofloridoside	1-O-α-D-Gal*p*-L-Glycerol	*Porphyra umbelicalis* & Genus *Rhodophyceae* (red algae) *Ochromonas malhamensis* (green brown algae)	Cabohydr. Reserve, in algae controling osmotic balance.
Floridoside	2-O-α-D-Gal*p*-L-Glycerol	Marine algae *Rhodymenia palmata*, & genus *Rhodophyceae*	Osmotic balance.
Galactinol	O-α-D-Gal*p*-(1-1)-myo-inositol	Sugar beet / tubers, potato/ tubers, *Lamium maculatum* / leaves	Reserve substance, D-galactosyl donor in biosynthesis of raffinose.
α-D-galacto-lipid:			
O-α-D-galactosyl-glyceride and its higher homologs.	1-O-[-O-α-D-Gal*p*-(1-6)-β-D-Gal*p*]-D-glycerol	Algae, Higher plants / leaves, flowers, fruit, roots	Carbohydrate reserve, sugar transport, protection of thylakoid membrane against frost injury.
Galacto-polysaccharides:			
D-galactans	[O-α-D-Gal*p*-(1-6)-α-D-Gal*p*]$_n$	Sugar beet	Carbohydrate reserve
D-galacto-mannans	-[- β-D-Man*p*-(1-4)-]$_n$ 6 1 α-D-Gal*p*	Plant families Leguminoceae, Anonaceae /seeds	Carbohydrate reserve

D-Galacto-glucomannan	Glu-man-man* Gal Gal		Gymnosperms, Angiospe- rms/ stem, leaves, seeds	Food reserve

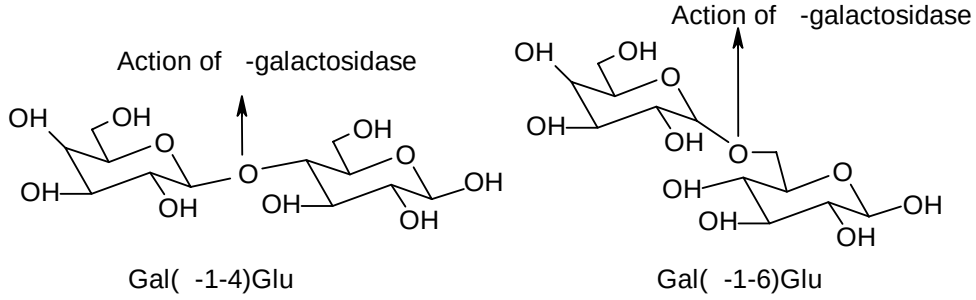
* β-D-Glu*p*-(1-4)- β-D-Man*p*-(1-4) - β-D-Man*p*-(1-4)-
6 6

1 1
Gal*p* Gal*p*

Gal*p*, Glu*p*, Man*p*, Fru*f* -represents, D-galactopyranosyl, D-glucopyranosyl, D-Manopyranosyl, D-Fructofuranosyl groups, respectively. Data adapted from Dey P.M. [30] and French D., [31].

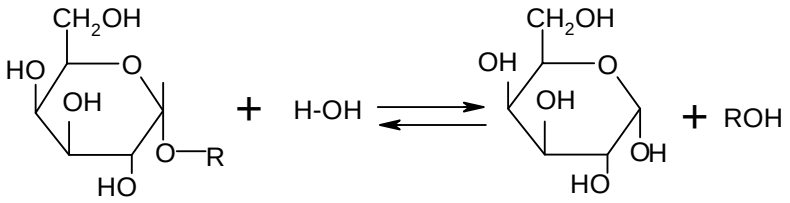
1.1.4. Galactosidases :

The enzymes responsible for hydrolyzing galactosidic linkages are termed as galactosidases. There are two types of galactosidases viz., **α-galactosidases** and **β-galactosidases** depending on the configuration of the anomeric carbon atom of galactose in the substrate molecule on which they act.



1.1.4.1 α-Galactosidases :

α-D-Galactose-galactohydrolase (E.C.3.2.1.22), commonly referred to as α-galactosidase, catalyzes the hydrolysis of α-galactosidic linkages in oligo-saccharide such as raffinose, melibiose, stachyose and verbascose, polysaccharides like galactomannans, and glycoconjugates such as glycoproteins and glycolipids. Ceramide trihexosides, its higher homologues and derivatives, are also cleaved by the action of α-galactosidase. Bau [32] and Fischer and Lindner [33] in 1895, have isolated for the first time, α-galactosidase from bottom yeast. Because of its action on melibiose it was named as melibiase, catalyzing the following reaction.



Where R is an alkyl or aryl group, or a glycosyl (mono or oligo) residue or group

1.1.4.1.1 Occurrence and physiological role :

α-Galactosidases are widely distributed in nature among plants, animals and microorganisms.

a) *Plants :*

The enzyme α-galactosidase is ubiquitous in legume seeds and for the first time it was reported from sweet almond emulsion [34], latter on it was isolated from many plants (Table 4). In addition, this enzyme was also found in leaves of *Cucurbita pepo* [35], stalks of the *Saccharum officinarum* [36] and endosperm of Coconut [37] etc. Very recently it has been isolated form tomato fruit, [38], grape flesh [39] cultured rice [40] and sunflower seeds [41].

Table 4. Occurrence of α-galactosidase activity among plants :

Source	Location	Reference
Absidia corymbifera	seeds	42
Acer pseudoplatanus	seeds	43
Cajanus indicus	seeds	43,44
Coffea canephora	seeds	45
Cyamopsis tetragonolobus	seeds	46
Glycine max sps.	seeds	47
Lens esculanta	seeds	48
Vicia faba	seeds	49
Maize	roots	50
Phaseolus vulgaris	seeds	51
Trifolium repens	seeds	52
Sweet almond	seeds	53
Vigna radiata	seeds	54
Runner bean	leaves	55

Significance : In plants, α-galactosidase is believed to be involved in a variety of processes, most importantly in the hydrolysis of oligo-saccharides such as raffinose and stachyose during the early germinative period, resulting in the liberation of free sugars, which may serve as a ready energy source for the growing plant [56]. The role of α-galactosidase and β-mannanase in the hydrolysis of cell wall storage polysaccharides such as mannan and galactomannans at the latter period of germination of seed has been established [57]. α-Galactosidase is an important enzyme in coconut and plays role during germination and cell wall development [37]. The enzyme may have a synthetic function, as transgalactosylation reactions have been reported for α-galactosidases [58]. Its role has been implicated in the removal of toxic accumulants and to hydrolyze phenolic glycosides could provide a mean of control of the levels of plant growth substances [59]. More over role of α-galactosidase in the metabolism of storage glycoproteins and galactolipids is tentatively suggested [55,60].

b) Animals :

There are very few reports of α-galactosidase from animals (Table 5). Among animals it was first reported from *Helix promatia* [61]. The presence of α-galactosidase was also reported from human; spleen, placenta, plasma and liver [62,63]. It was found in higher titers in rats especially in the cytoplasm of epithelial cells of Brunner's glands in the intestine [64]. Blood cells and bone marrow of some animals were found to contain α-galactosidase [65,66].

Table 5. Occurrence of α-galactosidase among animals :

Source	Reference
Sheep	67
Helix pomatia	61
Human	62
Chineese hamsters	68
Mouse	69
Bovine	70
Turbo cornutus	71

Significance : In animal kingdom, enzyme is reported to be involved in the hydrolysis of galactolipids. The deficiency of enzyme has been implicated in the manifestation of a disorder named Fabry's disease in humans. Fabry's disease belongs to the group of hereditary lysosomal storage disease and is caused by a reduced activity of

α-galactosidase-A [72], as α-galactosidase A and B are present in normal tissues. The enzyme also occurs in brain tissues with possible involved in the hydrolysis of monogalactosyl diglycerides and digalactosyl diglycerides [73].

c) Microorganisms :

Among microorganisms α-galactosidase activity was first detected in brewers' yeast [32,33], Subsequently it was also detected in *Saccharomyces carlsbergensis* [74], *Pichia guilliermondii* [75], *Candida javanica* [76], *Aureobasidium pullulans* [77] etc. Many bacteria have also been reported to contain α-galactosidase activity (Table 6). Recently its presence is reported in extreme thermophilic eubacterium *Rhodothermus marinus* [78], marine bacterium *Pseudoalteromonas sp.* [79] and lactic acid bacterium *Carnobacterium piscicola* [80]. In fungi it is found in *Aspergillus* [81,82], *Cephalosporium* [83], *Corticium* [84], *Gibberella*, [85], *Mortierella* [86], *Penicillium* [87], *Monascus* [88], *Pycnoporous* [89], *Rhizopus* [90], *Scopulariopsis* [91], *Trichoderma* [92], *Humicola* [93], *Thermomyces* [94] and many other fungi imperfecti [95]. Presence of α-galactosidase in higher fungi has also been reported [96].

Table 6. α-Galactosidases producing microorganisms :

Organism	Reference
Aerobacter aerogenes	97
Azotobacter vinelandii	98
Bacillus stearothermophilus	99,100
Bacteriodes ovatus	101
Bacteroides fragilis	102
Bifidobacterium adolescentis	103
Bifidobacterium breve	104
Clostridium perfringens	105
Diplococcus pneumoniae	106
Escherichia coli	107
Lactobacillus sp.	108,109,110
Micrococcus	111
Pseudomonas fluorescens	112
Streptococcus bovis	113
Streptococcus mutans	114
Thermotoga neapolitana	115
Thermus sp	116
Actinomycetes sp	117

Significance : Among microorganisms the physiological function of α-galactosidase could be in the hydrolysis of galactose containing oligo and polysaccharides present in the growth medium or in the natural environment, liberating an assimiable end products like galactose, glucose, mannose, which serve as a carbon source for growth of organism.

1.1.4.1.2 Assay of α-galactosidase :

p-Nitrophenyl α-D-galactopyranoside, *o*-nitrophenyl α-D-galactopyranoside, melibiose, raffinose and stachyose have mainly been employed as substrates for estimating the activity of α-galactosidase. For rapid and routine assay *p*-nitrophenyl α-D-galactopyranoside and *o*-nitrophenyl α-D-galactopyranoside are used as a substrate [93,109] and the *p*-nitrophenol liberated after hydrolysis of these substrates was estimated spectrophotometrically at 400-410 nm [118,119]. In few instances, phenyl glycoside was also used for routine α-galactosidase assay [120] & the phenol liberated was estimated by Folin and Denis method [121]. Many times melibiose, raffinose, stachyose and methyl-α-D-galactopyranoside are also used as a substrate [47,101,122,123] and the sugars liberated (glucose or galactose) after hydrolysis was estimated either by reducing sugar method or coupled enzymatic method [124,125,126,127,128,129,130,131]. In few cases HPLC analysis was also done to estimate the liberated galactose upon hydrolysis of raffinose and stachyose [132]. The most sensitive assay method described in the literature involves the use of fluorogenic substrate methylumbelliferyl-α-D-galactoside; the liberated aglycone was measured fluoremetrically [133,134]. Galactomannans, consisting of a backbone of β-1,4-D-mannosyl residues linked to D-galactosyl residues via α-1,6 linkages were also used as the substrates [106,117]. The galactose residues, which are cleaved by the action of α-galactosidase, are estimated as above. The guar gum degrading activity of enzyme was also assayed by measuring the reduction in the viscosity of the reaction mixture [135].

Apart from hydrolytic activity towards various oligo and polysaccharides, some of the α-galactosidases exhibited blood group B substance degrading activity [79], estimated by hemagglutination inhibition test [120,136]. Moreover in contrast few α-galactosidases exhibited hemagglutinin (Lectin like) activity, usually quantitated on trypsinized rabbit erythrocytes [137].

The transferase activity of α-galactosidase can be detected qualitatively by analyzing products through paper chromatography [106] or thin layer chromatography (TLC) [94] or quantitatively by HPLC analysis [94].

***In situ* determination of activity :** Methods have been developed for in *vivo* or *in situ* determination of α-galactosidase activity. For determination of intracellular fraction of α-galactosidase in bacterial cells, solvent treatment (toluene or acetone) is given to the

cells in order to increase the membrane permeability. Treated cells were then suspended in a substrate solution and assayed [111,138]. For screening of microorganisms producing α-galactosidase, chromogenic substrate 6-bromo-2-naphthyl-α-D-galactopyranoside and Fast Blue RR overlaid on the plates containing the colonies. α-Galactosidase producing colonies develop an intensive brown colour zone within 1-10 minutes [123]. Same chromogenic substrate was also used for qualitative detection of α-galactosidase activity in non-denaturing polyacrylamide gel [50,123]. Another chromogenic substrate, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside was also used for detection of enzyme activity on isoelectric focusing gels [139]. Methylumbelliferyl-α-D-galactoside has also been used for the detection of α-galactosidase activity in polyacrylamide gel [48].

1.1.4.1.3 Genetic regulation of α-galactosidase biosynthesis :

The lactose operon of *Escherichia coli* is a paradigm for gene regulation [140]. Studying *lac* regulation led to fundamental concepts of how a set of genes may be coordinately regulated depending upon the concentration of metabolizable compounds in the growth medium. Molecular characterization of sugar utilization systems has provided valuable knowledge in gene regulation in bacteria. [141,142].

Usually, sugar utilization systems are subject to carbon catabolite repression, the mechanism of which is clearly different in bacilli and enteric bacteria, as no cyclic AMP and cyclic AMP receptor protein homologue was found in *Bacillus* species [143,144]**.** Carbon catabolite repression in bacilli and other gram-positive bacteria is mediated by negative regulation [142,145]; the GalR/LacI family of negative transcriptional regulators [146,147,148] and AraC/XylS-type proteins [141,149].

Numerous studies have been carried out with the metabolic regulation of α-galactosidase in bacteria and yeasts including transmissible plasmids, which participate in metabolism of raffinose [141,150,151,152]. As the natural substrates of the α-galactosidase are galactose containing oligo and polysaccharides most of the microbial α-galactosidases are subject to induction by galactose and its derivatives [76,88,95,99,112].

The utilization of melibiose by cells of *E. coli* K_{12} is controlled at least by two inducible functions, a galactoside transport system (melibiose permease) and hydrolyzing enzyme (α-galactosidase) [151]. Genes of melibiose operon, *melB* and *melA* govern both the functions, respectively. In addition to this, two permeases have been demonstrated in *E. coli***,** these are Thiomethyl galactoside permease (TMG)-I and (TMG)-II, both are inducible by melibiose, but TMG-II differs from TMG-I in that it is inducible by galactinol, and is not active in cells grown at 37 °C [151,153].

The wild type strain of *E. coli* K_{12} cannot utilize raffinose as a sole carbon source, since it is unable to induce specific transport or hydrolyzing activity. However, the class of transmissible plasmids confers *E. coli* the capacity to grow on raffinose as a sole carbon source [152]. The system consists of regulatory gene and structural gene for the enzyme; raffinose permease, α-galactosidase and invertase, designated as the *raf* system [138]. The operon is subject to dual regulation; to negative control by the binding of RafR repressor to twin operators O1 and O2, and to positive control by the cAMP binding proteins, CAP [154].

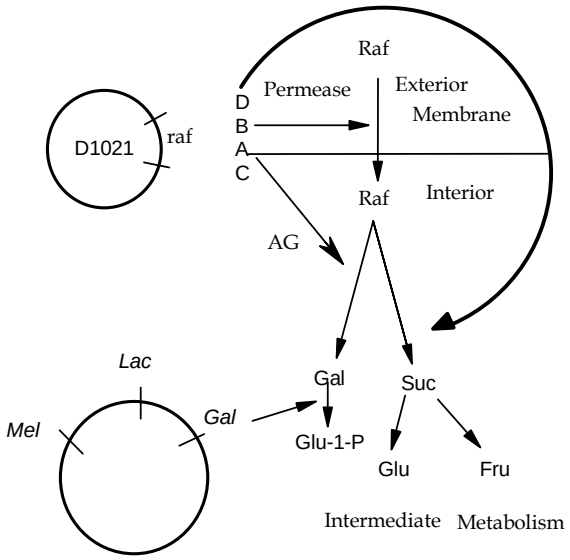


Figure 2. Gene-enzyme relationship in raffinose metabolism by *E. coli* K_{12} containing *Raf* plasmid and scheme for the regulation of raffinose metabolism according to Schmid and Schmitt [138]. The host chromosome and plasmid with relevant genetic loci are regulating the utilization of galactose, lactose, melibiose, and raffinose, respectively. Structural genes A, B and D which code for the three known *raf* -functions and the regulatory gene *raf C* are indicated. The suffices 'AG' α-galactosidase; Glu, glucose; Gal, galactose; Fru, fructose; Suc, Sucrose; Raf, raffinose.

Little is known about the mechanism of utilization of sugars by *S. pneumoniae*. Only characterized sugar transport system in *S. pneumoniae* is the maltodextrin (Mal) utilization system [155]. However, very recently a gene region was identified in the *S. pneumoniae* genome sequence that contains eight genes involved in regulation and metabolism of raffinose (Figure 3). The expression of aga (structural gene for α-galactosidase) positively regulated by raffinose and negatively by sucrose in the growth medium is consistent with the catabolite repression [150].

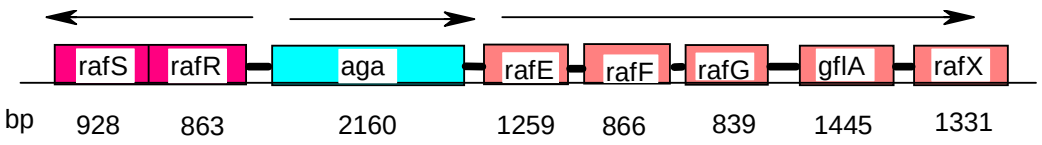


Figure 3. Map of the *raf* genes in the *S. pneumoniae* containing, Arrows indicate operon and the orientation of transcription, the number of base pair bp. The genes *rafR* and *rafS* are transcribed as one operon, and their gene products regulate the raffinose dependent stimulation of promoter, directing the expression of *aga*. Remaining five genes (*rafE, rafF, rafG, rafA,* and *rafX*) might be involved in transport and metabolism of raffinose (reproduced from Rosenow C., 1999 [150]).

In contrast, carbohydrate utilization by *Streptococcus mutans* is well studied [156,157]. A well-characterized sugar utilization system in *S. mutans* is the multiple-sugar metabolism operon (*msm*) [141]. This gene cluster contains eight genes, products of which are involved in the uptake of melibiose, raffinose, isomaltotriose and the metabolism of melibiose, sucrose, and isomaltosaccharides. The cluster also contains the gene for a regulatory protein that acts as a positive effector.

Recently galactoside utilization system; organization and regulation of α-galactosidase gene in *Thermus* sp has been studied. [158]. It shows close similarity with the component of bacterial binding protein dependent transport system for the uptake of galactosides. [141].

The genetic characterization of α-galactosidase system in *Lactobacillus plantarum* [159], *Lactococcus raffinolactis* [160] and *Carnobacterium piscicola* [80] has also been reported. The expression and transcriptional studies of *melA* and *rafP* (structural gene for α-galactosidase and putative raffinose transporter) in *Lactobacillus plantarum* demonstrated that *melA* is transcribed from its own promoter and not a part of operon in contrast to *melA* gene from other microorganisms [161]. Moreover the *melA* gene is regulated at the transcriptional level, i.e., it is induced by melibiose but not totally repressed by glucose, suggesting that *melA* regulation is mediated by an induction mechanism rather than by catabolite repression [159].

Genetic analysis of *Lactococcus raffinolactis* showed that the genes of galactoside metabolism are organized into an operon, containing aga (alpha-galactosidase), galK (galactokinase), and galT (galactose 1-phosphate). The expression of the aga-galKT operon was modulated by a regulator; GalR. The product of gene galR belongs to the LacI/GalR family of transcriptional regulators, suggesting that the expression of the aga operon in *lactococci* is negatively controlled by GalR and induced by a metabolite derived from the metabolism of galactosides [160].

In *Saccharomyces sp*. the ability to use melibiose depends on the presence of a MEL gene, encoding α-galactosidase [162]. The MEL1 gene in *Saccharomyces cerevisiae* is required for the production of α-galactosidase and for the catabolism of melibiose. Production of α-galactosidase is induced by galactose or melibiose and repressed by glucose. The positive and negative regulatory proteins GAL4 and GAL80 control inducibility, respectively [163].

Such regulatory mechanisms provide bacteria with the ability to select their preferred carbon and energy source from the environment by switching off genes that code for transport and metabolizing enzymes of other systems.

1.1.4.1.4 Microbial production of α-galactosidase :

Microorganisms in particular have been regarded as treasure sources of useful enzymes. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons**:** they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts, highly biodegradable, pose no threat to the environment, and are economically viable. The ability of α-galactosidase to hydrolyze the α-1-6-galactosidic linkages is one of the most promising applications of enzyme in soy food processing, animal feed processing, and in beet sugar industry. α-Galactosidase is widely distributed in nature and is produced by plants, animals and microorganisms (Table no. 4, 5 & 6), however; only microbial sources have been reviewed here with respect to this enzyme. The microorganisms preferred for commercial production of α-galactosidase are *Circinella muscae*, *Absidia griseola, Absidia hyalospora* (Hokkaido Sugar Co. Ltd., Tokyo, Japan) [164,165], *Mortierella vinacea* [166] and *Bacillus stearothermophilus* (Monsanto Company, St. Louis, Mo) [167]. Recently it is being produced commercially by *Aspergillus niger* (Novo Nordisk A/S, Bagsvaerd, Denmark) [168].

1.1.4.1.4.1 Fermentation techniques :

The submerged fermentation for aerobic microorganisms is now well known and widely used method for the production of α-galactosidase [166,167,169]. Although several α-galactosidase preparations are produced by solid-state fermentation method [170,171], 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.

a) α-Galactosidase production by submerge fermentation :

Microorganisms in submerged fermentation (SmF) thrive in a liquid environment. Generally, the submerged fermentation processes are carried out in shake flasks or aerated agitated fermentor equipped with controls of fermentation parameters. Submerged fermentation has several advantages over solid state fermentation such as **:** it requires less man power, gives higher yield and productivity, low cost, less contamination and better temperature control during fermentation [172]. A classical example of production of α-galactosidase by submerged fermentation method has been the batch fermentation by thermophilic and aerobic Bacillus sp JF_2 strain [173]. The design of fermentation system utilized in above said process is shown in Figure 4.

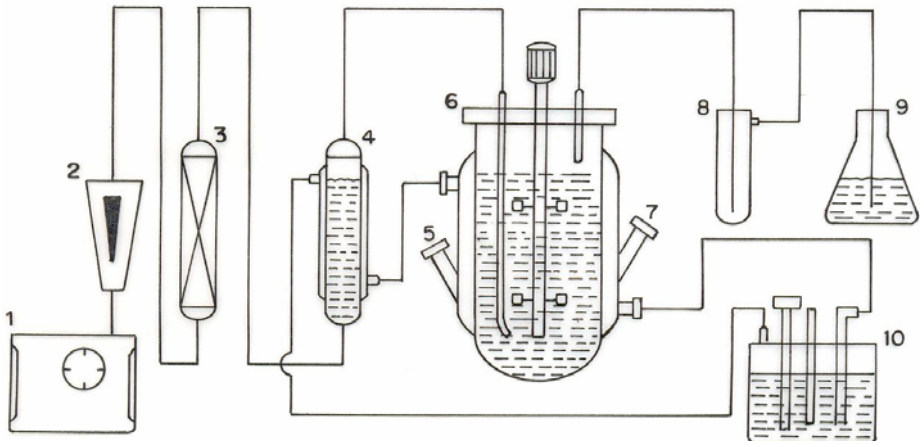


Figure 4. Schematic diagram of submerged fermentation process; (1) air pressure, (2) air flow meter (3) air filter (4) water vapour saturation (5) pH sensor (6) fermentor (7) take sample (8) air buffer (9) dilutent sulphuric acid (10) temperature control unit [Ref.-173].

Under submerged fermentation conditions various factors such as physical, chemical and nutritional parameters are known to have significant effect on productivity of α-galactosidase.

i) Medium Formulation :

To obtain a suitable medium for enzyme production, effect of various carbon, nitrogen sources in combination with mineral salts on growth and enzyme production must be studied to achieve maximal product formation [174].

The carbon source employed in microbial enzyme production is one of the most important factors in determining feasibility of the process. Usually assimiable sources of

carbon are suitable for use in fermentation. For α-galactosidase production, glucose, galactose, lactose, melibiose, raffinose and stachyose have been used at laboratory scale as well as at commercial scale [164,169,175,176,177]. Cheap agriculture residues like, wheat bran or wheat flour, rice bran, soy flour or soybean cake, sorghum, corn, millet etc, are also used for enzyme production [80,173,178,179]. Optionally, an inducer; raffinose or melibiose can be added to induce the α-galactosidase formation, when agricultural residues were used as a carbon sources in the medium [167]. In few cases, waste effluent and waste byproducts have been utilized for cultivating the organism and producing α-galactosidase in the fermentation medium [180,181]. In addition to above carbon sources, few microorganisms were found to assimilate complex carbohydrate containing substrates, like guar gum, locust bean gums and polygalacturonate to synthesize α-galactosidase [91,115,182].

The type of nitrogen source to be used depends essentially upon the nutritional requirement of the organism. A variety of nitrogen sources including both organic and inorganic have been evaluated for the production of α-galactosidase. *Bacillus stearothermophilus* [99], *Bacillus sp* [173], *Lactobacilli* [110], *Streptomyces sp* [120] utilize exclusively organic nitrogen sources like tryptone, peptone, polypeptone, yeast extract, beef extract, casamino acids, soybean meal etc for their growth and α-galactosidase production. Since the soybean meal acts both as a source of carbon as well as nitrogen, it could be economical substrate for commercial enzyme production [99]. The inorganic nitrogen sources like ammonium sulfate, ammonium nitrate, ammonium acetate, urea etc have been used in the fermentation medium for the growth of *Azotobacter* [98] and *Trichoderma reesei* [92]. Many times combination of organic and inorganic nitrogen sources has been practiced [76,88].

Apart from C & N sources, many other essential elements such as phosphorus, magnesium, calcium and numerous trace elements such as iron, copper, cobalt, zinc, manganese may be required in the medium to support active cellular function. Trace elements often serve as cofactors in enzymatic reactions. However very little information is available regarding effect of many of these trace elements on α-galactosidase production. In few cases Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} have been used in the fermentation medium for fungal growth and α-galactosidase production [85,86,182]. Like wise Mg^{2+} ions have been added in the medium for α-galactosidase production by gram-positive bacteria [123]. Moreover, when hyperthermophilic bacteria were grown for α-galactosidase production, the fermentation medium is fortified with mixture of metal ion solutions [115]. Some times vitamins (thiamine, ascorbic acid) have been incorporated in the medium for microbial growth and subsequent enzyme production [110].

ii) *Culture Conditions :*

The principal physical parameters, which affect α-galactosidase production in submerged fermentation, are pH, temperature, aeration and agitation.

The pH is particularly critical for microbial growth and enzyme production. Control of pH is important to maintain an optimal environment for growth and product formation [172]. Most of the bacterial fermentations for α-galactosidase production are carried out in the pH range 6.0-7.5. To maintain this pH, phosphate buffer or phosphate ions have been added in the fermentation medium [99,173,]. For production of α-galactosidase by alkalophilic organisms; pH of the medium was adjusted to 9~11, by sodium carbonate. [111,176]. Most of the fungal fermentations for α-galactosidase production have been carried out in the acidic pH range, 4.5-6.0 [178,183].

In order to obtain optimal yields, fermentations must be carried out at constant temperature. The microorganisms are broadly classified in to three groups; psychrophiles, mesophiles and thermophiles depending on their temperature requirement for growth. Therefore incubation temperature for production of enzyme must depend on the type of organism used. The optimum incubation temperature reported for α-galactosidase production ranges between 30-37 ºC [104,122]. However in few cases incubation temperature range of 40-60 ºC has also been reported, when thermophilic microorganisms were used for the production of α-galactosidase [94,100]. Moreover the hyperthermophilic microorganisms were cultivated at temperature up to above 85 ºC [184]. For alkalophilic microorganisms, growth temperature reported is in between 40-45 ºC [176].

Aeration rate and agitation speed on microbial enzyme production are important factors affecting successful progress of fermentation. Aeration could be beneficial for the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product and oxygen [185].

Agitation is also an important parameter for adequate mixing, mass and heat transfer. In laboratory, most common practice is to employ shake flask cultures. Small jar fermentors provided with the mechanical agitators and air spargers are also used. There are few reports [88,186], describing the conditions of aeration and agitation for the production of α-galactosidase. The conditions of agitation and aeration vary from strain to strain and the type of fermentors used. Delente *et al* [167] have employed aeration rate of 0.8 vvm (volume of air per volume of medium) and agitation at 400 rpm to produce α-galactosidase maximally from *Bacillus stearothermophilus* in 14 L fermentor. In another case the aeration rate of 0.5 vvm and agitation at 240 rpm was used by Narita *et al* [164], to produce α-galactosidase from *Circenella musae* in 20 L fermentor. When *Corticium rolfsii* was grown for enzyme production in 200-liter fermentor, 1 liter of air per liter of medium per minute was provided [183]. On the other hand, agitation creates

shear forces on the cells causing morphological changes, variation in their growth and product formation, and also damages to the cell structure. This effect was clearly seen in *Lactobacillus fermenti,* where biomass was higher for agitated cultures due to the improved mixing conditions and better oxygen transfer. However, higher enzyme activity was observed for static cultures. Lower activity in agitated culture was attributed to the shearing effect [109].

b) α-Galactosidase production by solid-state fermentation :

Solid-State Fermentation (SSF) is the cultivation of microorganisms on water saturated solid raw materials, such as grains, beans or wheat bran in the absence of free flowing water [187]. This is an alternative to the cultivation of microorganisms in liquid nutrient broth (SmF). SSF has been used for centuries and is still being used to produce foods, such as soy sauce, tempe or mushrooms. In Japan, it is the principal technology used to make enzymes. It is also known as Koji (Japanese). In many cases, the microorganisms used in SSF are fungi, because they are ideally suited to colonize and penetrate solid particles. The distinct advantage of SSF for fungal enzyme production are**:** lower capital investment, high product concentration, reduced expenditure on down stream processing, minimized waste disposal, simpler reaction design with minimum controls and fermentor volume [187,188]. Other advantages of SSF technique include: (a) a simplified procedure for inoculum development, (b) easier scale up of process, (c) reduced solvent requirement for product recovery etc [189].

There have been numerous reports on the production of α-galactosidase by solid-state fermentation processes [171,190]. The first report on α-galactosidase production from *Mortierella vinacea* by 'koji' method appeared in 1969 [191]. The organisms reported to be producing α-galactosidase in wheat bran based solid-state fermentation are *Aspergillus awamori* [192], *Aspergillus oryzae* [91] and *Aspergillus niger* [193]. Cruz and Park [194] and Kotwal and Gote [170] reported production of α-galactosidase in SSF system by *Aspergillus oryzae* and *Humicola* sp., respectively. Its application in the hydrolysis of galacto-oligosaccharides present in soybean milk has also been demonstrated. Furthermore Srinivas *et al* [195] described 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.

1.1.4.1.5. Strain improvement :

Improvement of microbial strains for the over production of industrial products has been the hallmark of all commercial fermentation processes. Conventionally strain

improvement has been achieved through mutation and selection and also by genetic modification or genetic recombination [172]**.**

The use of mutation and selection to improve the productivity of culture has been strongly established for more than 50 years and is still being used as a valuable tool for industrial strain improvement. An attempt was made by Delente *et al.* [99] to isolate derepressed UV mutant of ***B. stearothermophilus,*** producer of α-galactosidase. However when this mutant was tested in fermentation medium, reverse mutation took place regularly and cells with characteristics of the original strain eventually took over the whole population. Thus further approach could be needed to obtain a stable mutant.

Genetic modification imparts new properties and capabilities in organisms including plants, animals and microorganisms, which can be harnessed for a variety of useful applications. In contrast to conventional techniques, recombinant DNA technology enables the incorporation of genes from different and unrelated species and such modified organism are called transgenic. The enzymes with industrially useful special functions can be located in the natural environment. To improve the productivity-to-cost ratio, it is now possible to modify genes for increase in enzyme productivity in microorganisms.

Number of genes from animals, plants and microorganisms encoding α-galactosidase have been cloned and expressed in heterogeneous host. *E. coli* and S*accharomyces* have been used as a suitable expression host for production of high titres of α-galactosidase activity [196,197,198,199]. The agaA and agaN genes from *Bacillus stearothermophilus* strain KVE39 [123] and NUB3621 [198], respectively, have been cloned and constitutively expressed in *E. coli* at a high levels. Similarly genes encoding thermostable α-galactosidase from hyperthermophilic microorganisms; *Thermus* sp. strain T_2 [116], *Thermus brockianus* ITI 360 (agaT) [132] and *Thermotoga nepolitana* (agaA) [200], are also expressed and recombinant proteins have been studied with respect to their application in high temperature processes in biotechnology. Cloning and expression of α-galactosidase gene from *Pseudomonas fluorescens* subsp. *cellulosa* has been demonstrated and product has been used for galactomannan hydrolysis [197].

The synthesis of yeast α-galactosidase is encoded by MEL gene present in *Saccharomyces carlsbergensis* [162]. However, strains of *S. cerevisiae* that are used commercially for baking are MEL^-. Introduction of MEL gene in *S. cerevisiae* has been reported and as a consequence bakers yeast started utilizing raffinose present in the beet molasses and producing higher biomass and α-galactosidase commercially [201]. Moreover heterogeneous gene encoding guar α-galactosidases reported to be expressed in *Hansenula polymorpha* and *S. cerevisiae* SU50B [202].

Cloning and high level expression of α-galactosidase cDNA from fungi *Mortierella vinacea* [199] and *Penicillium purpurogenum* [203] has been studied in

details. Further the construction of recombinant strain of *Aspergillus niger* has also been reported [204].

1.1.4.1.6 Isolation and purification of α-galactosidase :

The first step in the purification of enzyme is the isolation of enzyme from cultured cells. Number of fungal, bacterial and yeast α-galactosidases reported are intracellular in nature and in such cases recovery of intracellular α-galactosidase was carried out either by French press cell homogenizer [75,88,115,122] or by grinding with abrasives [86]. Sometimes ultrasonicator was also used to disintegrate the cells [109,110,205]. In addition to this, in few cases organic solvents have been used for the extraction of intracellular α-galactosidase [93]. However, under submerged fermentation conditions, number of microorganisms secreting α-galactosidase out side the cell and is harvested simply by filtration or centrifugation [100,102,112,186]

Purification of proteins usually occurs by a series of independent steps in which the various physiochemical properties of the proteins of interest are utilized to separate it progressively from other unwanted constituents. The characteristics of the proteins that are utilized in purification include solubility, ionic charge, molecular size, adsorption properties, and binding affinity to other biological molecules [206].

α-Galactosidases from various sources have been purified by multistep conventional purification procedures. Majority of procedures involved concentration of culture filtrate either by ammonium sulfate fractionation, ethanol or acetone precipitation or by membrane filtration [207,208,209]. In case of thermostable enzymes, heat treatment step is generally introduced to precipitate & separate the unwanted thermolabile proteins to facilitate the further purification [116,198]. Subsequently combination of chromatographic steps including ion exchange, gel filtration and hydrophobic interactions were used to obtain purified α-galactosidase [75,94,115,186,210,211]. In addition to above techniques, in few cases, preparative gel electrophoresis [212], isoelectric focusing [92,102] or chromatofocusing [100,101] was used to achieve the highest purity of enzyme. The above methods have also been used to separate the multi-molecular forms of α-galactosidases [101,212]. Purification by more advanced tool like FPLC has also been reported [132].

Apart from the conventional methods, in several instances, affinity chromatography has proved to be a successful technique in the purification of human [213], plant [139,214,215] and microbial α-galactosidases [106]. Moreover, in few cases, affinity chromatography has been used effectively for the separation of multi-molecular forms of plant α-galactosidases [216]. Recently some of the recombinant α-galactosidases have been produced as fusion proteins with affinity tags, which enabled simpler one step purification, by its respective affinity ligand. [116,197].

1.1.4.1.7 Properties of purified α-galactosidases :

a) Molecular properties :

<u>i) Molecular mass :</u>

Bacterial α-galactosidases has more complex structure and their molecular mass varies considerably between 45 kDa to 400 kDa [197,116]. Thermostable α-galactosidase produced by *Bacillus stearothermophilus* has homotrimeric version of this enzyme with subunit molecular mass of 82 kDa [100]. Similarly α-galactosidase from *Corynebacterium murisepticum* is a homotetramer of 320-kDa protein [217]. Most complex structure of α-galactosidase has been detected in *Thermus sp*. strain $T_{2,}$ having molecular mass of 400 kDa and existing in solution as an octameric form [116]. However the α-galactosidase from *Thermotoga neapolitana* is active as a monomer of 61-kDa protein [200]. On the basis of subunit molecular size, bacterial α-galactosidases have been classified in to two groups [116]. The first group contained α-galactosidases from S*treptococcus mutans*, *Bacillus stearothermophilus*, *Pediococcus pentosaceus*, *E. coli* Raf A, *Bifidobacterium breve* and *Pseudomonas fluorescens,* which has molecular size of more than 80 kDa. While α-galactosidases from *Thermus* sp. strain T_2, *Thermus brockianus*, *Thermotoga maritima*, *Thermotoga neapolitana* and *E. coli (Mel A,* chromosome) belong to second group, possessing molecular size from 53-65 kDa (Table 7).

Most of the fungal α-galactosidases are monomeric proteins, with an average molecular size of 50 kDa [94,207,218]; nevertheless few multimeric forms are also reported from fungi and yeast [89,122,162]. The molecular properties of plant α-galactosidases have been well documented in various reviews [56,219,220].

<u>ii) Multimolecular forms and isoelectric point :</u>

The multimolecular forms of α-galactosidase are predominantly found in plants [56,219,220]. The existence of multimolecular forms of α-galactosidase has also been reported form few microorganisms (Table 8). These multimolecular forms of the enzyme appears due to the proteolytic cleavage or may be due to the differential glycosylation of proteins (post translational modifications) [219]. Microheterogeneity in case of *Thermomyces lanuginosus* α-galactosidase resulted from differential glycosylation of protein [94]. Some times isoenzymes are the products of two distinct genes. Two different molecular forms of α-galactosidase were detected in *Bacteroides ovatus* during growth of culture on two different carbon sources; guar gum and galactose. Their synthesis was regulated independently [101]. Similarly two isoenzymes, regulated by two different genes, *agaA* and *agaB* were detected in *Bacillus stearothermophilus* KVE39 [123]. In humans, two forms of the α-galactosidase; A and B, are present in normal

tissues but the thermolabile 'A' enzyme is absent in Fabry's disease [221].

The pI values of some of the microbial α-galactosidases reported in literature are given in Table no 7 & 8. The isoenzymes having similar molecular mass always differing in their charge properties and can be separated on the basis of their isoelectric point [82].

Table 7. Molecular mass of some of the bacterial α-galactosidases :

Source	pI	Molecular mass (kDa)		Ref.
		Native	Subunit (Nos.)	
Bacillus stearothermophilus (NUB3621)	--	320	80.3 (4)	198
E. coli K_{12} (RafA plasmid)	5.1	329	82 (4)	138
Streptococcus mutans	6.1	300	80 (4)	114
Pediococcus pentosaceus	--	--	80.5 (-)	222
Bifidobacterium breve	--	160	80 (2)	104
Puseudomonas fluorescens	6.3	390	86 (4)	112
E. coli (Mel A, chromosome)	--	100	50 (2)	223
Thermotoga nepolitana	4.6	61	61 (1)	115
Thermotoga maritima	--	125	63.7 (2)	224
Thermus brockianus ITI 360	--	200	53.8 (4)	132
Thermus sp. T_2	--	400	55 (8)	116

Table 8. Multimolecular forms of the α-galactosidases from various sources :

Source	Form	pI	Molecular mass (kDa)	Ref
Bacillus stearothermophilus AT-7	I	--	280	212
	II	--	325	
Bacteroides ovatus	I	5.6	250	101
	II	6.9		
E. coli K_{12} *	I	5.1	329	138
	II	--	200	
Aspergillus niger	I	4.15	350	82
	II	4.5	117	
	II	4.7	117	
	IV	4.8	117	
Aspergillus tamarii	I	--	265	225,226
	II	--	254	
	III	--	56	
Mortierella vinacea	I	5.4	240	218
	II	8.5	60	
Human (placenta)	A	4.7	103	221
	B	4.4	117	
Candida guilliermondii	I	6.16	270	227
	II	6.21	270	

* α-Galactosidases I- specified by plasmid (D1021), II- specified by *mel* gene (chromosomal).

iii) Amino acid analysis and glycoprotein nature :

The amino acid composition of few microbial α-galactosidases has been reported [138,208,223,228,229]. Considerable variation exists in the total amino acid composition of the α-galactosidases from different microbial sources. Most of the α-galactosidases mentioned above contains large amount (20-25 %) of acidic amino acids; aspartic acid

and glutamic acid, whereas sulfur-containing amino acids; cysteine and methionine are relatively low. In addition the α-galactosidase from *E. coli* [138,223] and *Humicola* sp [208] contain large amount of hydrophobic amino acids (40-45 %) whereas *Corynebacterium murisepticum* [217], *Cephalosporium acremonium* [228] and *Saccharomyces carlsbergensis* [229] contain large amount of polar amino acids (40-45 %). The amino acid analysis of few plant α-galactosidases has also been reported [37,56,220] and showed high acidic amino acid content and contains little cysteine. The variation in the amino acid composition among different α-galactosidases from various sources could be attributed to the genetic variation or proteolytic modification of the native protein [230,231].

Most of the fungal and plant α-galactosidases are glycosylated proteins [208,219]. However, generally bacterial α-galactosidases are non-glycosylated. In few cases the carbohydrate content of α-galactosidases has been estimated and very few studies on its composition and structural analysis have been carried out. The α-galactosidases from *Cephalosporium acremonium* [228] 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. Similarly α-galactosidases from *Aspergillus tamarii* [225] contains N-acetylglucosamine, mannose, glucose and galactose in the molar proportion of 1:9:5:8. However the enzyme from *Aspergillus niger* [232] 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. A similar structure was also reported for the glycopeptide from the lentil enzyme [233]. The carbohydrate moieties were also detected in human [221] and few yeast α-galactosidases [74,76].

The carbohydrates are believed to play an important role in stabilizing the enzyme structure, activity and stability [94].

b) Biochemical properties :

i) Effect of pH :

α-Galactosidase from yeast, mold and plant seeds, showed broad pH optima, ranging between pH 3 and 6 [75,235,234,56]. Most acidic form of α-galactosidase has been observed in fungus *Corticium rolfsii* [84] and *Aspergillus niger* [82], active at pH 2.5-4.5. However bacterial α-galactosidases have pH optima at a rather narrow and neutral pH range, 6.5-7.5 [198,200,236]. Shift in the pH optima in α-galactosidase from *Lactobacillus fermenti* [109] appeared to be temperature dependent. Like wise substrate

dependent pH optima was observed in α-galactosidases from *Aspergillus niger* [237] and *Penicillium duponti* [238].

Generally α-galactosidases are stable over a broad pH range [88,92,211]. Plant α-galactosidases are most stable towards the acidic side of pH, thus acidification step is often introduced to precipitate unwanted proteins in initial steps of purification [37,48]. The phenomenon of subunit dissociation and association as a function of pH was reported in few plant α-galactosidases [239,240].

Studies on effect of pH on K_m and V_{max} yield some useful information about type of ionizable amino acid residues in the catalysis. Few such studies are reported in α-galactosidase from plant origin [54,241,242,243] and showed that dissociable groups in the active site have pKa_1 in the range of 3.8-4.7 and pKa_2 in the range of 5.6-7.0, pointing towards the presence of carboxyl and histidine imidazolium group at or near the active site.

ii) Effect of temperature :

α-Galactosidases display varying degree of temperature stability depending upon their origin. The temperature optima and thermal stability of α-galactosidases from various sources are given in Table 9. The α-galactosidase of *E. coli* [244], *Diplococcus* [106] and Human (isozyme-A) [221] are thermolabile. However enzymes from *Bacillus stearothermophilus* [123], *Thermotoga* [245] and *Thermus sp* [116], are thermostable up to above 70 °C. Most thermostable version of α-galactosidase has been found in *Thermoanaerobacterium polysaccharolyticum* [211], remain stable at 70 °C for 36 hours.

A study of variation with temperature on velocity maximum (V_{max}) and substrate affinity (K_m), are expected to throw valuable light on the mechanism of enzymatic reactions. Such studies have been carried out according to the Arrhenius relationship. Calculated values of activation energies for α-galactosidase catalyzed reactions from various sources are summarized in Table no 7. Dey and Pridham [241] have studied the kinetic properties of α-galactosidase from *Vicia faba* and calculated the entropy values from the effect of temperature and suggested that the high negative entropy values (Enzyme-I,-32.4 and Enzyme-II, -43.6 cal./deg/mol) could be due to considerable conformational changes in the enzyme protein during the formation of ES complex. In another report, α-galactosidase from C*icer arietinum* [246], the entropy values of -10.3 and -24.7 (cal./deg/mol) have been reported for α-galactosidase-I and II, respectively.

Table 9. Temperature stability of some of the α-galactosidases :

Source	Temperature*		Activation energy (Kcal/mole)	Ref
	Optima	Stability		
Phaseolus vulgaris	---	30 °C, 60 m, compl. Stab.	13.6	247
Vicia faba -- I	---	60 °C, 15 m., retains 82 %,	15.4	241,248
--II	---	60 °C, 15 m., retains 42 %,	27.2	
Thermus sp.strain T_2	75 °C	70 °C, t1/2, 1 h	----	116
Lactobacillus fermentum	45 °C	50 °C, compl. Stab.	12.0	209
B. stearothermophilus	75 °C	70 °C, t1/2 19 h.	----	198
Thermotoga neapolitana	93 °C	85 °C, 4 h., retains 75 %,	----	200
Mortierella vinacea	60 °C	60 °C, 1h., retains 70 %, In presence of BSA	12.4	218
Thermomyces lanuginosus	65-70 °C	65 °C, t1/2, 3 h.	----	94
Humicola sp.	60 °C	65 °C, t1/2, 30 m.	35.87	208
Phanerochaete chrysosporium	---	80 °C 3 h. compl. Stab	----	249
Aspergillus niger	40 °C	55 °C, 60 m., retains 65 %,	16.4	237
Candida javanica	70 °C	70 °C, 15 m., retains 70 %,	----	76

*Thermal stability is given as % of activity retains after incubation at particular temperature for a given time period.

iii) Effect of inhibitors :

A number of substances may cause a reduction in the rate of enzyme-catalyzed reactions. Some of these (eg. Urea, Guanidinium chloride etc) are non-specific protein denaturants. Others, which generally act in a fairly specific manner, either reversibly or irreversibly are known as inhibitors.

Some of the divalent ions; Hg^{2+}, Ag^{2+}, and Cu^{2+} are potent inhibitors of α-galactosidases [36,186,198]. The inhibition of α-galactosidase with Hg^{2+} ions is usually attributed to its reaction with thiol, carboxyl, amino and imidazolium groups, whereas inhibition with silver ions may be attributed to the reaction with only carboxyl and or histidine residues at the active site of α-galactosidase [56,250]. Most of the metal ions tested for the effect on catalytic activity of α-galactosidase do not appear to have stabilizing/activating effect. However, Mn^{2+} ions have been found to be stabilizing/activating α-galactosidase from *Escherichia coli* [251] and *Penicillium janthinellum* [252].

The inhibition of α-galactosidases by various sugars and their derivatives has also been reported [36,253,254]. Generally D-galactose is a competitive inhibitor of many α-galactosidases [255,256], may be due to this sugar is a substrate analogue. However inhibition of α-galactosidase from *Mortierella vinacea* by D-galactose was found to be of mixed type, suggesting both competitive and non-competitive binding of D-galactose to the enzyme [257]. α-Galactosidase from *Aspergillus ficcum* was noncompetitively inhibited by glucose and by mannose uncompetitively [186], whereas D-galactose, D-glucose, melibiose and raffinose act as competitive inhibitors of α-galactosidase from *Aspergillus nidulans* [122]. Three different types of the inhibition pattern; competitive, noncompetitive and mixed, was observed in α-galactosidase from *Bacillus stearothermophilus* [212], when incubated with galactose, lactose and cellobiose, respectively.

***c) Catalytic properties of α-galactosidases* :**

Number of α-galactosidases from various sources has diverse preference for their substrates. The main function of α-galactosidase is to catalyze the hydrolysis of α-1,6 linked D-galactose residues from galacto-oligosaccharides and polysaccharides [258]. However, some of the α-galactosidases are known to catalyze the transgalactosylation reactions, especially at high concentration of substrate [259]. Besides these two functions few α-galactosidases of plant origin have hemagglutinin (Lectin) like activity [260].

i) Hydrolase activity : Hydrolase activity of the α-galactosidase from several microbial and plant sources has been studied extensively [100,112,48,261]. α-Galactosidases studied so far showed strict anomer selectivity and some flexibility in its regio, stereo,

glycone and aglycone specificity. The anomeric configuration of the product liberated by the action of α-galactosidase is specifically α-, irrespective of the type of the α-galactosidic linkage in the substrate, e.g. α-1-2, α-1-3, α-1-4, α-1-6 etc [210,228]. However many enzymes are not absolute specific for glycone residues, and hydrolyze structural analogues β-L-arabinopyranosides and α-D-fucopyranosides [48,102,262]. The aglycone group of the substrate may or may not have marked effect on hydrolysis; hence number of α-galactosidases can hydrolyze for e.g. methyl-, ethyl-, n-propyl, α-naphthyl-galactose, *p*-nitophenyl-α-D-galactopyrnoside, melibiose and raffinose [53,60,197,263]. The quantitative evaluation of glycone, aglycone and stereospecificity of α-galactosidase from various sources has been reported [47,48,94,112,264]. The natural substrates melibiose, raffinose, stachyose showed lower affinity and lower rate of hydrolysis as compared to *p*NPG and *o*NPG [132], indicating that aryl glycosides are the better substrates than the alkyl derivatives.

On the basis of size of the substrate hydrolyzed, α-galactosidases may be classified into two groups [265]. The first group contained enzymes active on oligo-saccharides with low degree of polymerization (DP 3-5) [116]. The second group of α-galactosidases consists of enzymes active on polymeric substrates [82,266]. A few α-galactosidases, however can utilize both groups of substrates [41,267]. Generally the affinity of α-galactosidase towards the substrate decreases with increase in chain length [122].

ii) Transferase activity **:** The process of hydrolysis take place when water is a acceptor molecule. Where as the process of transglycosylation takes place when acceptor is another glycosyl or alcohol. Many microbial and plant α-galactosidases have been reported to exhibit transgalactosylation reactions, in addition to hydrolysis of α-galactosidic linkages [268,269,270,271]. The D-galactose moiety is preferentially transferred to a primary alcoholic group in the acceptor molecule. Many investigators have studied acceptor specificity and kinetics of transfer reactions in detail [52,58,264] and found that hexoses are 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 [58].

The α-galactosidases are also known to synthesize (*de-novo-synthesis*) oligo-saccharides when incubated with high concentrations of monosaccharides [272,273], and this procedure has been used for the preparation of several glucose and galactose derivatives [274].

iii) Hemagglutinin (Lectin) activity: Proteins or glycoproteins of non-immune origin possesing the ability to agglutinate erythrocytes and/or precipitate glycoconjugates are termed as hemagglutinins or lectins. Carbohydrate binding proteins, which agglutinate erythrocytes and precipitate glycoconjugates, are very common in plant and animal tissues [275]. Some specific enzymes with multiple sugar binding sites have the ability to agglutinate erythrocytes [276]. The International Union of Biochemistry recommends the inclusion of such proteins (enzymes) under the definition "Lectins". α-Galactosidases from several sources exhibit this unique property of lectins, i.e., hemagglutination of erythrocytes [216,277].

Generally agglutination requires the presence of multiple binding sites. The binding of lectin to the erythrocytes membrane can be reversed by structurally complimentary sugars. Thus many α-galactosidases, which are glycoprotein, possessing high molecular weight and multiple binding sites, functions as lectins [278]. Whereas, α-galactosidases, which are monomers having single binding site, may not be able to cause visible agglutination [279]. In the case of soybean [216] and mungbean [278] α-galactosidases, only the tetrameric form can cause hemagglutination and have catalytic functions. In some cases, though hemagglutination occurs initially, clot gets dissolved on longer incubation due to the subsequent hydrolytic function of enzyme, an effect not observed with classical lectins. This clot dissolution phenomenon may also be influenced by conditions such as pH, temperature, etc. A model to explain hemagglutination and subsequent clot dissolution of α-galactosidase has been proposed by Dey [54].

1.1.4.1.8 Structure and function :

a) Primary structure :

The complete and N-terminal amino acid sequence of many α-galactosidases from plants, animals and microorganisms have been known [207,218,280,281]. On the basis of sequence and structural similarity, eukaryotic & prokaryotic α-galactosidases has been placed in family GH 27 & 36 of the classification of glycosyl hydrolases, respectively. However *E. coli* melibiase (gene *melA*) [282], assigned to the GH family 4 [27,28]. The *E. coli* melibiase requires NAD^+ and Manganese ions as cofactor [251] and is structurally related to neither family 27 nor 36

The amino acid sequence of α-galactosidase from *Bacillus stearothermophilus* NUB 3621 [198] showed significant homology with α-galactosidases of family 36 and shares 71 % sequence identity with the *Thermoanaerobacter ethanolicus* (GeneBank accession No.Y08557) [283], 50.7 % identity with AgaR of *Pediococcus pentosaceus* (L32093) [222], and 41.1 % identity with the Aga of *Streptococcus mutans* (M77351) [114]. Consequently *Bacillus stearothermophilus* NUB 3621 has been placed in family 36 of glycosyl hydrolases.

Although the bacterial α-galactosidases [except *E. coli* melibiase (gene *melA*)], are the members of family 36 of glycosyl hydrolases, the primary structure of α-galactosidases (*aga27A)* of *Pseudomonas fluorescens* subsp. *cellulosa* [197] and *Clostridium josui* [284] shows close similarity with the eukaryotic α-galactosidases and consists of two distinct functional domains, l.e., a family 27 catalytic domain of glycosyl hydrolases and a dockerin domain involved in cellulosome assembly, therefore they have been placed in family 27 of glycosyl hydrolases. Similarly the primary structure of the eukaryotic α-galactosidases AGLII from *Trichoderma reesei* [285] and AglB from *Aspergillus niger* [204] resemble bacterial α-galactosidases of family 36.

The amino acid sequence of the α-galactosidases from hyperthermophilic bacteria, *Thermotoga neapolitana* [200], *Thermotoga maritima* [286] and *Thermus brockianus* ITI 360 [132] displayed a low level of sequence similarity (20-25 %) with the existing families of α-galactosidases; 27 and 36. The only shared consensus pattern, [LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-[DS]-x-[WY] is near the amino terminal end of the eukaryotic enzymes (Family 27) and within the central region of the bacterial enzymes (Family 36). Its presence indicated a similar reaction mechanism or substrate-binding site of the enzymes of both families, however it is still doubtful to assign these enzymes to GH families 27 or 36, therefore they have been recommended for a new family of glycosyl hydrolases.

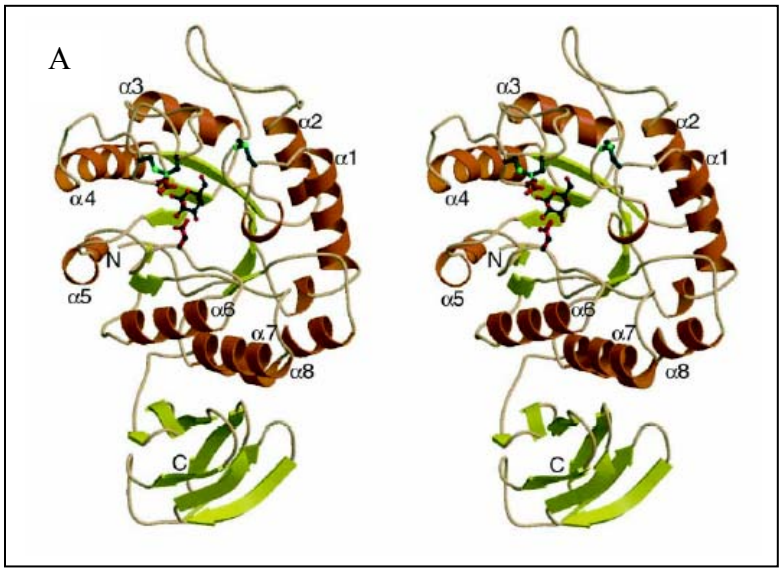
According to the Shibuya *et.al,* [203], comparison of primary structure of *Trichoderma reesei* (AGLI) [285], *Penicillium purpurogenum* [203], *Mortierella vinacea* [287], *Saccharomyces carlsbergensis* [162], *Cyamopsis tetragonoloba* [280] and human [288] α-galactosidase, indicated the presence of nine cysteine residues at identical positions, including two cysteine residues in the insertion and C-terminal regions. Thus, it is likely that these enzymes have similar tertiary structure and it was presumed that these cysteine residues might have role in maintaining the stability of these enzymes by forming an S-S bridge in the molecule.

b) Crystal structure :

Very recently crystal structure of rice α-galactosidase [289] and chicken α-N-acetylgalactosaminidase [290] has been determined & both the enzymes are the members of family 27 of glycosyl hydrolases. The crystal structure of rice α-galactosidase consisted of a catalytic domain and a C-terminal domain and was essentially the same as that of α-N-acetylgalactosaminidase. The catalytic domain has a $(\beta/\alpha)_8$-barrel structure, and the C-terminal domain was made up of eight β-strands containing a Greek key motif (Figure 5A). The structure was determined in presence of D-galactose, providing a mode of substrate binding in detail. The D-galactose molecule was found attached in the active site pocket on the C-terminal side of the central β-barrel of the catalytic domain

(Figure 5B). Catalysis by this enzyme seems to involve a double displacement mechanism, in which Asp-185 works as a catalytic acid/base and Asp-130 acts as a nucleophile.

A structural comparison of rice α-galactosidase with chicken α-N-acetylgalacto-saminidase has provided important information on the substrate recognition mechanism in these enzymes.



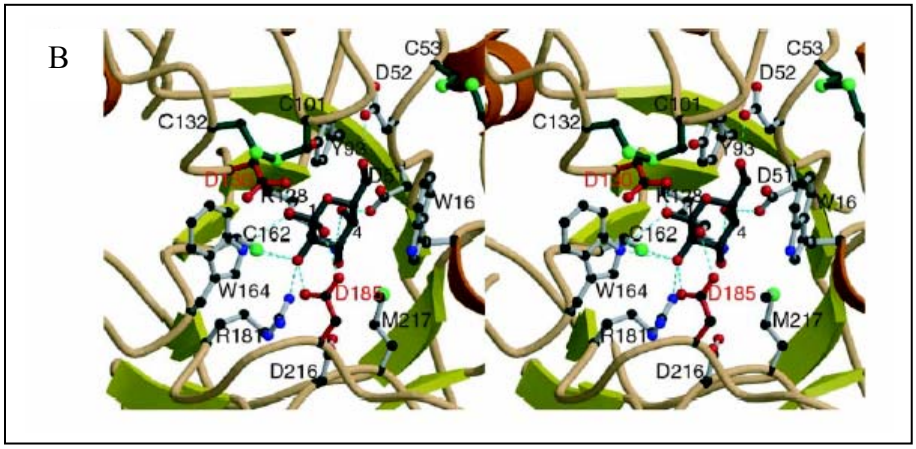


Figure 5. Stereo view of the ribbon model of rice a-galactosidase: The bound D-galactose, two catalytic residues, and two disulfide bonds are indicated by ball-and -stick drawings and shown in black, red, and green, respectively. Coordinating hydrogen bonds are shown in broken green lines (Figure A & B). [Figure adapted from Fujimoto Z (289)]

c) Modification of enzyme and mechanism of substrate hydrolysis :

Modification of protein or enzyme can be defined as the use of genetic or chemical approach to change the structure and function of a protein thus producing a novel product with specific, desired, properties. This method is also used to modify the active site of an enzyme, which gives important information on the amino acid residues in the active site and its structure function relationship in enzyme [291,292]. The recombinant DNA technology [293], protein engineering [294] and directed protein evolution [295] have been used to modify the substrate specificity of enzymes, improve catalytic properties or broaden the spectrum of reaction conditions under which enzymes can function, so that they are more compatible with existing industrial processes.

Two methods of protein engineering viz: site directed mutagenesis [296] and active site directed chemical modification [297] are now widely used to alter structure and function of enzymes and also to elucidate the chemical mechanism of enzymic catalysis. A few studies have been reported in the literature on modification of α-galactosidases and elucidation of its mechanism of hydrolysis.

Site directed mutagenesis of coffee bean α-galactosidase suggested the presence of tryptophan-16 at the active site [298]. The potential role of this residue in substrate binding and in catalytic mechanism has also been reported [299]. In contrast, the role of methionine at the active site of *Trichoderma reesei* α-galactosidase has been shown by oxidation of methionine by H_2O_2 [300]. 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. Chemical modification studies of coconut kernel α-galactosidase has indicated the probable presence of a tyrosine, tryptophan and two carboxyl groups at or near the enzyme's active site [301]; thus, an acid base catalytic mechanism of action for α-galactosidase was proposed (Figure 6) [242].

Aspartate-130 has been identified as a catalytic nucleophile in *Phanerochaete chrysosporium* α-galactosidase by labeling with mechanism based inactivator 2', 4', 6'-trinitrophenyl-2-deoxy-2-2-difluoro-α-D-*lyxo*-hexopyranoside [302].

Participation of tryptophan, lysine and carboxylate at or near the active site of α-galactosidase from thermophilic fungus *Humicola* sp. has also been suggested. [303]. The pH dependent inactivation and kinetic study of α-galactosidase from mung bean [54], *Vicia faba* [241], and sweet almond [243] 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 and *Vicia faba* α-galactosidase [54,241]. 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 [54].

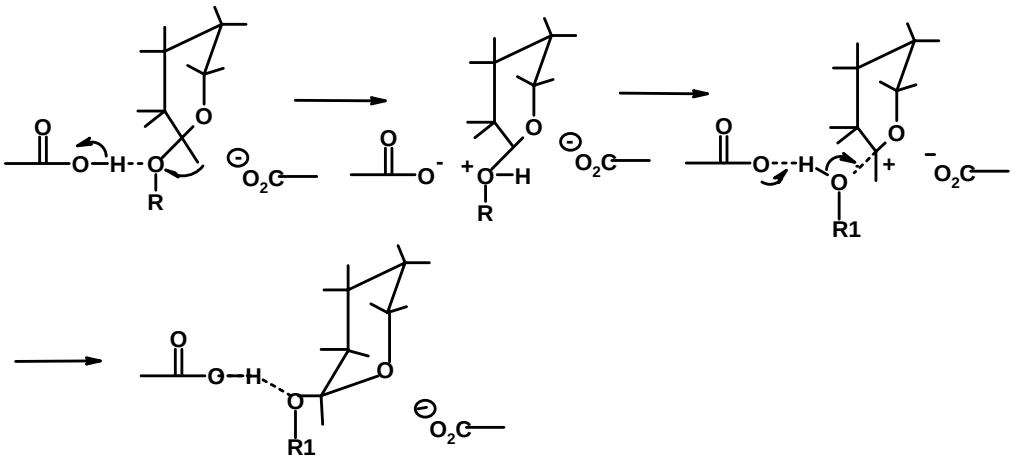


Figure 6. Mechanism of action of α-galactosidase [Ref.-242].

In conclusion, from various studies Dey P.M. [250] has proposed two-step and one-step mechanism of action of plant α-galactosidases (Figure 7.).

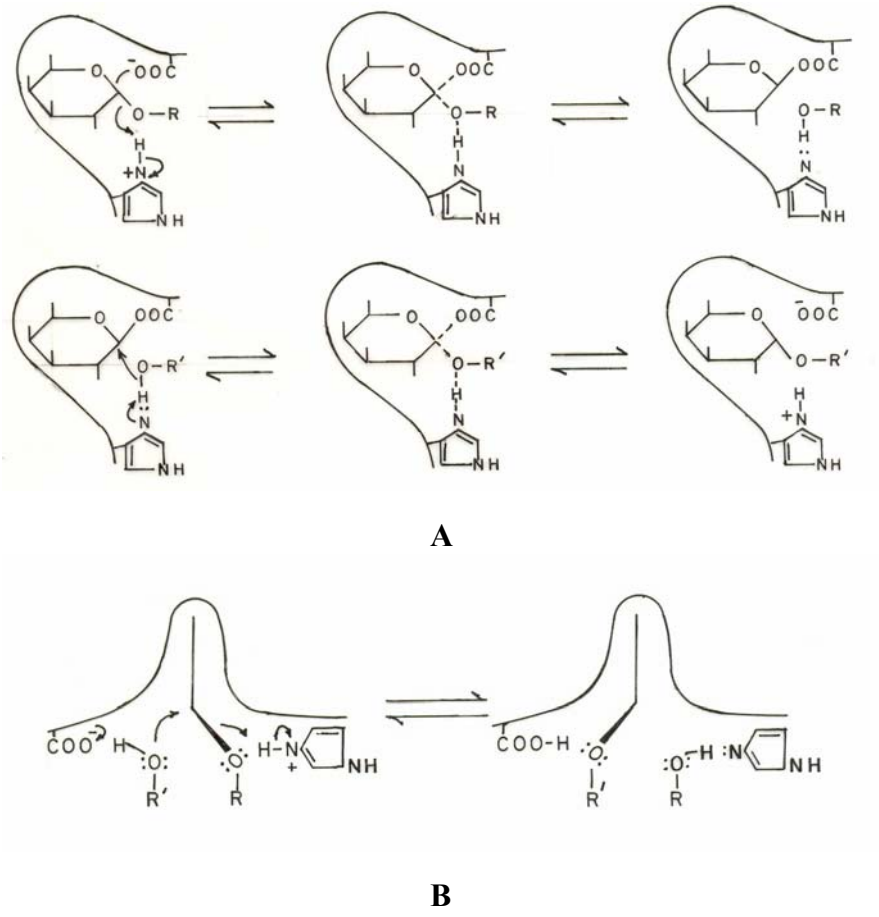


Figure 7. Two step (A) and one step (B) mechanism of action of α-galactosidase [Ref.-250].

One of the important investigations on the active site of α-galactosidase is the determination of role of proline-40 at the activity of lysosomal α-galactosidase-A, an enzyme responsible for Fabry's disease [304]. Fabry's disease is an X-linked, recessive, genetic disorder caused by a deficiency of activity of lysosomal α-galactosidase-A. Site directed mutagenesis study indicated that the reduced activity of lysosomal α-galactosidase-A in patients of Fabry's disease is due to the substitution of proline-40 by serine residue and this substitution was caused by C to T transition in the nucleotide sequence [304].

d) Directed enzyme evolution :

Directed evolution techniques allowed the generation of enzymes with greatly enhanced characteristics, and in some examples, enzymes with new and completely novel substrate specificities [305, 306].

There is single report of modulation of regioselectivity of *Bacillus* α-galactosidase by directed evolution. The evolved enzymes lost most of the activity towards the cleavage of 1-6 linkages and retained only ability to cleave 1-3 bonds from α-galactosides [307]. This is the first report of modification of glycosylhydrolase regioselectivity by directed evolution.

1.1.4.1.9 Applications of α-galactosidases :

The important property of α-galactosidase is the hydrolysis of α-1,6 linked D-galactosyl residues from galactose containing oligo and polysaccharides, has many potential applications in biotechnology and medicine. These applications have been reported by using α-galactosidases from several microbial and plant sources [58,79,81,85,123,266,308,309]. Moreover many α-galactosidase preparations are available commercially under the trade name; Beano, Terrainzyme, Jarro-Zymes-Plus, EZ-Gest, Bean-Zyme etc, used as a dietary supplement in humans diets. Recently, Genzyme Corporation (Cambridge, MA) has received US, FDA approval for marketing of Fabrazym, a α-galactosidase preparation for enzyme replacement therapy for Fabry's patients. The enzyme from *Mortierella vinaceae* strain *raffinoseutilizer* [179] and *Aspergillus niger* (produced by Novo Nordisk A/S, Denmark) [168] is used in beet sugar industry and in food and feed processing.

a) Processing of legume based foods :

Legumes play an important role in the traditional diet of many regions throughout the world. Legumes include peas, beans, lentils, peanuts, and other podded plants are used as a food in Asia, India, South America, the Middle East and Mexico. Beans have long been recognized for their protein content and more recently have been noted for their soluble fiber content and are used largely as low cost, high quality protein supplement [310]. Beans, however, contain large amount of antinutritional factors, mainly galacto-oligosaccharides of raffinose family sugars (Figure 8).

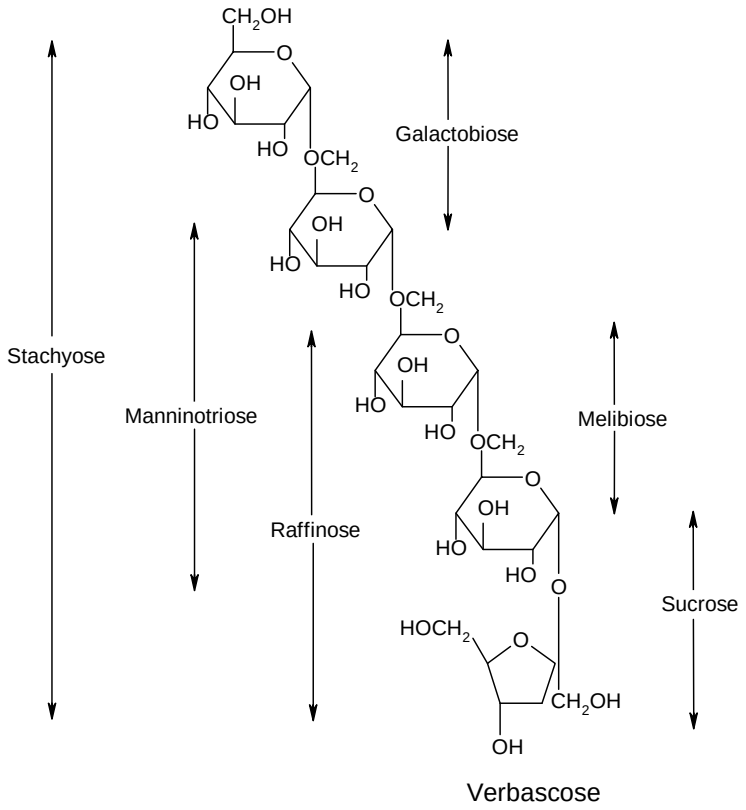


Figure 8. The structural interrelations of the principal members of the raffinose family oligo-saccharides present in legumes.

These galacto-oligosaccharides are responsible for the flatulence in humans. The absence of α-galactosidase in human intestinal track prevents the hydrolysis of these sugars present in soybeans and other legumes [313]. The undigested sugars pass into the large intestine where they are fermented anaerobically by α-galactosidase producing bacteria, thereby resulting in production of gas and gastro-intestinal distress [314,315].

Many attempts have been made to eliminate these oligo-saccharides from legume seeds by soaking and germination [316], fermentation process [317] etc. Enzymatic treatment of soybeans by microbial α-galactosidase offers promising solution for elimination of these oligo-saccharides, especially in soymilk [170,194]. Recently, Somiari and Balogh [193] have reported the hydrolysis of stachyose in cowpea flours using a crude preparation of α-galactosidase from *Aspergillus niger*. They found that enzyme treatment is more effective than soaking and cooking. The immobilized α-galactosidase has also been used for the processing of soymilk, the enzyme from *Mortierella vinacea* [318] and *Gibberella fujikuroi* [319] was entrapped in polyacrylamide gel, packed in a fluidized bed reactor and used for continuous hydrolysis of raffinose and stachyose in soymilk.

b) Processing of sugar beet molasses :

Raffinose is also an important component of beet molasses. In beet sugar industry, during concentration of beet sugar molasses, the content of raffinose increases gradually to 6-10 % and the crystallization of beet sugar is inhibited at this stage and thereby reducing sucrose yield [191]. D-Raffinose elimination by the action of α-galactosidase in beet syrups facilitates crystallization and consequently improves the yield of the sucrose [320].

Many reports describe enzymic process for the hydrolysis of raffinose by exploiting enzymes extracted from number of species of microorganisms of the genera *Absidia*, *Aspergillus*, Saccharomyces, *Bacillus*, *Circinella*, *Mortierella*, *Penicillium* etc, [164,165,166,167,169,177,179]. The immobilized α-galactosidase preparations has also been extensively employed in the beet sugar industry for the elimination of raffinose [119,308, 321].

c) Guar gum processing :

Galactomannan occurs in varying amounts in the endosperm of a wide range of leguminous seeds [322]. These polysaccharides from different species have different proportions of D-galactose and D-mannose, but always consist of a β-(1-4) mannan backbone with single D-galactose stubs linked with α-(1-6) bond. The complete hydrolysis of galactomannan requires the action of three enzymes; α-galactosiase, β-mannosidase and exo-β-mannanse (Figure 9) [115]. In particular, galactomannans such

as guar and locus bean gum are used as rheology modifiers in food products [323]. In addition, they have many industrial applications in pharmaceuticals, cosmetics, paper products, paints and plastics, well drilling and mining and explosives [265]. Moreover, these galactomannans possess non-cytotoxic antitumor activity and act as inhibitors of viruses [324]. 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.

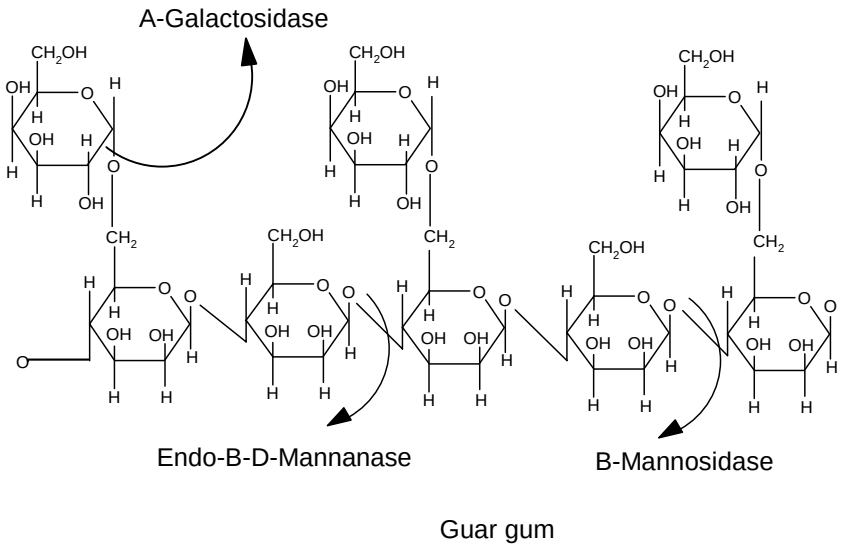


Figure 9. Chemical nature of galactomannan (guar gum) shown with possible sites for enzyme hydrolysis [115].

The commercially produced guar galactomannan has greater proportion of galactose side chains (38 % galactose and 62 % mannose), therefore, guar 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 [325]. Hence, the potential applications of guar gum are limited. Owing to the relatively high cost of locust bean gum, it is of commercial interest to exploit the guar gum by enzymatic modification.

To convert guar galactomannan into a locust bean galactomannan equivalent, some of the side chains (1-6)-α-linked-D-galactosyl residues need to be removed without significant cleavage of the galactomannan back bone [326]. This can be achieved by partial enzymatic hydrolysis of guar gum by α-galactosidase [327]. Many investigators

have developed processes for the modification of galactomannan polymers by using plant and microbial α-galactosidases [197,266,328,329,330]. Studies with purified α-galactosidase have shown that modified guar galactomannan exhibits physical and functional properties similar to that of locust bean gum [331].

d) Animal feed processing :

Among the leguminosae, soy in particular is used in large quantities for feeding pigs, cattle's and poultry animals. Most legume seeds contain measurable amounts of α-galactosides, such as raffinose and stachyose [332]. These oligo-saccharides have been proved to pass undigested through the small intestine of monogastric animals, and depress to some extent the digestibility of other nutrients [333].

The use of exogenous enzymes extracted from microbes has expanded recently and different enzymatic additives are being used to improve the nutritive value of monogastric diets [334]. Many attempts have been made to assess the effect of adding a high dose α-galactosidase to cereal-soybean-pea based diets on performance and digestive efficiency of growing pigs and broilers [335,336].

e) Clinical significance and applications :

At present time there is increased interest of α-galactosidase in human medicine. It plays crucial role in treatment of Fabry's disease, xenotransplantation and in blood group transformation.

i) Treatment of Fabry's disease :

In animals, α-galactosidase-A is a lysosomal enzyme, which catalyzes the hydrolysis of the glycolipid, globotriaosylceramide (GL-3), to galactose and ceramide dihexoside [337]. Further metabolism of ceramide dihexoside 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 signaling mechanisms [338,339].

Fabry's disease is a recessive, X-linked, lysosomal storage disorder, caused by a deficiency of the enzyme α-galactosidase-A (α-Gal A) [340], leading to an accumulation of the glycosphingolipid, mainly globotriaosylceramide (GL-3) in most tissues of the body, causing multisystem disease. The early clinical features include acroparesthesia, angiokeratoma, hypohidrosis, and corneal and lenticular opacities [341, 342].

Since Fabry disease cannot be cured, at present clinical management is symptomatic. Enzyme replacement therapy (ERT) with recombinant alpha-Gal A has been introduced as a new therapeutic option for the treatment of Fabry patients. Enzyme

replacement therapy mediated by gene transfer may become a promising alternative treatment strategy in the future [342,343].

ii) Xenotransplantation :

Xenotransplantation from pigs to human beings is actively pursued as a potential solution to the current acute shortage of donor organs for human beings [344]. A major obstacle to xenotransplantation is the presence of xenoreactive natural antibodies (XNA) in human beings that react with molecules on foreign cells from animals, particularly foreign endothelial cells in vascularized organs, which is known as xenorejection [345]. It has been demonstrated that most of the xenorejection is caused by the carbohydrate epitope Gal α-(1,3) Gal (Galα1-3Galβ-4GlcNAc-R) [346]. The treatment of porcine endothelial cells with α-galactosidase *in vitro* resulted in reduction of the xenorejection induced by human natural antibodies [347]. These studies imply great potential applications of α-galactosidase in overcoming xenorejection for xenotransplantation [348,349].

iii) Blood group transformation :

In 1900 Karl Landsteiner demonstrated the existence of blood group antigens; A, B and H on human red blood cells as well as antibodies directed against those antigens in human sera [350]. The ABH blood group specificity is determined by the nature and linkage of monosaccharides at the ends of the carbohydrate chains. The carbohydrate chains are attached to peptide (glycoprotein) or lipid (glycosphingolipid) backbones, which are attached to the cell membrane of the cells (Figure 10). 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. Thus type O blood is considered “universal” and may be used for transfusion in any individual of blood type A, B, AB and O.

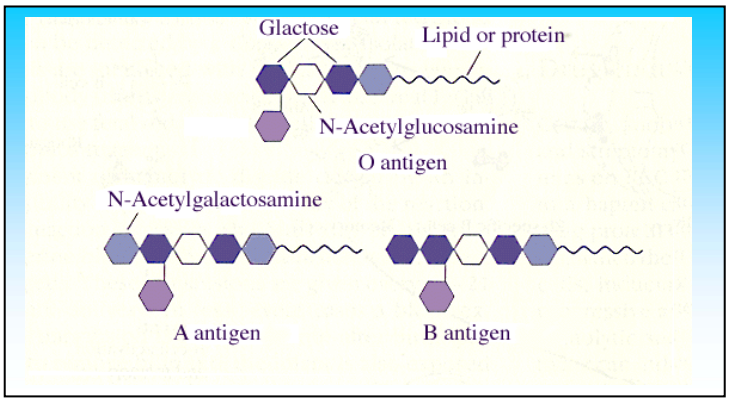


Figure 1. ABO blood group. Structure of terminal sugars, which constitute the distinguishing epitopes, in the A, B, and O blood antigens.

In order to increase the supply of type O blood, efforts have been made to develop methods for converting group A and B blood to group O blood [351]. Enzymatic conversion of type B to O erythrocytes with *Coffea* (coffee bean) α-D-galactosidase was first described by Harpaz and Flowers [352] and subsequently adopted by Goldstein et al [353]. α-Galactosidase with activity against blood group B epitope has been also studied from *Glycine max* [354] and microorganisms, *Pseudoalteromonas* sp [79] *Penicillium* sp [210], *Streptomyces* sp [355] etc.

f) Other potential applications of α-galactosidases :

1. The enzyme is also useful for structural analysis and for elucidation of the biological functions of complex natural compounds, since alpha-linked galactosyl units are constituents of many oligo-saccharides polysaccharides glycoproteins and glycolipids [228,356].
2. Transgalactosylation activity of α-galactosidases has frequently been used for the synthesis of new saccharides [357].
3. In paper and pulp industry α-galactosidases could enhance the bleaching effect of β-1, 4 mannanase on soft wood Kraft pulp [358].

1.2 Aims and objectives :

Due to our interest in thermostable α-galactosidases with reference to the required properties for industrial applications, we decided to study further the enzyme accounting for α-galactoside hydrolyzing activity. Although number of α-galactosidases has been studied from plants animals and microorganisms, only few are available commercially. A commercial enzyme should be stable and fast reacting during reaction, should have a low transferase activity and should be produced by organisms free of toxicity. *Bacillus stearothermophilus* have been widely used in food fermentation in the orient for hundred of years. They are generally recognized as safe (GRAS) by many investigators. Therefore we decided to isolate and study the α-galactosidases from *Bacillus stearothermophilus.*

The specific aim of this thesis was:

1. Screening of microorganism producing thermostable α-galactosidase.

2. Optimization of culture conditions for maximal enzyme productivity.

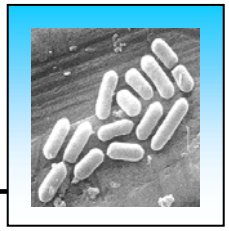
3. Purification of α-galactosidase in minimum possible steps by using conventional and advance techniques of protein purification.

4. To study the biochemical properties of purified α-galactosidase.

5. Chemical modification studies for the determination of amino acid residues present at the active site.

6. Evaluation of α-galactosidase for the removal of anti-nutritional factors present in food and feed.

CHAPTER-2



Production of Thermostable α-Galactosidase by *Bacillus stearothermophilus* (NCIM-5146).

2.1 Summary :

An extracellular α-galactosidase with wide pH and high temperature stability was produced by newly isolated strain of *Bacillus stearothermophilus*, (NCIM 5146), grown on agricultural residues, at 60 °C, under submerged fermentation. The maximum α-galactosidase activity (2.0 U/ml) was found when water extract of soybean meal (2.0 % w/v) was used as a carbon source along with yeast extract (0.3 % w/v) and ammonium sulfate (0.3 % w/v) as a nitrogen sources. The pH & temperature optimum of α-galactosidase was 6.5-7.0 and 65 °C, respectively. The enzyme was stable in the pH range 3-10. The half-life of the enzyme at 70 °C was 80 minutes. The enzyme α-galactosidase was active against synthetic as well as natural glycosides. The K_m values determined for *p*NPG, stachyose, raffinose and melibiose were 0.625, 3.33, 6.66 and 13.33 mM and the V_{max} values were 4.3 x 10^7, 18.03, 12.65, and 6.3 x 10^3 µmoles $min^{-1}mg^{-1}$ of protein, respectively for above substrates. The enzyme activity was strongly inhibited by Ag^+, Hg^{2+}, and Cu^{2+} metal ions. No significant inhibition was found with different sugars.

2.2 Introduction :

Although mesophiles are used for the production of industrially important enzymes, thermophiles offer some major advantages to biotechnology and this is the theme for their extensive exploration [359,360]. There is also a considerable interest in enzymes that catalyzes the hydrolysis of glycosidic bonds, due to their extensive industrial, therapeutic and biochemical applications [235, 361,362].

α-Galactosidase (α-D-galactoside galactohydrolase, E.C.3.2.1.22) catalyzes the hydrolysis of terminal α-1,6-linked D-galactose residues from simple galactose containing oligo-saccharides; melibiose, raffinose, stachyose, and verbascose as well as more complex polysaccharides including galactomannans. Interest in this class of enzymes stems from their potential technological applications. The most important industrial application of α-galactosidase is presently in beet sugar industry [308] and in legume based food processing industry [81,170]. Moreover, recently it has been used in the animal feed processing [333] and in pulp and paper industry [358]. As the most of

these processes operate at high temperature the use of thermostable enzymes appears to be ideal. Furthermore for the development of a technology for the enzymic hydrolysis of raffinose in beet sugar molasses, it is absolutely essential to use α-galactosidase, which is totally free from invertase, otherwise it catalyzes the undesirable hydrolysis of sucrose [165].

The α-galactosidases are found in plants [36,49] and animals [62,67] but the microorganisms are the most promising source [76,86,100]. Commercially, the enzyme is isolated from *Mortierella* [179], *Aspergillus* [168], *Saccharomyces* [169] *Absidia* [165] etc. In addition, although, few α-galactosidases are produced from thermophilic and hyperthermophilic microorganisms [167, 200], there is always a quest for the enzyme having high temperature stability with desired process efficiency. The present study was therefore undertaken to provide a method for production of thermostable α-galactosidase suitable for application in beet sugar, soymilk and animal feed processing.

In this chapter, studies on the optimization of culture conditions for maximum production of α-galactosidase in submerged (SmF) fermentation by *Bacillus stearothermophilus* (NCIM 5146) has been described in detail. The properties of the enzyme have been studied to determine the suitability for its industrial application.

2.3 Materials and Methods :

Chemicals :

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG), *o*-nitrophenyl-α-D-galactopyranoside (*o*NPG), *p*-nitrophenyl-β-D-galactopyranoside, raffinose, stachyose, melibiose were obtained from Sigma Chemical Company, USA. Yeast extract, malt extract, beef extract, peptone were purchased from Difco Laboratories, USA. All other chemicals used were of analytical grade.

Isolation of microorganism :

Microorganism producing high amount of thermostable α-galactosidase was isolated from soil and compost samples. The enrichment medium (Medium-I) used for isolation contains (per liters of distilled water) soya flour, 10.0 g; peptone, 10.0 g; beef extract, 5.0 g; and NaCl, 5.0 g; pH of the medium was adjusted to 7.0, if necessary. The flasks containing 50 ml of medium were inoculated with the soil or compost samples and incubated at 60 ºC for the period of 24 hours. The population was enriched by giving three successive subcultures and the broth in the each flask was tested for α-galactosidase activity. Flasks showing relatively high α-galactosidase activity were selected and samples (serially diluted) were spread on the plates containing nutrient agar medium supplemented with water extract of soya flour. The medium contained (per liters of distilled water) peptone, 10.0 g; beef extract, 5.0 g; NaCl, 5.0 g; water extract of 20.0 g,

of soya flour and agar 20.0 g, pH of the medium was adjusted to 7.0 if necessary (Medium-II). Plates were incubated at 60 ºC for 12 hours. Colonies were picked up from the plates and were checked for α-galactosidase production using liquid medium (Medium-I) in a shake flask culture, at 60 ºC. The colony secreting high amount of thermostable α-galactosidase was selected for further use.

Identification of Isolate :

The above, selected isolate was identified on the basis of its morphological and biochemical characteristics using Bergey's Manual of Determinative Bacteriology 8th edition [363], Bergey's Manual of Systemic Bacteriology (vol-2) [364] and Methods in Microbiology (vol-16,1984) [365]. The isolate was maintained on nutrient agar slants containing 2 % (w/v) water extract of soya flour (Medium-II).

Scanning Electron Microscopic study :

Lag, log and stationary phase culture of the organism was collected, washed thrice with physiological saline and spread on an aluminum foil and air dried. The samples were then fixed with gluteraldehyde (2.0 % v/v), for 4 hours at 25 ºC. The fixed samples were then washed serially with 10-90 % of acetone, each fraction of acetone was kept for the period of 10 minutes. The film was air dried and coated with thin layer of gold in a gold coating unit (model E-5000, Polaron equipment Ltd.) and viewed with SEM Leica Stereoscan 440 model (Leica Cambridge Ltd. UK) at an accelerating voltage of 10 kV and beam current of 25 Pa. The photographs were recorded by a 35 mm camera attached to the resolution recording unit.

Cell growth determination :

Bacterial growth was determined by measuring the optical density (OD) of the cell suspension at 660 nm. An OD value of 1.00 was equal to 425 mg of cell dry weight per/L.

Enzyme assays :

α-Galactosidase activity was determined by incubating 100 µl of suitably diluted enzyme with 50 µl of 6.66 mM of chromogenic substrate (*p*-nitrophenyl-α-D-galactopyranoside) and 850 µl of 100 mM phosphate buffer (pH 7.0) at 65 °C for 10 minutes. The reaction was terminated by adding 2 ml of 1 M sodium carbonate and the *p*-nitrophenol released was determined from absorbance at 405 nm [366]. When raffinose, stachyose, guar and locust bean gum and yeast mannan were used as the substrates, the reducing sugar produced was determined by the method of Somogyi and

Nelson [124,367], whereas when melibiose was used as a substrate, glucose liberated in the reaction was estimated by Glucose Oxidase Peroxidase Method (GOD-POD) [368]. One unit (IU) of enzyme activity was expressed as the amount of enzyme required to liberate 1 μmole of product (*p*-nitrophenol or reducing sugar) per min under the assay conditions.

β-Galactosidase and α-glucosidase activity was assayed by incubating 100 μl of suitably diluted enzyme with 50 μl of 6.66 mM substrate (*p*-nitrophenyl-β-D-galactopyranoside or *p*-nitrophenyl-α-D-glucopyranoside) and 850 μl of 100 mM of phosphate buffer (pH 7.0) at 50 °C for 10 minutes. The reaction was terminated by adding 2 ml of 1M sodium carbonate and the *p*-nitrophenol released was determined from absorbance at 405 nm as described above. One unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 μmole of *p*-nitrophenol per min under the assay conditions.

Invertase activity in the culture supernatant was assayed at 50 °C, using 0.5 M sucrose in 50 mM acetate buffer, (pH 5.0) [369], and reducing sugar released was estimated by Somogyi and Nelson's method [124,367]. One unit was defined as the amount of enzyme that catalyzes hydrolysis of 1 μmol of sucrose per minute under above conditions.

Xylanase activity from the culture broth was determined by incubating 0.5 ml of 1 % (w/v) xylan solution and 0.5 ml of suitably diluted enzyme in 50 mM phosphate buffer, pH 6.0. The reaction mixture was incubated at 50 °C for 30 min and then terminated by the addition of 1 ml DNSA (1 %, w/v) [370]. The reducing sugar liberated was determined at 540 nm, according to Miller [126]. One unit of xylanase activity is defined as the amount of enzyme required to liberate 1 μmole of xylose per min under assay conditions.

Protease activity in the culture supernatant was checked according to the method described by Kunitz. [371] and Laskowski [372]. One unit of protease activity is defined as the amount of enzyme which releases 1 μmol of tyrosine per minute at 50 °C and at pH 7.0 and 9.0 in 50 mM sodium phosphate and sodium carbonate buffer, respectively, when 1 % (w/v) casein was used as a substrate. Protease activity on hemoglobin was also determined similarly at pH 5.0 in 50 mM sodium acetate buffer.

Amylase activity was determined according to Bernfeld [373]. The reducing sugar liberated following the hydrolysis of 1 % (w/v) soluble starch at pH 5.0 and 8.0 and 50

°C was estimated by the DNSA reagent (1 %, w/v). One unit of the enzyme activity is defined as the amount of enzyme required to liberate 1 μmole of reducing sugar glucose per min under the assay conditions.

Protein determination :

Protein concentration in the culture filtrate was estimated according to the method of Lowry *et al* [374] using bovine serum albumin as a standard. The absorbance was read at 660 nm.

Inoculum preparation :

A loopful of culture from 24 hours fresh slant was inoculated in to a nutrient broth medium containing (per liters of distilled water) Difco beef extract, 10.0 g; Difco peptone, 10.0 g; and NaCl, 5.0 g . The medium was incubated at 60 ºC for 12 hours. 2 % of inoculum was used for the cultivation of organism in fermentation medium.

Medium and culture conditions :

The basal medium used for the preliminary study contained (per liter of distilled water): K_2HPO_4, 3.0 g; KH_2PO_4, 1.0 g; yeast extract, 3.0 g and water extract of defatted soybean meal, 20.0 g. The pH of the basal medium was 7.0. The medium was autoclaved at 121 ºC for 20 minutes. Cultivation of organism was carried out in 250 ml Erlenmeyer flasks containing 50 ml of basal medium. Flasks were inoculated with 2 % (v/v) of inoculum and incubated at 60 °C on a rotary shaker at 200 rpm. After fermentation, cells were removed by centrifugation at 7000 x g for 10 minutes and clear supernatant was used for the determination of extracellular enzyme activity.

Time course and distribution of α-galactosidase activity :

Time course of α-galactosidase production was studied in a basal medium containing 2 % (w/v) water extract of defatted soybean meal as a carbon source and 0.3 % (w/v) yeast extract as a nitrogen source at 60 ºC under submerged fermentation conditions. Samples were withdrawn at regular time intervals till 24 hours and checked for growth, α-galactosidase activity and pH. To determine distribution of α-galactosidase activity inside and out side the cell during the various stages of growth, 25 ml of culture broth was centrifuged at 7000 x g for 10 minutes and cell free culture broth was collected as extracellular enzyme. The centrifuged cells were washed and resuspended in the same volume of 50 mM phosphate buffer, pH 7.0. The same cell suspension was then sonicated using Ultrasonic Processor at 10 Kilocycle^{-s} for 3 minutes. After sonication cell debris were removed by centrifugation at 12000 x g for 20 minutes

and clear supernatant was used as a source of intracellular enzyme, where as cell debris were used for the determination of membrane bound activity.

Optimization of culture conditions for production of α-galactosidase :

Various carbon and nitrogen sources were supplemented in the basal medium to study their effect on production of α-galactosidase. Similarly the bacterium was also grown at different initial pHs (5-8) at 60 °C and enzyme production was monitored up to 24 hours. To check effect of temperature on production of enzyme, fermentation was carried out at 40, 50, 60 and 65 °C. Effect of metal ions on enzyme production was also determined by adding 1 mM metal salts to the fermentation medium and checked for growth and extracellular α-galactosidase production.

Characterization of crude α-galactosidase :

Effect of pH on enzyme activity was determined by standard assay method, by incubating 100 µl of suitably diluted enzyme in different buffers, (pH 3-10) at 65 °C for 10 minutes. The buffers used were 100 mM of Citrate phosphate buffer (pH 3.0-6.0), potassium phosphate buffer (pH 6.5-7.5), barbitone buffer (pH 8.0-9.0) and glycine NaOH buffer (pH 9.0-10.0). The pH stability was also determined by incubating 100 µl of the enzyme in above buffers at room temperature for 12 hours. Subsequently the residual activity was determined under standard assay conditions. Optimum temperature was determined by standard assay method at different temperatures (50-75 °C) for 10 minutes. Temperature stability was also investigated by incubating 100 µl of the crude enzyme at different temperatures for 2 hours, and residual activity was determined by standard assay method. Effect of metal ions on α-galactosidase activity was determined by incubating enzyme in presence of 1 mM metal salts at room temperature for 10 minutes, followed by α-galactosidase activity. The kinetic parameters were determined under standard assay conditions by using substrate concentrations in the range of 0.066-0.6 mM for synthetic substrates and 0.5-5.0 mM for natural substrates. The K_m and V_{max} values were determined by Lineweaver Burk double reciprocal plot [375].

2.4 Results :

Identification of the Isolate :

The isolate NCIM 5146 is Gram positive, elongated motile rods (0.6-1.0 x 2.0-5.0 µm), has an optimum growth temperature at 60-65 ºC and does not grow above 70 ºC. Glucose metabolism was facultative with acid but no gas produced under anaerobic conditions. Acid is also produced from galactose, maltose, sucrose and melibiose but not from xylose and arabinose, Isolate was catalase positive, degrades starch. Citrate was not utilized and there was no formation of indole and urease was observed. Casein was not hydrolyzed and nitrate was not reduced to nitrite. Spores were observed, their cellular

location was terminal (Figure 1, C). From these properties isolate was tentatively identified as strain of *Bacillus stearothermophilus*. The strain is deposited in the National Collection of Industrial Microorganisms, Division of Biochemical Sciences, National Chemical Laboratory, Pune, India, as a strain of *B. stearothermophilus* with accession no. NCIM 5146.

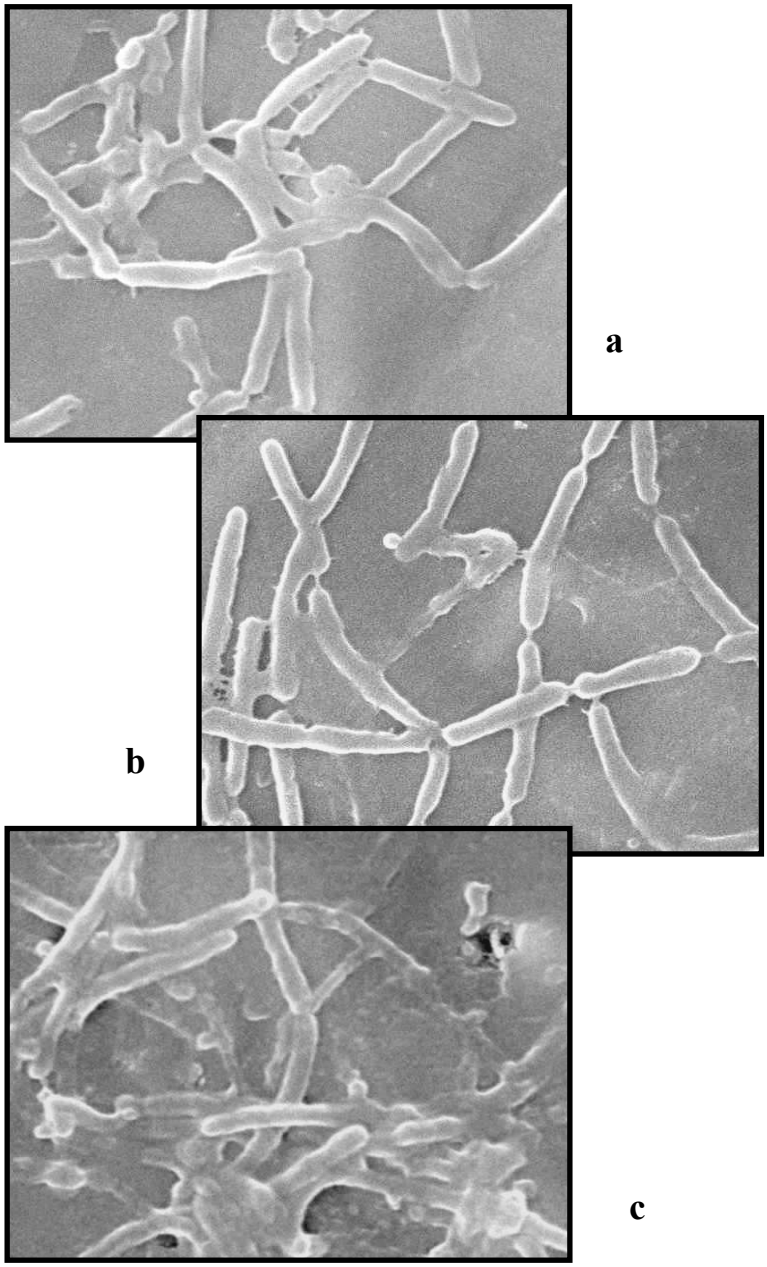


Figure 1. Scanning electron microscopy of strain of *B. stearothermophilus* (NCIM 5146) at magnification of 10 Kx. (**a**) Lag phase culture (**b**) Log phase culture (**c**) stationary phase culture.

Optimum conditions for growth and enzyme production :

Preliminary experiments revealed that *B. stearothermophilus* (NCIM 5146) grows and produces α-galactosidase in a medium containing 2.0 % water extract of defatted soybean meal as a carbon source and 0.3 % yeast extract as a nitrogen source at pH 6.5-7.0 and at temperature 60 °C, under submerged fermentation. Additionally, it was also observed that potassium salts (K_2HPO_4 0.3 % and KH_2PO_4 0.1 %) were required in the fermentation medium to maintain the pH. Hence above culture conditions were used for the further study. Under these culture conditions other activities; xylanase, protease and invertase were not detected. However amylase (2.0 U/ml) and α-glucosidase (0.345 U/ml) activities were detected in the cell free broth.

Time course of α-galactosidase production :

Time course of α-galactosidase production using basal medium in submerged fermentation is shown in Figure 2. Organism grows rapidly during the initial hours of cultivation. The maximum biomass was observed at 10-12 hours of fermentation after which cell mass declined slowly. The α-galactosidase activity appeared in the culture broth after 4 hours of cultivation and increased rapidly up to 16 hours, for the maximum of 1.08 U/ml.

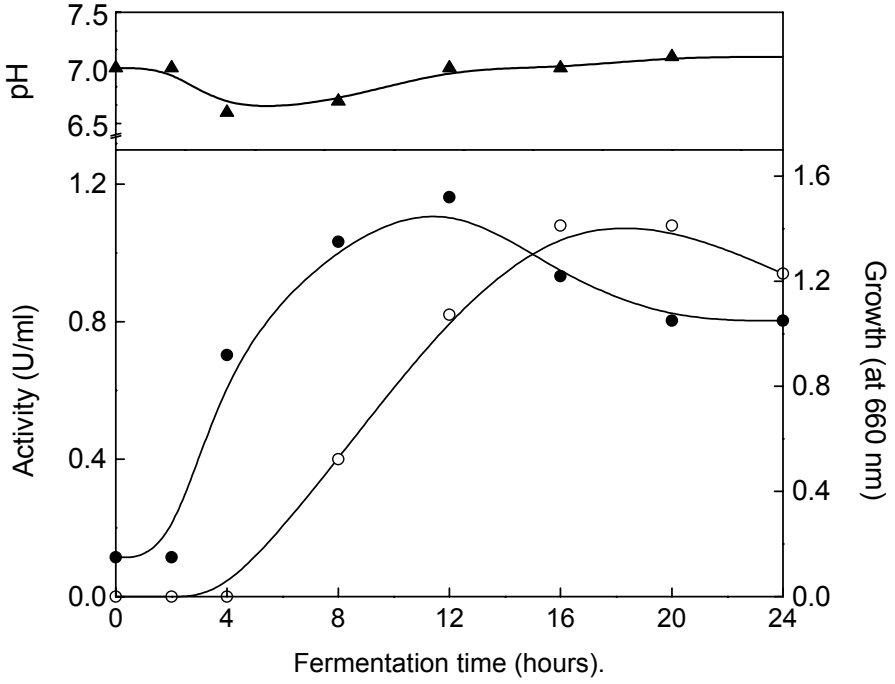


Figure 2. Time course of a-galactosidase production: *B. stearothermophilus* (NCIM 5146) was grown in a basal medium and under submerged fermentation conditions as described in methods. Symbols; (●) growth, (o) activity, (▲) pH.

Distribution of α-galactosidase activity during growth :

To check whether the α-galactosidase activity is truly extracellular or intracellular, distribution of α-galactosidase inside and out side the cell was determined during the fermentation (Table 1). Initially α-galactosidase activity was found inside the cell (cell homogenate), which was further secreted in the fermentation medium during the late exponential to stationary phase of the growth. Maximum activity (86%) was found in the cell free medium after 16 hours of fermentation. Moreover ratio of extracellular to intracellular activity at this stage was quite high (6.66) indicating that secretion of α-galactosidase occurs at high rate.

Table 1. Distribution of α-galactosidase activity in *B. stearothermophilus* (NCIM-5146) :

Hours of fermentation	*A* (660 nm)	α-Galactosidase activity U/ml.[a]		Total Activity [b]	Activity in supernatant (%)
		Cell free medium	Cell homogenate		
6	1.13	0.212	0.478	0.69	17.0
8	1.35	0.40	0.564	0.964	32.20
12	1.52	0.82	0.337	1.157	66.00
16	1.22	1.08	0.162	1.242	86.95
24	1.04	0.94	0.089	1.029	75.68

[a] Fraction of membrane bound activity was very less and not mentioned here. [b-] Maximum total activity (in a cell free medium and cell homogenate) was considered as 100 %.

Effect of pH, temperature and metal ions on α-galactosidase production :

Influence of initial pH of the medium on growth and enzyme production is shown in Figure 3. Growth and α-galactosidase activity was observed at neutral pH. The

optimum growth and enzyme secretion was observed in the pH range 6.5-7.5. No growth and enzyme secretion was detected in acidic medium.

Figure 4 shows that the incubation temperature significantly affected the production of α-galactosidase. The highest activity (1.08 U/ml) was achieved by growing NCIM 5146 at 60 ºC for 16 hours. Longer fermentation time was required to attain maximal activity when incubation was carried out at 40 & 50 ºC. The profile also shows that further increase in temperature above 60 ºC concomitantly decreases production of α-galactosidase although biomass was not decreased significantly.

Effect of various metal ions on bacterial growth and α-galactosidase production is shown in Table 2. Growth and enzyme production was affected considerably by presence of Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{+} and Fe^{2+} metal ions, whereas partial inhibition of growth and enzyme production was observed when Ni^{2+} and Ca^{2+} were present in the medium.

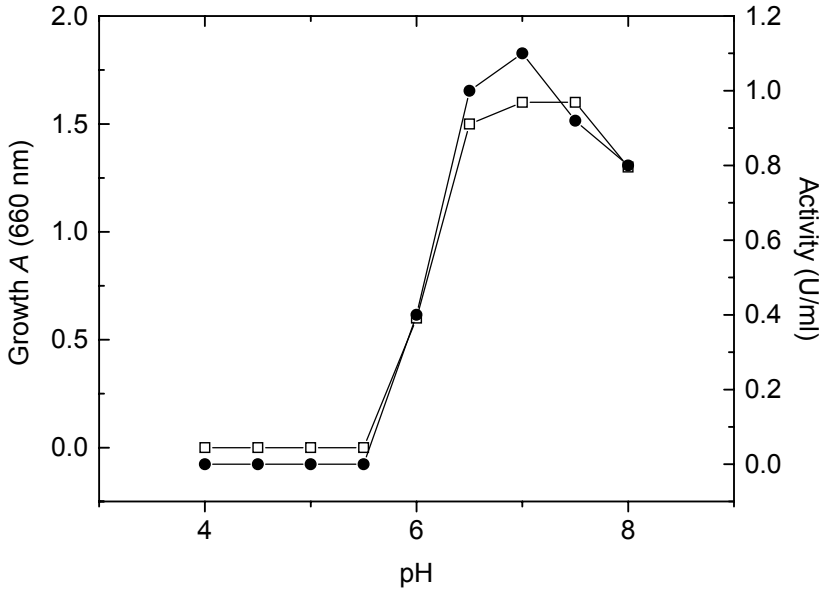


Figure 3 . Effect of pH on growth and α-galactosidase production: Bacterial growth (□) and α-galactosidase secretion (●) was monitored up to 24 hours at various pH as described in methods.

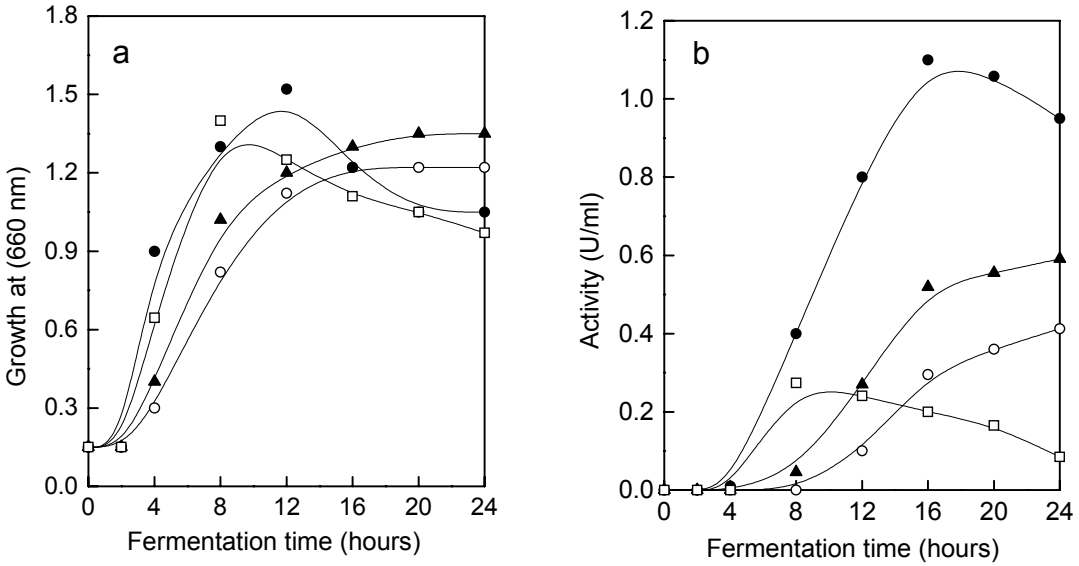


Figure 4. Effect of temperature on growth (**a**) and α-galactosidase production (**b**): Bacterial growth and α-galactosidase secretion was monitored up to 24 hours at temperatures 40 (○), 50 (▲), 60 (●) and 65 °C (□) as described in methods.

Table 2. Effect of various metal ions on growth and α-galactosidase production :

Metal ions (1 Mm)	Relative growth (%)	Relative activity (%)
None	100	100
Mn^{2+}	100	100
Mg^{2+}	100	100
Ca^{2+}	87	80
Co^{2+}	20	5
Zn^{2+}	13	0
Fe^{2+}	26	0
Cu^{2+}	0	0
Hg^{+}	0	0
Ni^{2+}	75	70

Effect of various carbon sources on α-galactosidase production :

The effect of various carbon sources on α-galactosidase production is summarized in Table 3. Enzyme production was maximum when melibiose and galactose (0.2-0.4 % w/v) were used as a carbon source. Raffinose also gave substantial α-galactosidase activity. All other monosaccharides and disaccharides used had a very little effect on α-galactosidase production, whereas supplementation of glucose in the medium inhibited the α-galactosidase production.

The effect of various cheaper agricultural residues on α-galactosidase production was also studied and is shown in Table 4. Among these, soy flour (2.0 % w/v) gave maximum activity (1.1U/ml) whereas cotton seed cake, red bean flour and mustard cake had a moderate effect on α-galactosidase production. Use of high concentration of soya flour (above 2.0 % w/v) gradually decreases the α-galactosidase production.

Table 3. Effect of various carbon sources on α-galactosidase production. :

Carbon sources[a]	*A* 660 nm	Activity U/ml[b]
Glucose	0.854	0.05
Galactose	1.10	0.90
Lactose	0.469	0.095
Maltose	0.808	0.06
Sucrose	0.50	0.05
Raffinose	0.992	0.60
Melibiose	0.897	1.00
Xylose	0.677	0.075
Galactose +glucose[c]	1.29	0.25
Raffinose + galactose[c]	1.3	0.95
Melibiose + galactose[c]	1.649	0.80

[a] Soybean meal in the basal medium was replaced with 0.3 % w/v of the carbon sources. [b]Activity was checked after 24 hours of fermentation. [c] 0.3 % (w/v) each was added in the medium.

Table 4. Effect of various agricultural residues on α-galactosidase production :

Agricultural residues[a]	Final pH	α-Galactosidase activity (U/ml)
Soy flour		
1.0 %	7.20	0.70
2.0 %	7.30	1.10
3.0 %	7.35	1.00
Wheat bran	7.10	0.45
Rice bran	7.03	0.10
Coconut cake	6.00	0.09
Mustard cake	6.83	0.60
Cotton seed cake	7.03	0.60
Red bean flour	7.20	0.70
Chana bran	6.93	0.30
Guar gum*	6.92	0.07
Locust bean gum*	7.55	0.20

[a] Water extract of soybean meal in the basal medium was replaced with 2.0 % (w/v) water extract of all the agricultural residues. When 3 %. (w/v) soybean flour was used, fermentation was monitored up to 48 hours, other wise it was terminated after 24 hours. * 0.2 % (w/v) was used in the basal medium.

Effect of various nitrogen sources on α-galactosidase production :

Effect of various nitrogen sources on α-galactosidase production is shown in Table 5. Organic nitrogen sources such as yeast extract, casamino acids and corn steep liquor supported good growth and enzyme production. 0.3 % (w/v) yeast extract was the best nitrogen source for growth and α-galactosidase production. Increasing the level of organic nitrogen sources in the fermentation medium above 0.3 % did not significantly influence the of α-galactosidase production, although biomass was found to be greatly increased. Among the inorganic nitrogen sources, 0.3 % ammonium sulfate and ammonium nitrate supported moderate growth and enzyme production. However when these inorganic nitrogen sources were used along with yeast extract a 100 % increase in α-galactosidase production was observed (Table 5) .

Table 5. Effect of various organic and inorganic nitrogen sources on α-galactosidase production :

Nitrogen Sources[a]	*A* 660 nm	α-Galactosidase activity (U/ml)
Yeast extract		
0.1 %	0.805	0.75
0.3 %	1.52	1.05
0.5 %	2.20	1.1
Peptone	1.02	0.45
Tryptone	1.10	0.40
Beef extract	1.35	0.20
Malt extract	1.45	ND
Casamino acid	1.40	0.85
CSL	1.42	0.90
Ammonium sulfate		
0.1 %	0.687	0.45
0.3 %	0.966	0.70
0.5 %	0.780	0.60
Ammonium nitrate (0.3 %)	0.915	0.65
Ammonium sulfate +Yeast extract[b]	2.10	2.0
Ammonium nitrate +Yeast extract[b]	2.0	1.95
Control	0.515	0.048

[a] Yeast extract in the basal medium was replaced with 0.3 % (w/v) of nitrogen sources. If necessary pH of the medium was adjusted with 0.1N HCl or 0.1N NaOH. ND -Activity not detected. Control – No N_2 source was added . [b] 0.3 % (w/v) each was added in the medium.

Characterization of crude α-galactosidase :

The relative activity and stability of α-galactosidase at different pH values are shown in Figure 5. The optimum pH of the α-galactosidase was 6.5-7.0 and it was stable over a pH range 3–10. Enzyme exhibited 70 % of residual activity at pH 3.0 and 90 % at pH 10.

The effect of temperature on α-galactosidase activity is shown in Figure 6. The enzyme was most active at 65 ºC and exhibited 96 % of activity at 70 ºC. The

thermostability of crude α-galactosidase is shown in Figure 7. The enzyme was thermostable with half-life of 80 minutes at 70 ºC and retained more than 80 % activity after 120 minutes of incubation at 65 ºC. Enzyme activity decreased rapidly at 75 ºC.

Effect of various metal ions and sugars on α-galactosidase activity is shown in Table 6. α-Galactosidase was strongly inhibited by Ag^{+}, Hg^{2+} and Cu^{2+} metal salts at 1mM concentration, while EDTA did not affect the α-galactosidase activity. When tested for the effect of different sugars, only partial inhibition of enzyme activity was observed with lactose and galactose (Table 6).

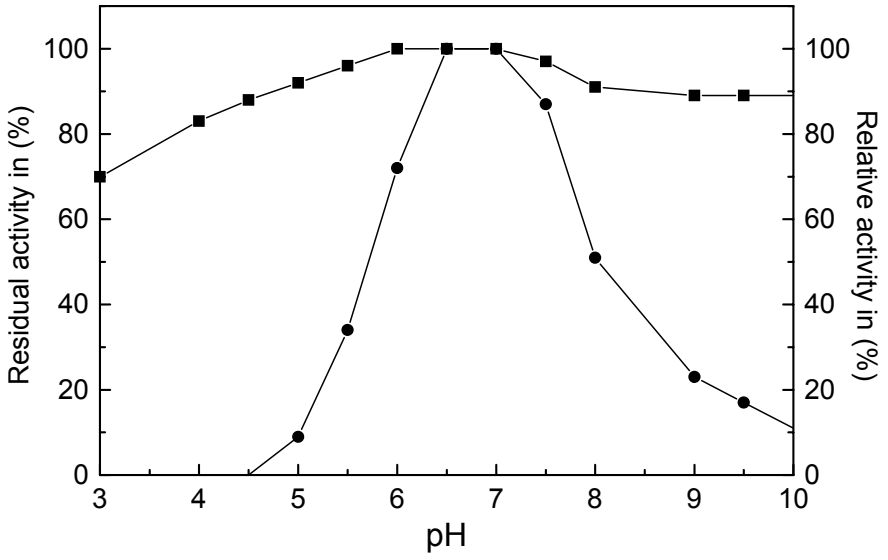


Figure 5. Effect of pH on α-galactosidase activity (●) and stability (■): Enzyme activity and stability was determined at various pH's as described in methods.

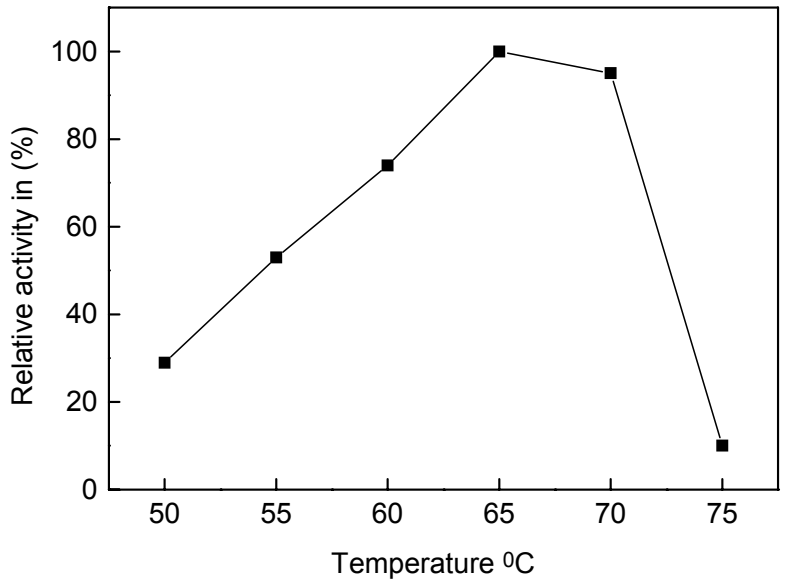


Figure 6. Effect of temperature on α-galactosidase activity: Enzyme activity was determined at various temperatures as described in methods.

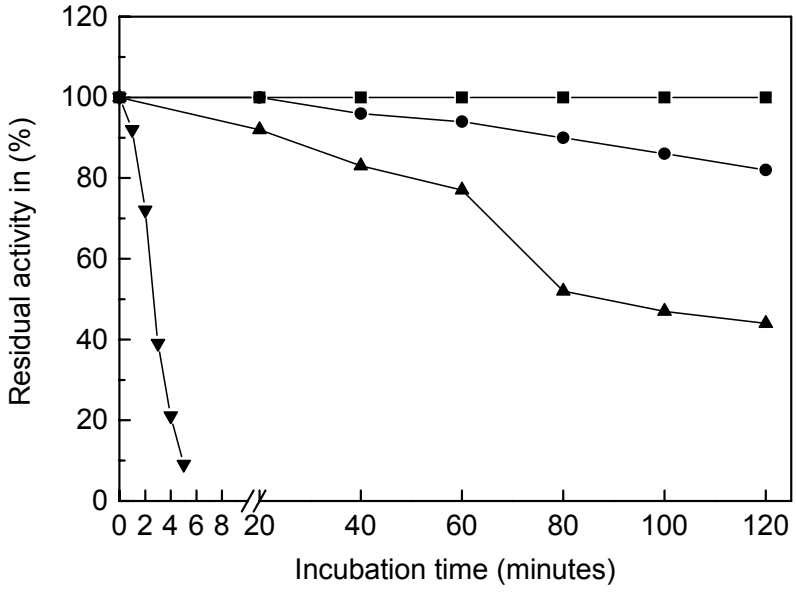


Figure 7. Effect of temperature on stability of α-galactosidase: Enzyme solutions were incubated at temperatures 60 (■), 65 (●), 70 (▲) and 75 °C (▼) for varying time period and residual activity was determined as described in methods.

Table 6. Effect of various metal ions and sugars on α-galactosidase activity :

Metal ions[a]	Residual activity (%)	Sugars[b]	Residual activity (%)
None	100	Glucose	100
Ag^{+}	0	Galactose	90
Ca^{2+}	97	Lactose	85
Cu^{2+}	0	Sucrose	105
Hg^{2+}	0	Fructose	100
Mg^{2+}	100	Mannose	100
Mn^{2+}	100	Arabinose	100
Ni^{2+}	96	xylose	100
Zn^{2+}	95		
EDTA[b]	100		

[a] Concentration used was 1mM. [b] Concentration used was 20 mM.

Substrate specificity of crude α-galactosidase :

Kinetic constants (K_m and V_{max}) for *p*NPG, melibiose, raffinose and stachyose were determined and values are given in Table 7. Synthetic substrate *p*NPG was more preferentially hydrolyzed. Among natural substrates, tetrasaccharide; stachyose was preferred to trisaccharide; raffinose. Whereas enzyme had low affinity (K_m 13.33 mM) but high activity (V_{max} 6.3 x 10^3) towards disaccharide; melibiose. Polysaccharides; guar gum and locust bean gum were hydrolyzed very slowly and therefore affinity and activity on that substrates was not calculated. Yeast mannan was not attacked.

Table 7. Substrate specificity of α- galactosidase :

Substrates	V_{max} (μMoles/min/mg)	K_m (mM)
p-Nitrophenyl-α-D-	4.3×10^7	0.625
galactopyranoside.	6.3×10^3	13.33
Melibiose	12.65	6.66
Raffinose	18.03	3.33
Stachyose	Hydrolyzed	__
Guar gum*	Hydrolyzed	__
Locust bean gum*	Not hydrolyzed	__
Yeast mannan*		

All the substrates were prepared in 50 mM phosphate buffer pH 7.0 and the kinetic parameters were determined under standard assay conditions by using concentrations in the range of 0.05-0.5 mM for synthetic substrate and 0.5-5.0 mM for natural substrates. * 0.2 % (w/v) solution of the substrates (1ml) prepared in phosphate buffer (pH 7.0) was used in the assay mixture and incubated for 30 minutes in presence of 1 IU of α-galactosidases activity.

2.5 Discussion :

The phenotypic properties of a new isolate; (NCIM 5146) are identical with the reported strain of *B. stearothermophilus* KVE 39 [123] and NRRL-B-5407 [167], thus the isolate was identified as a strain of *B. stearothermophilus.*

Growth profile of a isolate exhibited typical growth pattern, produces significant amount of thermostable α-galactosidase activity in the cell free medium, with no detectable invertase and protease activity. Generally bacterial α-galactosidases are located intracellularly, when grown in submerged cultivation [107,104,111], nevertheless, few extracellular enzymes are also reported from bacteria but activity produced by them is comparatively less [100,115]. Most of the fungal α-galactosidases are extracellular in nature [91,92], however, they requires longer fermentation period, produces strong invertase activity along with α-galactosidase activity, their enzymes are thermolabile and active at acidic pH and thus could not meet the desired properties for industrial use [177,179,376].

Biosynthesis of α-galactosidase was induced in presence of sugars viz. raffinose, melibiose and galactose as found in many reports [173,178] while lactose and other sugars had a very little influence on enzyme production, suggesting the specificity for α-galactosides. Glucose inhibition of α-galactosidase production might be due to catabolite repression [99]. When cheap agricultural residues were used, 2.0 % (w/v) soybean flour gave highest level of enzyme activity possibly due to high level of galacto-oligosaccharides. Mustard cake, cotton seed cake and red bean flour also gave relatively high yield; 50-70 % of the yield with soybean flour. Few bacterial α-galactosidases have been produced by using soybean meal and other cheap agricultural residues [173]. However production of α-galactosidase by fungi on waste agricultural residues is a common practice [171,181].

Studies on the effect of nitrogen sources revealed that combination of organic and inorganic nitrogen sources gives higher α-galactosidase activity, since increasing the level of organic nitrogen sources in the fermentation medium above 0.3 % did not influence the production of α-galactosidase, though biomass was found to be greatly increased.

The α-galactosidase activity (2.0 U/ml) produced by *B. stearothermophilus* (NCIM 5146) on agriculture residues (soya flour/ soybean meal) is quite higher (100 fold) than the extracellular α-galactosidase reported earlier from *Bacillus stearothermophilus* (0.02 U/ml) by Talbot [100] and intracellular α-galactosidase from the same genus, (0.276 U/ml) reported by Kocabas [205], (0.427 U/ml) by Delente [99] and (0.09 U/ml) and by Pederson [212]. However, for higher productivity cloning and expression of α-galactosidase gene from *Bacillus stearothermophilus* has been reported recently by Olafur Fridjonsson [198] and Christiane Ganter [123], who claimed production of 52 U/mg and 18.2 U/mg of α-galactosidase activity, respectively by recombinant *E coli* cells.

The pH optimum of the NCIM 5146 α-galactosidase lies in the pH range 6.5-7.0 and is stable over a broad pH range, 3-10. Generally bacterial α-galactosidases have pH optimum in the neutral to alkaline range (6.0-7.5), where as that of the fungal and yeast α-galactosidases has pH optima in the acidic range (3-5) [235]. The acidic form of α-galactosidases are not suitable for use in food processing industries and other biotechnological industries. For example, natural pH of the soymilk is in the range of 6.2-6.4 and lowering of this pH to the acidic side causes precipitation of proteins and leaves a sour taste to the milk. Similarly adjusting the pH of beet molasses to the acid side, in order to facilitate the functioning of acidic enzymes, causes inversion of sucrose and hence less productivity [377].

The α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) is fairly thermostable with half-life ($t_{1/2}$) of inactivation is 80 minutes at 70 ºC, which is higher

than other microbial sources [92,109,197]. High temperature (usually 65-70 ºC) is used in many biotechnological processes, such as in pasteurization of soybean milk, in sugar refining process, and in animal feed pelleting process, requires thermostable enzyme preparations, thus the present enzyme offers considerable advantage over the thermolabile counterpart. To date only α-galactosidase of hyperthermophilic bacteria *Thermotoga maritima* ($t_{1/2}$ 6 h at 85 ºC) [184], *Thermotoga neapolitana* ($t_{1/2}$ 9 h at 85 ºC) and (4 h at 85 ºC, retains 75 % activity) [115,200] and *Thermus brockianus* ($t_{1/2}$ 17 h 80 ºC) [132] have demonstrated activity and prolonged stability above 75 ºC. More over a α-galactosidase reported from the *Bacillus stearothermophilus* has temperature stability at 70 ºC ($t_{1/2}$ 19 h at 70 ºC) [198]. However the potential applications of these enzyme preparations in high temperature processes were either not demonstrated or briefly demonstrated [123,200].

Kinetic study exhibited that *p*NPG was the most susceptible substrate, while melibiose > stachyose > and raffinose preferred to hydrolysis in this order. Stachyose was found to have more affinity than raffinose. The K_m values determined for raffinose and stachyose (6.66, and 3.33 mM) were lower than those reported earlier for thermostable α-galactosidase from *Bacillus stearothermophilus* (raffinose, 16.4 mM) [198] and *Thermomyces lanuginosus* (raffinose 11.3 mM) [94]. The V_{max} values obtained for *p*NPG and melibiose (4.3 x10^7 and 6.3 ×10^3 U/mg/min) were quite higher, indicating a higher turn over number for these substrates.

No divalent cation tested stimulated enzyme activity nor was inhibited by EDTA [198]. However enzyme gets strongly inhibited by Ag^+, Hg^{2+} and Cu^{2+} metal ions [236]. No significant inhibition was observed by sugars. Only partial inhibition was found with galactose and lactose. This is an added advantage of α-galactosidase in its practical application, since product inhibition is common in α-galactosidases [212,237].

Thus the α-galactosidase from *Bacillus stearothermophilus (*NCIM 5146) is unique because it is extracellular in nature, exhibits high thermostability and high substrate specificity towards natural substrates; raffinose, stachyose and melibiose. Moreover the enzyme preparation is optimally active at neutral pH and contains no detectable protease as well as invertase activity. It displays minimal product inhibition by galactose, hence this enzyme has a great potential in the soymilk and beet sugar industry and related food industry.

CHAPTER-3

Purification and Characterization of Thermostable α-Galactosidase from *Bacillus stearothermophilus* (NCIM-5146).

3.1 Summary :

An extracellular thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM-5146) has been purified to homogeneity by single chromatographic step, using Phenyl Sepharose CL4B. The molecular mass of the enzyme as determined by SDS-PAGE and gel filtration column were 79.9 and 165.9 kDa, respectively, suggested that enzyme consists of two identical subunits. The purified α-galactosidase is a non-glycosylated protein with a pI value of 4.9. The enzyme is rich in acidic and hydrophobic amino acids and partial N-terminal sequence exhibited remarkable homology with the earlier reported α-galactosidase from *Bacillus stearothermophilus* (NUB 3621) of family 36 of glycosyl hydrolases. The secondary structure of the enzyme determined by CD spectroscopy and analyzed by prediction method, exhibited α/β class of protein. The pH and temperature optima for the purified enzyme are 6.5-7.0 and 65 ºC, respectively. The α-galactosidase was stable over a broad pH range (3-9) and its half-life of inactivation ($t_{1/2}$) at 70 ºC was 30 minutes. The enzyme exhibited strict anomer specificity towards the substrates and hydrolyzes only the α-galactosidic linkages and not the β-galactosidic linkages. The relative substrate specificity of α-galactosidase towards the various synthetic and natural galactosides is in the order of *p*NPG > *o*NPG > melibiose > stachyose > raffinose > methyl-α-D-galactopyranoside > locust bean gum and guar gum. The enzyme also hydrolyzes α-1-3 and α-1-4 galactosidic linkages from disaccharide sugars. The kinetics of hydrolysis of number of natural and synthetic substrates showed that *p*NPG and *o*NPG has lower K_m (higher affinity) and higher K_{cat} (higher catalytic efficiency). However natural substrates, melibiose, raffinose, and stachyose showed higher K_m (lower affinity) and lower K_{cat} (lower catalytic efficiency). Among the natural substrates, a disaccharide, melibiose (K_m = 5.0 mM) was hydrolyzed at higher rate than the trisaccharide, raffinose (K_m = 5.0 mM), although, both the substrates has similar K_m (similar affinity). However by contrast, although, trisaccharide, raffinose and tetrasaccharide, stachyose (K_m = 2.5 mM) showed similar K_{cat} (similar rate of hydrolysis), both the substrates has different K_m values (different affinities). Thermodynamic parameters calculated from the temperature dependence studies of binding and hydrolysis of different glycosides indicated that binding might be enthalpy as well as entropy driven process. Hence, it suggests that hydrophobic interactions as well as hydrogen bonds play

a predominant role in substrate binding. The high catalytic efficiency of *p*NPG and melibiose is due to the highest transition state stabilization and ground state destabilization, suggested that these substrates fit properly in the active site cleft. By contrast high entropy loss in binding and activation of raffinose and stachyose indicated strained substrates and hence hydrolyzes at lower rate due to lower transition state stabilization and ground state destabilization. Purified α-galactosidase showed biphasic Arrhenius plots with break point at 55 ºC for *p*NPG and 50 ºC for melibiose, raffinose and stachyose, suggested temperature dependent conformational states. Besides the hydrolytic activity, present α-galactosidase also exhibited weak transferase activity and produces two new sugars, tentatively identified as melibiose and stachyose. The enzyme was completely inhibited by Ag^{+}, Hg^{2+} and Cu^{2+} metal ions, nature of inhibition was non competitive. Galactose, melibiose (K_i = 16.25), stachyose (K_i = 33.0) inhibited α-galactosidase activity competitively, whereas lactose (K_i = 14.5) inhibition was uncompetitive.

3.2 Introduction :

α-Galactosidase (E.C.3.2.1.22) is an exo-glycosidase that mainly cleaves terminal α-1-6-linked D-galactosyl residues from galactose containing oligo-saccharides, polysaccharides and glycoconjugates [73,210,327]. α-Galactosidases have been isolated and purified from a variety of sources, including plants [40,41,139], animals [62,67] and microorganisms [82,89,104,227]. However only the plant and fungal α-galactosidases have been well studied with respect to their substrate specificity and have been classified into two groups [265]. The first group contains α-galactosidase active only on oligo-saccharides with low degree of polymerization (DP 2-5) for example, melibiose, raffinose, stachyose, verbascose and short fragments of galacto (gluco) mannans. These enzymes are usually very active on artificial substrates like aryl α-D-galactosides. The second group of α-galactosidases consists of enzymes active on polymeric substrates, like galactomannans. However similar to enzymes of first group, they attack short oligo-saccharides as well as artificial α-galactosides.

Besides these hydrolytic functions some of the α-galactosidases are also known to catalyze transgalactosylation reactions especially at a high concentration of substrate [274].

The use of these enzymes, which are specific in their action, is becoming more popular as tools, for structural investigations as well as degradation and synthesis of complex carbohydrate molecules [356,357].

Genes encoding α-galactosidase have also been cloned and sequenced from various prokaryotic and eukaryotic sources [114,200,280,281,287,288]. The known

eukaryotic and prokaryotic enzymes display a significant degree of amino acid sequence homology with their group members and have been placed in family 27 and 36, respectively in the classification of glycoside hydrolases [27,28]. However *E. coli* melibiase (gene *melA*) [282] represents the third family of α-galactosidases, family 4. The *E. coli* melibiase [251] requires NAD^+ and Manganese ions as cofactors and is structurally related to neither family 27 nor 36.

It is now well established that proteins purified from thermophilic and hyperthermophilic microorganisms are stable and functional at very high temperatures (up to 100 ºC) [359]. Some of the α-galactosidases from thermophilic and hyperthermophilic microorganisms have also been purified and studied as potential enzymes for hydrolyzing different substrates [94,115,116,132,200]. Moreover genes of these microorganisms have also been cloned and recombinant enzymes have been purified and characterized with respect to their required properties for industrial applications. However, very little information is available on the enzyme structure and catalytic mechanism as well as on the mechanism of heat resistance in α-galactosidases from thermophilic and hyperthermophilic microbial sources [303,378,379].

In order to study structure function relationship of enzyme, it is necessary to obtain it in a pure form and in large quantity through simple and efficient method of purification. In the present study, we have described a single chromatographic step purification of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146). The purified enzyme has been extensively characterized for its biochemical and molecular properties and also for its substrate specificity towards the oligomeric and polymeric substrates.

3.3 Materials and methods :

Materials :

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG), *o*NP-α-D-galactopyranoside, *m*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyrano side, *p*NP-α-D-mannopyranoside, *p*NP-N-acetyl-α-D-glucosaminide *p*NP-α-D-fucopyranoside *p*NP-α-L-arabinopyranoside, *p*NP-N-acetyl-α-D-galactosaminide, 3-O-α-D-galacto-pyranosyl-D-galactobiose, 4-O-α-D-galacopyranosyl-D-galactopyranose, Raffinose, Stachyose, Melibiose, Glucose, Mannose, Cellobiose, SDS and gel filtration markers, Coomassie Brilliant Blue R-250 and Bromophenol Blue were purchased from Sigma Chemical Company, U.S.A. Sephacryl-300, Phenyl Sepharose CL-4B were obtained from Pharmacia, Sweden, Glucose Oxidase Peroxidase (GOD-POD) reagent kit from a local chemical company. All other chemicals used were of analytical grade & of the highest purity available locally.

Methods :

Organism and culture conditions :

Thermophilic bacterium *Bacillus stearothermophilus* (NCIM 5146) is used in the present study. The organism was maintained as described earlier in the Chapter II. For purification of α-galactosidase, isolate was grown in a medium containing 0.2 % galactose as a carbon source, 0.3 % K_2HPO_4, 0.1 % KH_2PO_4 and 0.3 % Yeast extract as a nitrogen source. The initial pH of the medium was 7.0. Fermentation was carried out in 250-ml Erlenmeyer flasks containing 50 ml of medium. Flasks were inoculated with 12-hours old inoculum (2 %, prepared as mentioned in Chapter-II), and incubated at 60 ºC on a rotary shaker at 200 rpm for 24 hours. After fermentation, the biomass was separated by centrifugation (7000 x g, 10 minutes) and clear supernatant was used as source of extracellular enzyme.

α-Galactosidase assay :

α-Galactosidase activity on various synthetic and natural glycosides was determined by the method described earlier in the chapter II. The *p*-nitrophenol liberated following the hydrolysis of synthetic substrates was measured at 405 nm. When raffinose, stachyose, 3-O-α-D-galacopyranosyl-D-galactobiose, 4-O-α-D-galacopyranosyl-D-galactopyranose, methyl-α-D-galactopyranoside, locust and guar gum were used as substrates, the reducing sugar liberated was determined by the method of Somogi and Nelson [124,367]. Glucose released in the hydrolysis of melibiose, estimated by Glucose Oxidase Peroxidase (GOD-POD) reagent [368]. One unit of α-galactosidase activity was defined as the amount of enzyme required to liberate 1μmole of product (*p*-nitrophenol or galactose/glucose) under standard assay conditions.

Hemagglutinin (lectin) activity of purified α-galactosidase was performed in a microtiter plate by making two-fold serial dilutions of purified enzyme samples in phosphate buffer saline. To each well, 4 % trypsinized human erythrocytes (50 μl) were mixed with diluted enzyme samples (50 μl) and incubated at 30 ºC for 30 minutes. Hemagglutination unit (HU) was expressed as the reciprocal of the highest dilution showing detectable hemagglutination [380].

Protein determination :

Protein concentration in the fermentation broth and in the purified enzyme preparation was determined by the method of Lowry et al [374] using BSA as a standard. During the course of purification, protein concentration was determined by absorbance at 280 nm using the relation $1.7A_{280}$ nm = 1 mg/ml of α-galactosidase.

Purification of α-galactosidase :

Cell free culture broth was concentrated 10 times by an Amicon Ultrafiltration unit using YM-30 (Cut-off M_r 30,000 Da) membrane filter. The concentrated culture broth was then fractionated with chilled ethanol. Precipitate obtained in 50-90 % saturated fraction was collected by centrifugation and re-dissolved in a minimum volume of 25 mM phosphate buffer (pH 7.0). The same enzyme sample was then applied to hydrophobic column chromatography using Phenyl Sepharose CL-4B (30 ml bed volume), previously equilibrated with 25 mM phosphate buffer (pH 7.0). The column was washed thoroughly with 20 bed volumes of above buffer. Elution was done with 120 ml linear decreasing gradient of phosphate buffer (25-0 mM) with a flow rate of 20 ml per hour and approximately 3.0 ml fractions were collected. Fractions showing activity were pooled, concentrated by Ultrafiltration and used for further study. Unless otherwise mentioned all the purification procedures were carried out at 4 °C.

Electrophoresis :

Native PAGE (10 %) and SDS-PAGE (12 %) was performed in a vertical gel apparatus at pH 8.8, according to the method described by Laemmli [381]. Samples containing approximately 5-10 μg of protein were applied to the gel and electrophoresed at 200 V for 3-4 hours. Protein bands were visualized either by Coomassie Brilliant Blue R-250 (0.2 % w/v) or by Silver staining (0.4 % w/v) [382].

Activity staining :

α-Galactosidase activity in non-denaturing polyacrylamide gel was visualized by incubating gel at 50 ºC for 15 minutes in 100 mM potassium phosphate buffer, pH 7.0, containing fluorescent substrate, 4-Methyl-Umbeliferyl-α-D-galactopyranoside (MU-α-gal, 2.0 mM). The activity band was visualized as a fluorescent band under UV transilluminator. Corresponding band in other half of the gel was visualized by silver staining [382].

Isoelectric focusing :

The Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) was performed in a tube gel (7.5 %) using wide range Ampholytes (3-10) [383]. Approximately 50 μg of purified protein was applied to the gel and focused at 200-400 Volts for a period of 4-5 hours. Protein band was visualized with Coomassie Brilliant Blue R-250 stain [382]. The p*I* of purified enzyme was also determined by the method of Chinnathambi et al, [384] using a mini scale density gradient isoelectric focusing unit and α-galactosidase band was revealed by *p*NPG as a substrate.

Determination of molecular mass (M_r) :

Gel filtration : The native molecular mass of α-galactosidase was estimated by gel filtration, according to the method described by Andrews [385]. The gel filtration column (Sephacryl, S-300, 1x150 cm), was equilibrated in phosphate buffer (50 mM, pH 7.0) and calibrated using gel filtration standard molecular weight markers**:** Bovine serum albumin (M_r 66 kDa); Alcohol dehydrogenase (M_r 150 kDa); β-amylase (M_r 200 kDa), Apoferetin (M_r 443 kDa) and Thyroglobulin (M_r 669 kDa). The column void volume was determined with Blue dextran (M_r 2000 kDa). About 2 mg (500 µl) of samples were applied to the column, which was operated at a flow rate of 10-ml per hour. In order to achieve optimal calibration, the standards were chromatographed in two batches, the first included Bovine serum albumin and Alcohol dehydrogenase and the second β-amylase, Apoferetin and Thyroglobulin. The calibration graph was plotted as log M_r values versus K_{av} (partition coefficient) values calculated as follows.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad \text{Eq (1)}$$

Where: V_0 - Void volume, V_e - Protein elution volume, V_t – is the column bed volume.

SDS-PAGE :

The subunit molecular mass of the purified α-galactosidase was determined by SDS-PAGE according to Weber and Osborn [386]. For calibration of gel, Sigma high molecular weight standard markers (116.0, 66.2, 45.0, 29.0, 20.0 and 14.0 kDa proteins) were used. After electrophoresis proteins were visualized by staining with Coomassie Brilliant Blue R-250. Relative mobility (R_f) of the denatured proteins on the gel was calculated as per formula given below. 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 R_f values (Relative mobility).

$$\text{Mobility} = \frac{\text{Distance of protein migration} \quad X \quad \text{Length before staining}}{\text{Length after staining} \quad X \quad \text{Distance of dye migration}} \quad \text{Eq (2)}$$

Amino acid analysis and glycoprotein nature :

Amino acid analysis of α-galactosidase was done at commercial services (Commonwealth Biotechnologies, Inc., Richmond, Virginia, USA). For analysis, salt free lyophilized enzyme sample 100 μg was hydrolyzed in gas phase 6N HCl for 20 hours at 110 °C. Following hydrolysis, the sample was taken to dryness, dissolved in 200 μl sample loading buffer (0.4 M sodium borate pH 10.2) and 1 μl of undiluted sample was subjected to analysis on a Hewlett Packard Aminoquant method with Diode array detector. Tryptophan [387] and cysteine [388] contents were determined spectrophotometrically.

Glycoprotein nature was determined by estimating total sugar content of the purified enzyme sample by phenol sulfuric acid method [389] using a solution containing equal amounts of D-galactose and D-mannose as a standard.

Electroblotting and N-terminal sequence :

For N-terminal sequence analysis, the purified enzyme was electrophoresed on 10 % SDS-PAGE at pH 8.8. After electrophoresis, the gel and Polyvinylidenedifluoride (PVDF) membrane were sandwiched between Whatman filter paper strips. The strips and membrane were previously equilibrated with CAPS buffer (10 mM, pH 11.0) containing 10 % methanol. The protein was electrotransfered using a semi dry blotting system (Pharmacia Biotech, Nova Blot) under a constant current of 52 mA for 30 minutes. After transfer, the membrane was removed and washed thoroughly with milli-Q water and protein band was visualized by Coomassie Blue R-250 stain. The N-terminal amino acid sequence of the first 10 residues of the enzyme was determined by Edman degradation on an Applied Biosystems Procise sequencer at National Institute of Immunology, New Delhi, India. Reported sequences of α-galactosidases form various sources were searched from GenBank and SwissProt Databases and a sequence alignment were performed using a Clustal-W sequence alignment program [390].

Circular dichroism (CD) and analysis of secondary structure :

The secondary structure of native α-galactosidase at various temperatures was determined by CD spectroscopy. Spectra were recorded on a Jasco-710 Spectropolarimeter in the far UV region (190 to 250 nm) using a 250-μl-sample holder and 0.1 cm path length cell. The concentration of enzyme used was 125 μg/ml. The temperature of the samples was controlled at 30-75 ºC using a circulating water bath. Results are expressed as molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), based on mean amino acid residue weight (MRW) assuming average weight of 115 Da. The molar ellipticity was determined as follows [391]:

$$[\theta]_{\lambda} = (\theta_{obs} \times MRW / 10 \times d \times c)$$

Where θ_{obs} is observed ellipticity in degrees, MRW is mean residue weight, 10 is constant factor, *d* is path length in centimeter and *c* is the protein concentration in g/ml. The secondary structural components were determined by CONTINLL algorithm [392].

pH optimum and stability :

pH optimum of α-galactosidase was determined by assaying α-galactosidase activity over a pH range 3.0-9.0, using 50 mM citrate phosphate buffer (pH, 3.0-7.0) and potassium phosphate buffer (pH, 6.0-9.0) by standard assay method. The pH stability was also determined in a same pH range by pre-incubating enzyme samples in above buffers at room temperature for the period of 12 hours. Small aliquots (10 µl) were withdrawn from all the samples and residual enzyme activity was determined by standard assay method.

Temperature optimum and stability :

The optimum temperature of α-galactosidase was determined by performing assays at temperatures, 30 to 75 °C. The Temperature stability was also determined by incubating enzyme samples over a same temperature range for the period of one hour. Aliquots (100 µl) were withdrawn after suitable time interval and residual enzyme activity was determined by standard assay method.

Substrate specificity and Kinetic studies :

The relative substrate specificity of α-galactosidase towards various synthetic and natural glycosides was determined. Substrates were prepared in 100 mM phosphate buffer (pH 7.0) at final concentration of 5.85 mM. A suitable quantity of enzyme was incubated with fixed quantity of substrates (200 µl, 5.85 mM) and the reaction was carried out under standard assay conditions. Products released during the first 10 minutes of reaction were assayed and activity (U/ml) was determined. Relative activity on various substrates is expressed as percentage of the activity calculated with *p*-nitrophenyl α-D-galactopyranoside as a substrate (100 %) with which enzyme showed best performance.

The initial rate of hydrolysis of various glycosides was determined by varying the substrate concentrations in the range of 0.066-0.399 mM for *p*NPG and *o*NPG, 0.25-1.5 mM for melibiose, 0.4-2.4, 0.2-1.2 and 0.146-0.730 mM, respectively for raffinose, stachyose and 3-O-α-D-galactobiose. Assays were carried out at 65 °C under standard assay conditions. The kinetic rate constants, K_m and V_{max} were determined graphically from Lineweaver-Burk plotting [375].

Temperature dependence studies :

Rate constants K_m and K_{cat} for *p*NPG, melibiose, raffinose, and stachyose were also determined at different temperatures ranging from 40-65 °C by varying the substrate concentrations as mentioned above. The kinetic constants, K_m and K_{cat} were determined graphically from Lineweaver-Burk plots. Activation energies were calculated by rearranging the Arrhenius rate equation and by fitting the K_{cat} values to the linear regression as follows [393] :

$$K = Ae^{-Ea/RT} \quad (1)$$

Equation 1 is a Arrhenius rate equation, rearranging :

$$\ln(K_{cat}) = (-E_a/R) \times (1/T) + \ln(A) \quad (2)$$

Hence, a plot of ln K_{cat} as a function of 1/T gives a straight line with slope = (- E_a/R). The free energy change of activation ($\Delta G^{\#}$) was determined based on Eyring's absolute rate equation [394] :

$$\Delta G^{\#} = -RT.\ln(K_{cat}.h/K_B T) \quad (3)$$

Where K_B is the Boltzmann constant, [R/N] = 1.38 x 10^{-23} J K^{-1}, where N Avogadro's No. (6.02 x 10^{-23} mol^{-1}), R is gas constant (8.314 J K^{-1} mol^{-1}), *h* is Planck's constant (6.6256 x 10^{-34} Js). The activation enthalpy ($\Delta H^{\#}$) and entropy ($\Delta S^{\#}$) were calculated as follows :

$$\Delta H^{\#} = (E_a - RT) \quad (4)$$

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T \quad (5)$$

Binding energies of above substrates were also determined from van't Hoff equation. The van't Hoff equation is a linear equation relating the equilibrium constant to temperature. Thus for rapid equilibrium systems $K_m = K_d$ (dissociation rate constant). Hence temperature dependence of K_m values were fitted by linear regression to the following equation [393] :

$$d \ln K/dT = \Delta H/RT^2 \quad (6)$$

Equation 6 is a van't Hoff equation, rearranging,

$$-\log K_m = (\Delta H_b/2.303R)(1/T) + (\Delta S_b/2.303R) \quad (7)$$

Where K_m is a Michaelis constant. The slope of the van't Hoff plot is equal to (ΔH_b/2.303R). ΔH_b, and ΔS_b gives changes in binding enthalpy and entropy values, respectively. The free-energy change of binding (ΔG_b) of α-galactosides to the enzyme was calculated using the following equation :

$$\Delta G_b = -2.303\ RT \log K_a \quad (8)$$

Here $1/K_m$ was considered as association rate constant (K_a) of enzyme and substrate. The free energy of transition state binding (ΔG_{tb}) of α-galactosides to the enzyme was calculated using the following equation

$$\Delta G_{tb} = -2.303\ RT \log K_{cat}/K_m \quad (9)$$

Using the ΔG_b and ΔH_b the changes in entropy of binding, ΔS_b, was calculated for each substrate as follows :

$$\Delta G_b = (\Delta H_b - T\Delta S_b) \quad (10)$$

Further, the binding enthalpy values obtained from the slope of the van't Hoff plots were confirmed and corrected by substituting K_m values in the integrated Van't Hoff equation between the limits of *Keq* at higher and lower temperatures, as follows [395] :

$$2.303 \log_{10} K_2/K_1 = \Delta H / R \times (T_2-T_1)/ T_1T_2 \quad (11)$$

where K_2 and K_1 are the equilibrium constants at temperatures T_2 and T_1, respectively.

Transgalactosylation reactions :

Transgalactosylation reactions were carried out in a reaction mixture containing 50 µl of suitably diluted enzyme (1-2 Units), 50 µl of galactosyl donor, 50 µl of acceptor sugar and 50 µl of phosphate buffer (100 mM, pH 7.0). Reactions were carried out at 40-60 ºC for 0.5 to 5 hours. Aliquots were removed at suitable time interval and heated in a boiling water bath for 5 minutes to terminate the reaction. Galactosyl donors used were stachyose, raffinose and melibiose, at final concentrations of 5-100 mM in the reaction mixture. Acceptor sugar concentrations used were 100-500 mM of the monosaccharides; galactose, glucose, fructose and disaccharides; sucrose, cellobiose and lactose. Transfer products were detected by TLC on silica-gel-coated plastic sheets (Merck Co. Ltd.).

TLC analysis :

The hydrolysis and transfer products of galacto-oligosaccharides were analyzed by thin layer chromatography (TLC) (pre coated silica gel plates, Merck). Plates were developed at room temperature in a saturated chamber containing n-propanol: acetic acid: water (1:1:0.1 v/v/v) as a solvent system. After development, the TLC plates were sprayed with 1 % α-naphthol in absolute ethanol containing 10 % of orthophosphoric acid. The plates were then kept in an oven at 140 °C for 5 minutes for visualization of sugar spots [396].

Effect of various metal ions sugars and inhibitor reagents :

A suitably diluted enzyme (100 µl) in 50 mM phosphate buffer, pH 7.0, was pre-incubated with various metal salts, sugars and inhibitor reagents at room temperature for 10 minutes, subsequently residual activity of the samples was determined by standard assay method.

To determine the type of inhibition, kinetic constants K_m and V_{max} of hydrolysis of *p*NPG was determined under standard assay conditions in the absence and presence of fixed concentration of inhibitor. Several sets of such experiments, each with different inhibitor concentration were carried out and reciprocal of initial velocity was plotted against reciprocal of substrate concentrations used according to the Lineweaver-Burk.

Inhibition constants (K_i) for several inhibitors were determined by the Dixion method [397], by varying inhibitor concentration in the reaction mixture and assays were carried out at fixed concentration of substrate (*p*NPG) under standard assay conditions. Two such experiments at two different substrate concentrations (S1 & S2) were carried out and reciprocal of initial velocity was plotted against inhibitor concentrations (I) according to the Dixion.

3.4 Results :

Purification of α-galactosidase :

Activity staining of α-galactosidase on the concentrated culture broth revealed single active band (Figure 1), indicating that there could be single molecular form of α-galactosidase *in B. stearothermophilus* (NCIM 5146).

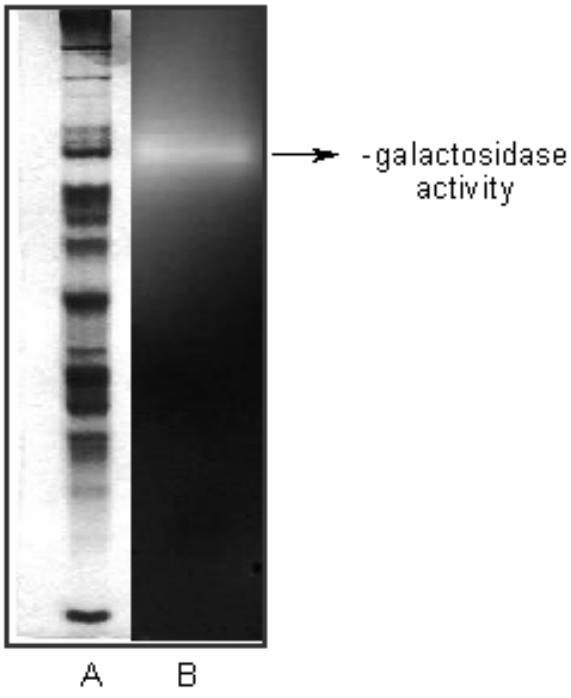


Figure 1. Activity staining of α-galactosidase on polyacrylamide gel: 50 µg of crude enzyme preparation was applied in two separate wells and electrophoresed under non-denaturing conditions as described in methods. After electrophoresis half of the gel was treated with 4-MU-α-galactoside and activity band was visualized as fluorescent band under transilluminator [**B**]. Corresponding band on remaining part of the gel was visualized by silver staining [***A***].

Single chromatographic step purification of α-galactosidase was achieved by hydrophobic chromatography on Phenyl Sepharose CL-4B. The enzyme was purified from a 24-hours old culture supernatant with a specific activity of 1.03 U/mg of protein. The ethanol-fractionated enzyme was introduced in to a Phenyl Sepharose CL-4B column in absence of ammonium sulfate. The elution profile of the enzyme from Phenyl Sepharose is shown in Figure 2. Purification steps of the α-galactosidase are summarized in Table 1 and the electrophoretic pattern of the proteins at each step of purification is

shown in Figure 3. Major activity was eluted in a single peak with a reverse gradient of potassium phosphate buffer 25-0 mM with a peak of activity was typically at 12.5 mM phosphate buffer. A total of 1.8 mg of α-galactosidase (yield 44 %) was obtained with a specific activity of 400 μmoles.min^{-1} mg^{-1} of protein, when measured at pH 7.0 and 65 °C with *p*NP-α-galactoside as a substrate. About 388-fold purification of α-galactosidase was achieved by this method.

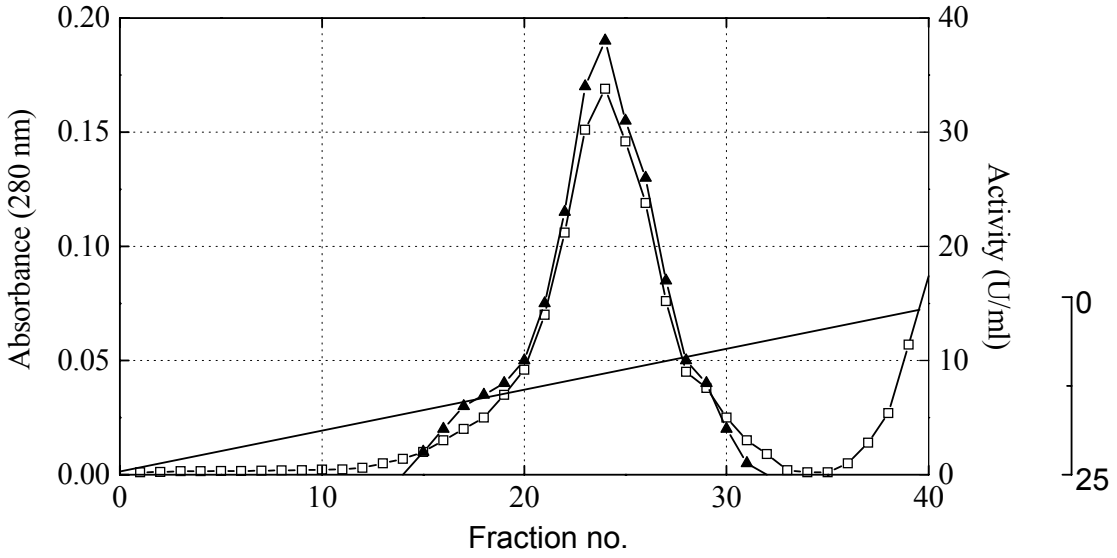


Figure 2. Elution profile of α-galactosidase from Phenyl Sepharose CL 4B column: Protein (--□--), Activity (--▲--), Buffer gradient (____).

Table 1. Summary of steps of purification of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) :

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Culture filtrate	1582	1634	1.03	0	100
Amicon concentration	266.3	1550	5.8	5.6	94.8
Alcohol precipitation	57.25	1085	18.9	18.4	66.5
Phenyl Sepharose CL 4B.	1.8	721	400	388.8	44.1

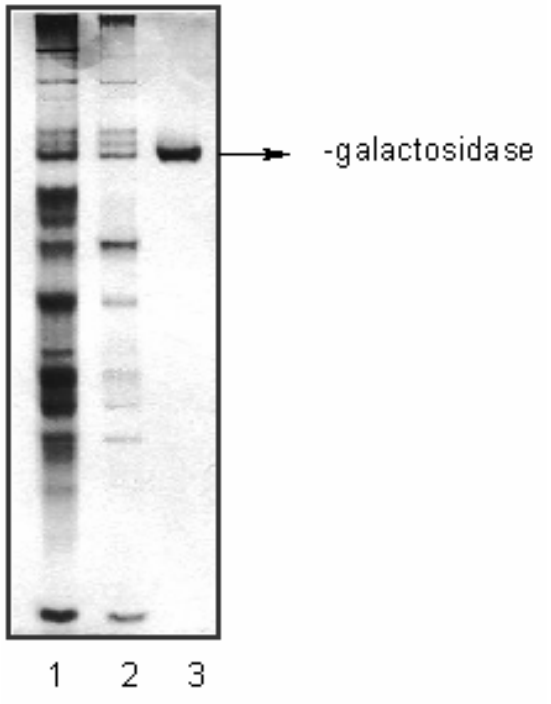


Figure 3. Electrophoretic pattern of the proteins obtained at each step of purification of α-galactosidase: Fractions of α-galactosidase activity obtained after each step of purification were electrophoresed at pH 8.8 under non-denaturing conditions as described in Methods, and gel was stained with silver staining. **Lane1:** Concentrated culture filtrate. **Lane 2:** Alcohol precipitated enzyme. **Lane 3:** Phenyl Sepharose eluted enzyme.

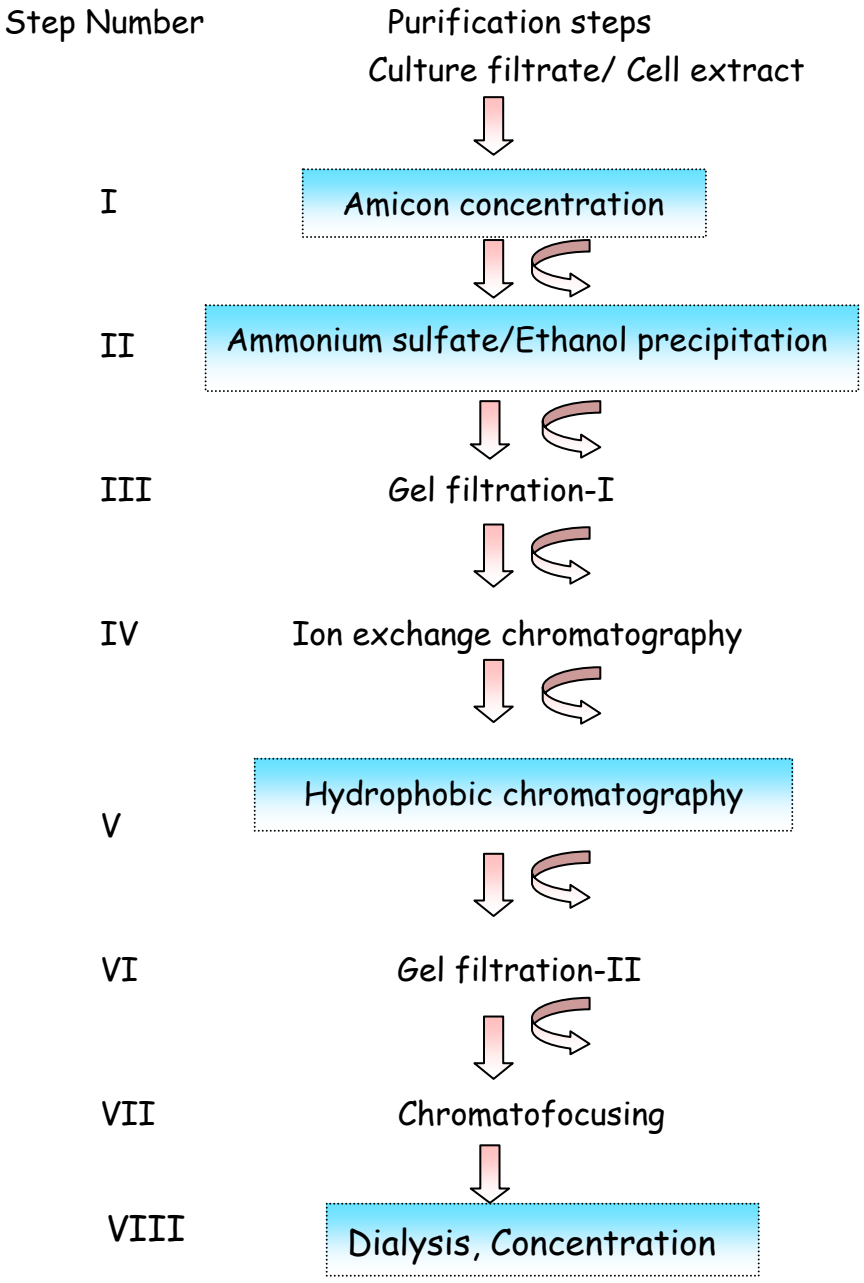


Figure 4. Schematic representation of the comparative analysis of the purification steps used for the purification of a-galactosidase by other investigators [92,100,101,256] with that used in the present study. The four steps used in the present study including single chromatographic step are boxed, while the concentration and dialysis steps eliminated are represented by curved arrows.

Molecular properties of α-galactosidase :

Isoelectric point (pI) :

Single protein band was observed on IEF-PAGE (Figure 5B) indicated that the enzyme is homogenous and contain single molecular form of α-galactosidase. The p*I* of the α-galactosidase estimated by isoelectric focusing was 4.9 (Figure 5A) revealing that the enzyme is an acidic protein.

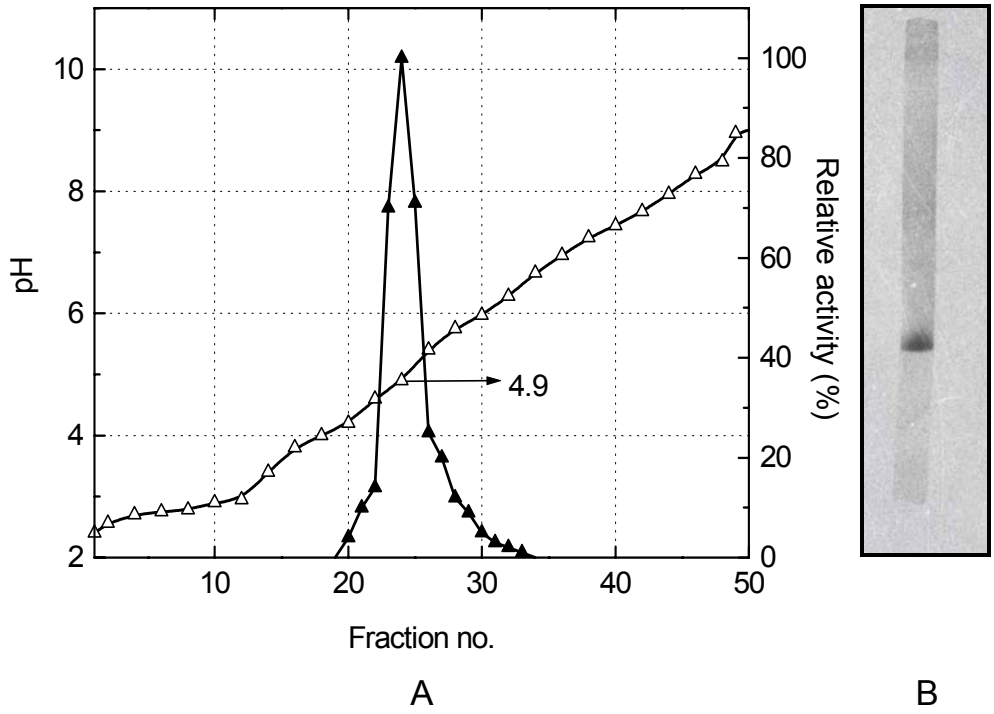


Figure 5. Isoelectric point (pI) and IEF-PAGE of purified enzyme from *Bacillus stearothermophilus* (NCIM- 5146) :

A) pI - Purified enzyme (200 µg) was focused in a mini scale isoelectric focusing unit as described in Methods. After focusing, fractions (200 µl) were collected, diluted suitably and were checked for its pH (∆) and activity (▲) profile. Maximum activity obtained was considered as 100 % and pH of the corresponding fraction was considered as a pI of protein.

B) IEF-PAGE- Purified enzyme (50 µg) was electrophoresed in a polyacrylamide tube gel in presence of ampholytes (pH 3-10) as described in Methods. After electrophoresis, gel was stained with Coomassie Brilliant Blue-R-250.

Molecular mass (M_r) :

The apparent molecular weight of the purified α-galactosidase estimated by a Sephacryl, S-300 gel filtration column and SDS-PAGE was 165.9 kDa and 79.9 kDa, respectively (Figure 6 and Figure 7). These results indicated that enzyme comprises two subunits with the same molecular mass.

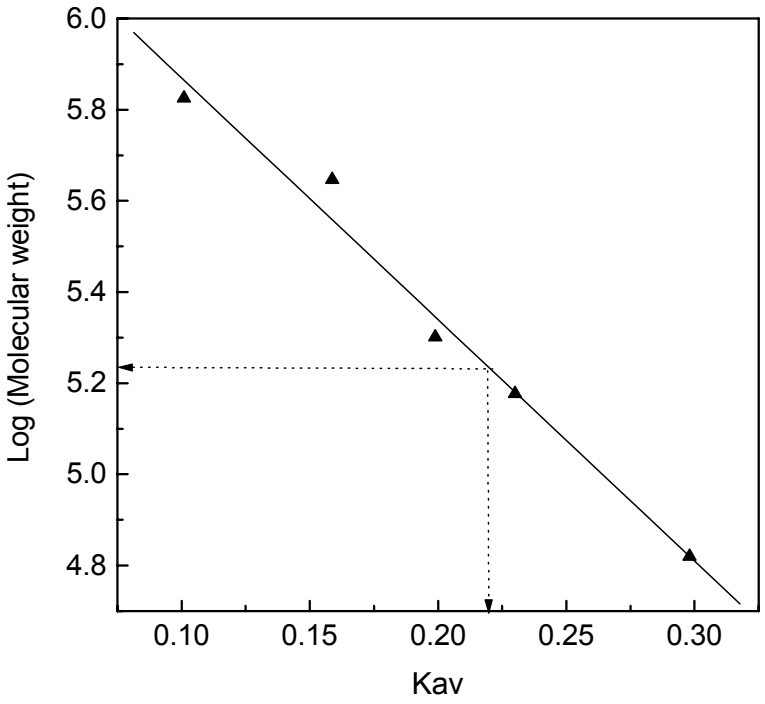


Figure 6. Native molecular mass of α-galactosidase estimated by gel filtration column : Sephacryl, S-300 column (1x150 cm) calibrated using standard gel filtration molecular weight markers as described in Methods. Partition coefficient (K_{av}) of the proteins was determined by the formula as given in the text and values are plotted against the log molecular mass (Mr). The best-fit line was drawn using Origin 4.0 (MicroCal).

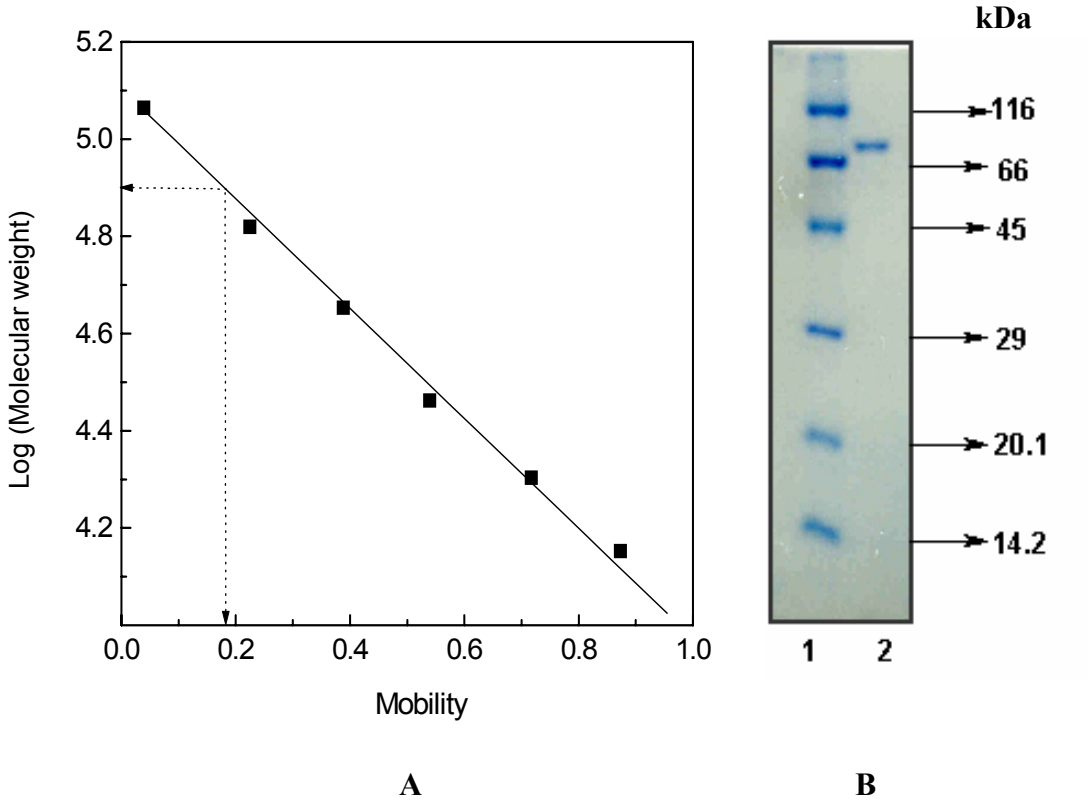


Figure 7. Subunit molecular mass of α-galactosidase by SDS-PAGE : **A)** Purified α-galactosidase was elelctrophoresed along with standard markers in 12.0 % (w/v) Polyacrylamide gel, under the denaturing conditions as described in Methods. The Mobility of proteins was determined as per the formula given in the text. The values are plotted against the log Mr and the best-fit line was drawn in Origin 4.0 (MicroCal).

B) Purified α-galactosidase was elelctrophoresed along with standard markers in 12.0 % (w/v) Polyacrylamide gel, under the denaturing conditions as described in Methods. After electrophoresis, gel was stained with Coomassie Brilliant Blue R-250. Markers are 116.0 (β-galactosidase), 66.0 (Albumin bovine serum), 45.0 (Albumin egg), 29.0 (Carbonic anhyrdrase), 20.1 (Soybean trypsine inhibitor), 14.2 (Lactalbumin).

Amino acid analysis :

The amino acid composition of the purified α-galactosidase (Table 2) revealed the enzyme of 1480 amino acid residues and a calculated molecular weight of about 162.8 kDa. The content of hydrophobic or apolar amino acids (L, I, V, M, P, A, F, W) of α-galactosidase is about 41.28 % (residues/mole). The Asx and Glx content is about 22.19 % and are lightly outnumbered the basic amino acids (Lys, Arg, His), about 13 %.

The enzyme had higher proline content (5%), however sulfur-containing amino acids are relatively low (2.63 %).

Table 2. Amino acid composition of purified α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) and other reported α-galactosidases :

Amino acids[a]	*E. coli* Raf,A[b]	*Humicola* sp[c]	*B. stearothermophilus* NCIM (5146)[d]
Ala	216	350	92
Val	152	242	95
Leu	284	294	145
Ile	136	132	68
Pro	148	116	74
Phe	120	153	74
Trp	96	32	26*[e]
Met	80	24	39
Gly	220	423	107
Ser	156	277	88
Thr	116	200	67
Cys	32	02	00*[f]
Tyr	96	109	75
Lys	72	135	57
Arg	248	142	100
His	116	26	44
Asx	252	397	153
Glx	308	363	176
Total	2848	3417	1480
Mol.wt (kDa).	329.0	354.8	162.8[g]

[a] Values are given in residues/mole, [b] Schmid K and Schmitt R [138], [c] Kotwal et al [208], [d] present enzyme, * Determined spectrophotometrically. [e] Spande T. F and Witkop B [387], [f] Habeeb A. F. S. A. [388], [g] Calculated molecular weight,

***N-terminal sequence* :**

The N-terminal sequence of first 10 amino acid residues of purified α-galactosidase was found to be **A-I-V-F-H-P-A-N-K-T**. The comparison of N-terminal amino acid sequence of α-galactosidase from *B. stearothermophilus* (NCIM 5146) with other selected examples available in a literature is given in Table 3. This sequence is distinct from known fungal, plant and human α-galactosidases. The closest sequence homology (70 % similarity) was observed with the α-galactosidase from *Bacillus stearothermophilus* (NUB3621) [198]. A highly conserved proline and lysine at position 7 and 10, respectively are present at N-terminal region of α-galactosidases from *Bacillus stearothermophilus*.

Table 3. N-terminal sequence alignment of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) with some of the known sequences of family 36/27/4 in glycoside hydrolases **:**

Source and gene name	**N-terminal Sequence**										**%**	
	1	2	3	4	5	6	7	8	9	10	**Similarity**	**(Ref)**
	11	12										
Guar	M	A	T	H	Y	S	I	I	G	G - -	10	(280)
Human AGAL	M	Q	L	R	-	N	P	E	L	H L -	10	(398)
Aspergillus niger AGLC	M	I	G	S	S	H	A	V	V	A - -	20	(329)
Saccharomyces cerevisiae MEL1	M	F	A	F	Y	F	L	T	A	C - -	10	(399)
E coli MELA	-	-	M	M	S	A	P	K	I	T F I	20	(282)
B. stearothermophilus (NCIM 5146)	-	A	I	V	F	H	P	A	N	K T -	100	(P)
B. stearothermophilus (NUB 3621) AGAN	M	A	I	V	F	D	P	T	N	K - -	70	(198)
B. stearothermophilus (KVE 39) AGAA	M	S	V	A	Y	N	P	Q	T	K - -	20	(400)
B. stearothermophilus (KVE 39) AGAB	M	A	V	T	Y	N	P	Q	T	K - -	30	(378)
B. stearothermophilus (MCA 2184) GALA	M	P	I	Q	V	N	E	E	T	K - -	10	(401)

Swiss Port database accession numbers for above sequences: Reference number and accession numbers are given respectively as follows : [280] P 14749, [398] PO 6280, [329] Q9UUZ4, [399] PO 4824, [282] PO 6720, [p] Present enzyme, [198] Q9X624, [400] Q9ALJ4, [378] Q934H7, [401] Q9LBD1.

Carbohydrate content :

Purified α-galactosidase was found to be a non-glycosylated protein, as determined by phenol sulfuric acid method.

Secondary structure :

Secondary structure of the α-galactosidase is determined by CD spectroscopy. Figure 8 shows the far UV CD spectra (250-190 nm) of α-galactosidase at 50 ºC, where the α-galactosidase acquires most ordered secondary structural conformation. There are clearly two negative elipticity minima's around 222 and 208 nm and positive band near 195 nm, at 50 ºC and at pH 7.0, indicated predominance of α-helical structure.

The effect of temperature on protein secondary structure was also monitored from 30-70 ºC with 10 ºC increment and 10 minutes incubation time. The analysis of temperature dependent CD data (240-190 nm) by continll algorithm (Table 4) shows approximately 15 % increase in β-sheets and 14 % decrease in unordered conformation, when temperature of protein sample was increased from 30 to 50 ºC. The content of α-helix and β-turns are essentially same in this temperature range. Further increasing the incubation temperature up to 70 ºC, causes concomitant decrease in α-helix content and increase in unordered or random coil structure by 17 %. The amount of β-turn is constantly 20-22 % at all the temperatures studied. α-Galactosidase completely losses its activity and secondary structure when incubated at 75 ºC (data is not given). Moreover the reversibility of structural changes was not studied.

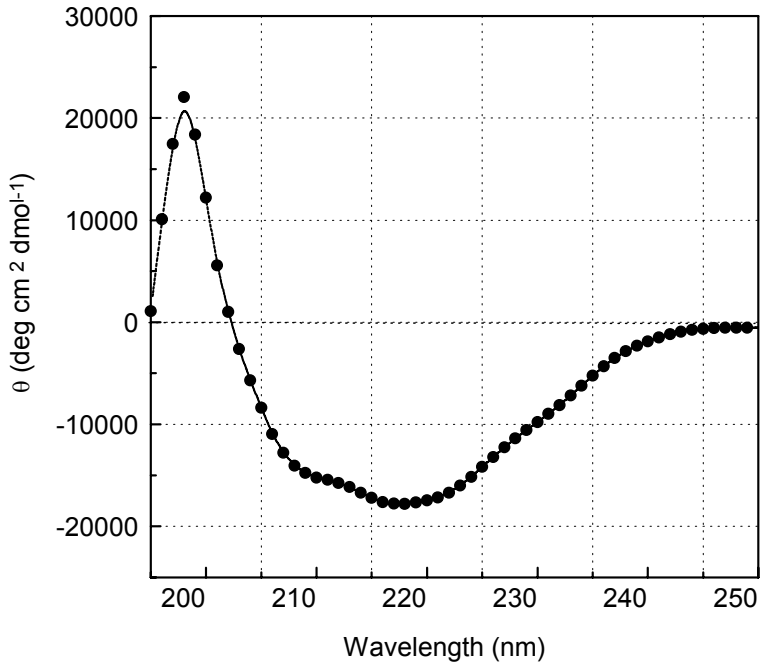


Figure 8. Far UV CD spectra of native α-galactosidase at 50 °C in 50 mM phosphate buffer, pH 7.0.

Table 4. Changes in secondary structure of α-galactosidase in accordance with temperature : Analysis of CD data by continll algorithm :

Temperature	Secondary structural content (%)			
	α-Helix	β-Sheet	β-Turn	Random coil
30 ºC	41.66	4.3	22.63	31.65
40 ºC	37.3	18.8	20.40	23.40
50 ºC	40.3	21.6	20.2	17.80
60 ºC	31.4	13.6	26.3	28.70
70 ºC	23.1	20.8	21.5	34.5

Biochemical properties of α-galactosidase :

***pH optimum and stability* :**

pH activity profile of α-galactosidase is shown in Figure 9. Purified α-galactosidase exhibited maximum activity (more than 70 %) in the pH range 5.5-8.0 with optimum at 6.5-7.0. However enzyme did not show any activity below pH 5.5.

The enzyme demonstrated wide pH stability and is completely stable in the alkaline pH range, 6.0-9.0, however it showed 60-80 % of its original activity, when incubated in the acidic pH buffers; 3.5 - 5.5.

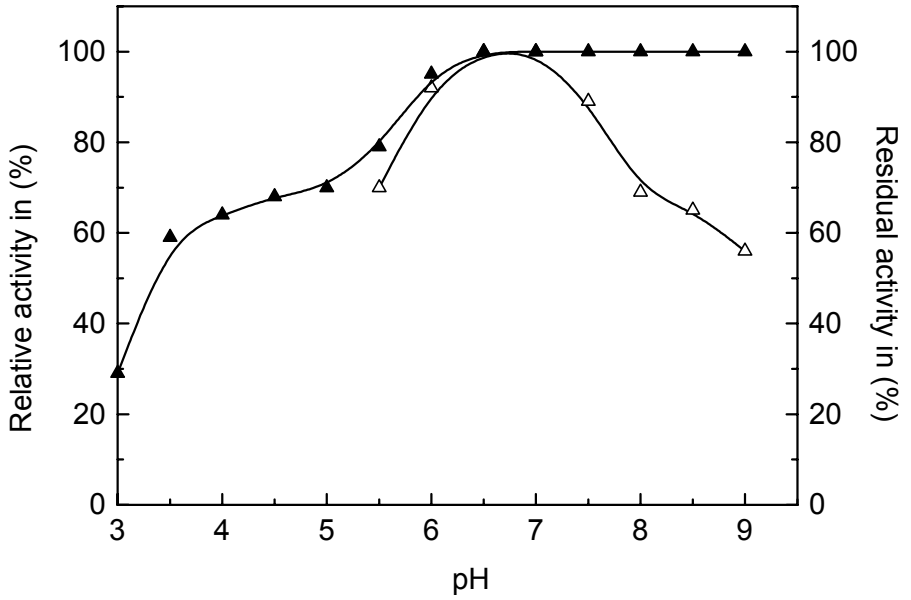


Figure 9. Effect of pH on activity and stability of purified α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) :

pH Optimum (Δ) : A Suitably diluted enzyme (~ 0.01 U) was assayed in a series of pH (3-9) at 65 °C as described in Methods and the relative enzyme activity was determined. Maximum enzyme activity obtained was taken as 100 %.

pH Stability (▲) : A suitable concentration of purified enzyme (~1.0 U/ml) was incubated in a series of pH (3-9) at R.T for the period of 12 hours. Aliquots (10 µl) were removed and residual enzyme activity was determined under standard assay conditions. Maximum activity obtained was taken as 100 %.

Temperature optimum and stability :

The purified α-galactosidase displayed a temperature optimum at 65 ºC and it showed 80 % of the relative activity at 60 ºC and 90 % at 70 ºC, when *p*NP-α-galactoside was used as a substrate (Figure 10). However enzyme showed only 3 to 30 % of the relative activity in the temperature ranges from 30-50 ºC.

Thermostability of α-galactosidase was determined by studying the time dependent thermal inactivation of enzyme as shown in Figure 11. Enzyme was found to be completely stable at 50 ºC for 60 minutes. The half-lives of inactivation of α-galactosidase at 65 and 70 ºC were 60 and 30 minutes, respectively.

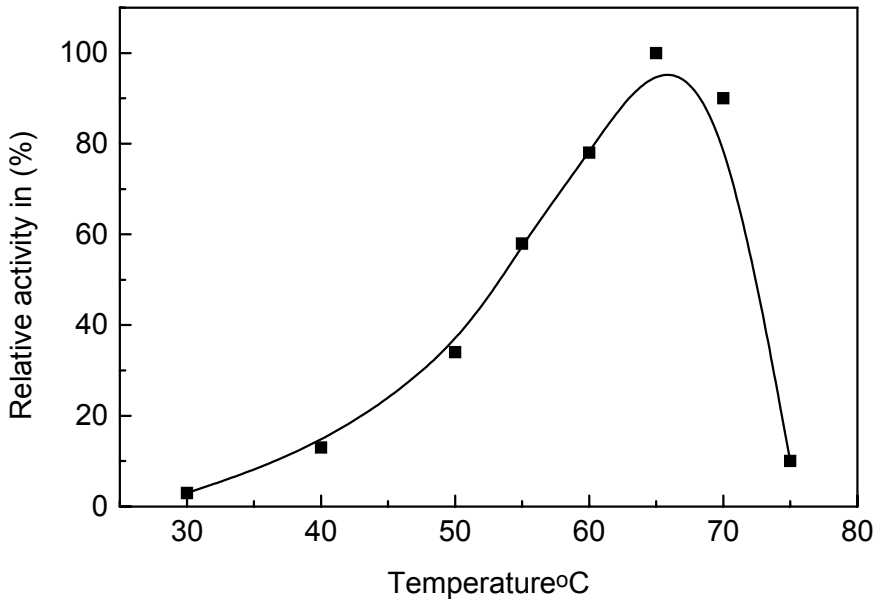


Figure 10. Temperature optimum of purified α-galactosidase: Purified α-galactosidase (~ 0.01U) was assayed at different temperatures ranging from 30-75 °C at optimum pH as described in Methods and relative enzyme activity was determined. Maximum enzyme activity obtained was considered as 100 %.

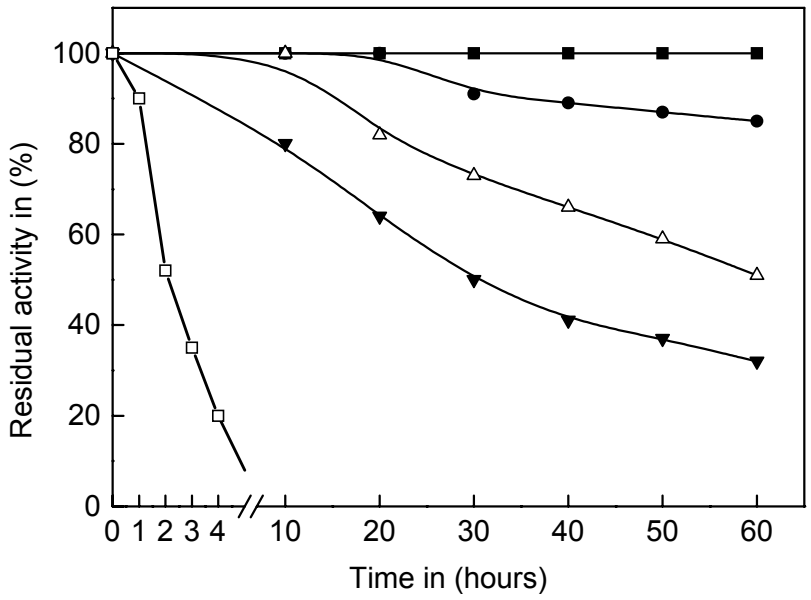


Figure 11. Temperature stability of purified α-galactosidase: Purified enzyme samples (1.0 U/ml) were incubated at various temperatures ranging from 30-75 °C. Aliquots (~10 µl) were removed at suitable time interval and assayed for residual enzyme activity. Maximum enzyme activity obtained was taken as 100 %. Symbols used are: 50 (■), 60 (●), 65 (Δ), 70 (▼), 75 °C (□).

Substrate specificity :

α-Galactosidase from *Bacillus stearothermophilus* (NCIM 5146) exhibits wide substrate specificity and can hydrolyze various synthetic as well as natural glycosides, containing α-1-6-galactosidic linkages at their non-reducing end (Table 5). The highest hydrolytic activity was found towards *p*-nitrophenyl-α-D-galactopyranoside, followed by *o*-nitrophenyl-α-D-galactopyranoside. It also hydrolyzes Methyl-D-galactopyranoside at a much slower rate. Whereas the *m*-nitrophenyl-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-fucopyranoside, *p*NP-β-L-arabinopyranoside and *p*NP-α-D-glucopyranoside were not hydrolyzed by the action of this enzyme. In order to investigate the substrate specificity of α-galactosidase on galacto-oligosaccharides and polysaccharides, purified enzyme was incubated with melibiose, raffinose, stachyose, guar and locust bean gum and yeast mannan. The relative rate of hydrolysis of these substrates was expressed as percentage of activity calculated with *p*-nitrophenyl-α-D-galactopyranoside (100%). The enzyme hydrolyzed these substrates in the order of melibiose > stachyose > raffinose > locust bean gum > and guar gum. However yeast mannan was not hydrolyzed by the action of this enzyme. The hydrolysis products of the galacto-oligosaccharides were visually assayed by TLC analysis (Figure 12). The results' clearly indicating that melibiose was rapidly hydrolyzed to galactose and glucose, whereas hydrolysis of raffinose and stachyose was relatively slow. During stachyose hydrolysis, raffinose was accumulated in the reaction mixture (Figure 12A).

Table 5. Relative substrate specificity of purified α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146) :

Substrates[a]	Relative velocity (%)
*p*NP-α-D-galactopyranoside	100
*o*NP-α-D-galactopyranoside	40.37
*m*NP-α-D-galactopyranoside	Not hydrolyzed
*p*NP-β-D-galactopyranoside	Not hydrolyzed
*p*NP-α-D-glucopyranoside	Not hydrolyzed
*p*NP-α-D-mannopyranoside	Not hydrolyzed
*p*NP-α-D-fucopyranoside	Not hydrolyzed
*p*NP-β-L-arabinopyranoside	Not hydrolyzed
*p*NP-N-acetyl-α-D-galactosaminide	Not hydrolyzed

Methyl-α-D-galactopyranoside	0.317
Melibiose.	3.15
Raffinose.	1.25
Stachyose.	2.82
Locust bean gum[b].	0.12
Guar gum[b].	0.057
Yeast mannan[b].	Not hydrolyzed

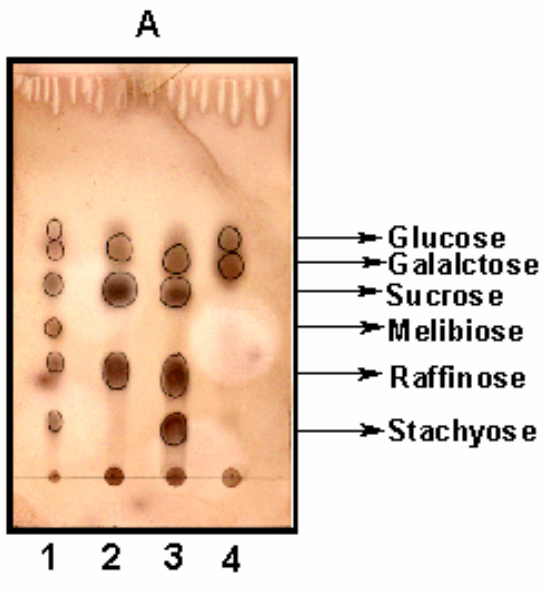
[a]Enzyme activity on above substrates was determined as described in Methods. Activities are expressed as percentage of the activity calculated with *p*-nitrophenyl α-D-galactopyranoside as substrate with which the enzyme showed the best performance. [b] Incubation time was 30 minutes.

Apart from α-1-6-galactosidic linkages, α-galactosidase from B*acillus stearothermophilus* (NCIM 5146) also cleaves terminal α-1-3 and α-1-4-galactosidic linkages present in disaccharide sugars (Figure 12B). The relative rate of hydrolysis of α-1-3-galactosidsidic linkages was found to be greater than that of the α-1-6 linkages (Table 6). However α-galactosidase from B*acillus stearothermophilus* (NCIM 5146) did not exhibit lectin or hemagglutinin like activity (data not shown).

Table 6. Linkage specificity of α-galactosidase from B*acillus stearothermophilus* (NCIM 5146) :

Substrates	Relative velocity
*p*NP-α-D-galactopyranoside	100
α-D-gal-(1-2)-D-gal	ND
α-D-gal-(1-3)-D-gal	3.45
α-D-gal-(1-4)-D-gal	0.148
α-D-gal-(1-6)-D-gal	3.00

ND-not determined



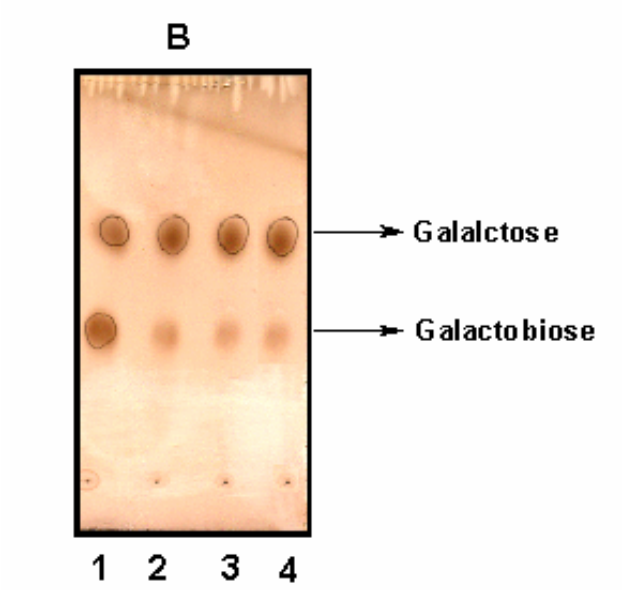


Figure 12. Action of a-galactosidase on galacto-oligosaccharides. The reaction mixture was composed of 100 µl of 10 mM substrate prepared in phosphate buffer (50 mM, pH 7.0) and 10 µl of enzyme (2.0 Units). Reaction was carried out at 65 °C for 30 minutes and 5 µl of the mixture was applied to the TLC and developed as described in methods.

Figure 12A: Lane 1. Standard mixture of sugars, **Lane 2.** Raffinose + enzyme, **Lane 3.** Stachyose + enzyme, **Lane 4.** Melibiose + enzyme.

Figure 12B: Lane 1. Standard sugars, **Lane 2.** α-D-gal-(1-6)-D-gal + enzyme, **Lane 3.** α-D-gal-(1-3)-D-gal + enzyme, **Lane 4.** α-D-gal-(1-4)-D-gal + enzyme.

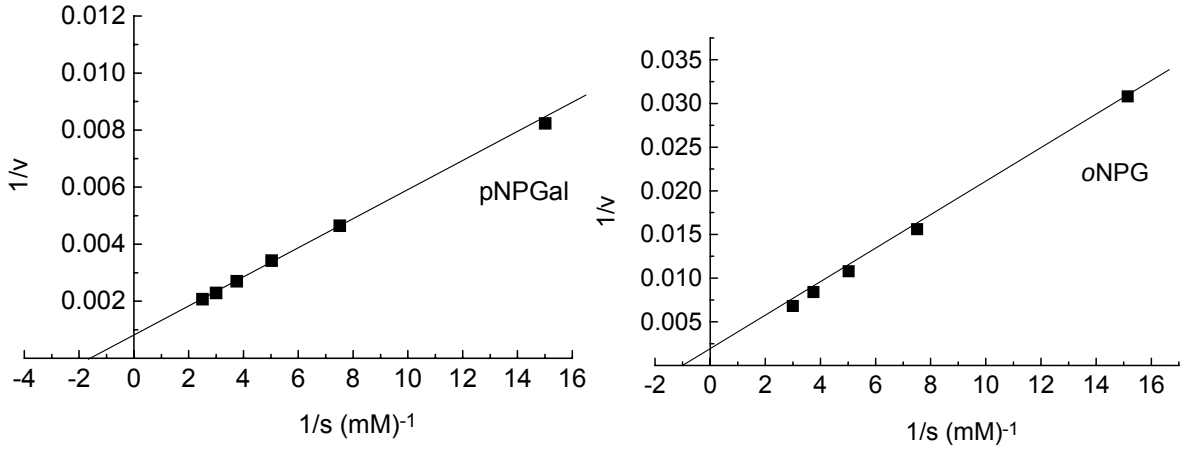
Kinetic studies of α-galactosidase :

The effects of varying substrate concentrations on the reaction rate were studied and Michaelis constants (K_m) and maximum velocity (V_{max}), turnover number (K_{cat}), and specificity constant (K_{cat}/K_m) were calculated for several substrates from Lineweaver-Burk plots (Figure 13).

Table 7. Kinetic parameters of the hydrolysis of different glycosides by α-galactosidase[a] :

Substrates	K_m	V_{max}	K_{cat}	K_{cat}/k_m
*p*NP-α-D-galactopyranoside	0.606	1250	2.0×10^5	3.3×10^5
*o*NP-α-D-galactopyranoside	1.0	627	1.0×10^5	1.0×10^5
Melibiose.	5.0	200	3.3×10^4	6.6×10^3
Raffinose[b].	5.0	25	4.1×10^3	830
Stachyose[b].	2.5	25	4.1×10^3	1660
α-D-gal-(1-3)-D-gal.	6.66	266	4.4×10^4	6.63×10^3

[a] Determined at 65 °C under standard assay conditions., [b] incubation time was 30 minutes. Units of the kinetic parameters are K_m = mM, V_{max} = μmoles min^{-1} mg^{-1}, K_{cat} = min^{-1}, K_{cat}/k_m = mM^{-1} min^{-1}.



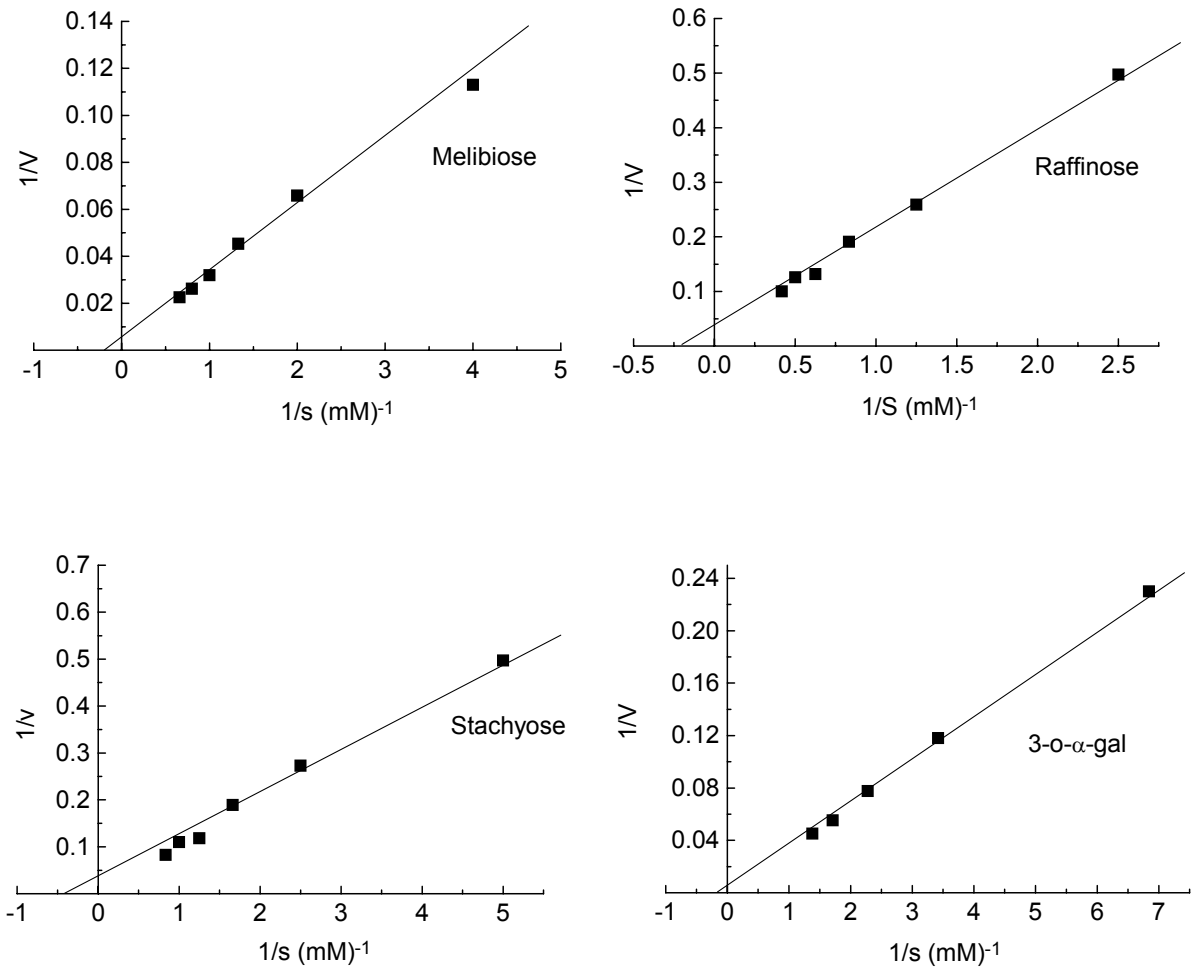


Figure 13. Lineweaver-Burk plots for the hydrolysis of several substrates by α-galactosidase of NCIM-5146. The assays were carried out at different substrate concentrations as described in methods under the standard assay conditions. V_{max} is expressed as µmoles $min^{-1}mg^{-1}$. Data was fitted to linear regression using Lineweaver-Burk plot in Origin 4.0 (Microcal).

Above plots indicated that the initial velocity of α-galactosidase was not affected by the substrate concentrations used, under the experimental conditions. Results of kinetic studies are summarized in Table 7. Data indicated that enzyme has a higher affinity for *p*NPG and *o*NPG (lower K_m) and also higher rate of hydrolysis (higher V_{max}) than for raffinose, stachyose and melibiose. Among the three alkyl galactosides, disaccharide, melibiose was hydrolyzed at a more rapid rate than the trisaccharide, raffinose, although, both the substrates has similar K_m. Moreover, although, trisaccharide, raffinose and tetrasaccharide, stachyose has similar rate of hydrolysis, they have different K_m values. On the other hand galactobiose, α-(1-3)-D-gal has higher K_m and higher rate

of hydrolysis as compare to alkyl galactosides. The question was then asked: why low affinity substrates were not hydrolyzed at higher rate and *vice versa*. One method to investigate this anomalous behavior of enzyme is with temperature dependence studies, which has become an important tool for elucidating the mechanism of catalysis and the linkage between catalysis and conformational changes.

Temperature dependence studies :

The kinetic parameters calculated for α-galactosidase with different substrates at various temperatures are summarized in Table 8. This indicated that the activity of α-galactosidase increased with increase in temperature. The turn over number, K_{cat} of *p*NPG increased about 11-fold from the minimal temperature (40 °C) to the maximal temperature (65 °C). Similarly K_{cat} of melibiose also increased by 5 fold and raffinose and stachyose by 3 fold. The Michaelis constant, K_m, also showed a similar trend but with only a 2-fold increase from the minimal to maximal temperature with no significant increase at any given temperature. It is therefore evident that K_m is independent of V_{max} and possibly represents the dissociation of enzyme substrate complex only (i.e. K_d).

The temperature variation of K_{cat} values obtained with different substrates were analyzed by Arrhenius plots, shown in Figure 14. Discontinuous Arrhenius plots were obtained for all the substrates with break point at 55 ºC for *p*NPG and 50 ºC for melibiose, raffinose and stachyose. The thermodynamic parameters of activation of different substrates calculated from Arrhenius plots are given in Table 9. The apparent activation energies (E_a) and activation enthalpies ($\Delta H^{\#}$) below the transition temperature were higher and above the transition temperature were lower in case of *p*NPG, raffinose and stachyose, whereas reverse values were obtained for melibiose. Below the transition temperature relative rate of hydrolysis of the substrates was very low (about 30 %), hence it is considered as a rate-limiting step in the hydrolysis of various glycosides. So we calculated thermodynamic parameters at 60 ºC. Hydrolysis of *p*NPG and melibiose was found to be enthalpy driven and compensated by favorable contributions from the entropy of activation (positive $\Delta S^{\#}$). Whereas hydrolysis of raffinose and stachyose was found to be entropy driven process (high negative $-\Delta S^{\#}$) and required less activation energy. The activation parameters obtained for raffinose and stachyose were similar, since both the substrates were hydrolyzed at similar rate. However they shows difference in the free energy of transition state stabilization.

Thermodynamic parameters of binding of different glycosides to the enzyme were calculated from the temperature variation of equilibrium constant. Here K_m is regarded as dissociation constant of the enzyme-substrate complex, hence the values of ΔH and ΔS would represent the changes occurring during the formation of Michaelis complex (ES

complex). Data were fitted to the van't Hoff equation (equation- 7 & 11) and analyzed by van't Hoff plots, shown in Figure 15. The thermodynamic parameters of binding are given in Table 10. The association constant of binding of *p*NPG, melibiose, raffinose and stachyose were 2000, 255, 250 and 500 M^{-1}, respectively at 60 ºC. This indicated that affinity of α-galactosidase for glycosides (oligo-saccharides) increased by increasing chain length of galacto-oligosaccharides by one galactose unit, irrespective of degree of polymerization (DP) of the substrate (total chain length). Synthetic substrate, *p*NPG shows high affinity for enzyme. The binding enthalpy values calculated from van't Hoff plots are similar for all the substrates (-27.0 kJ mol^{-1}). However relative binding of the galacto-oligosaccharides is determined by ΔΔS contributions.

Table 8. Kinetic parameters of the hydrolysis of different substrates by α-galactosidase as a function of temperature[a] :

Temp.	*p*NPG		Melibiose		Raffinose[b]		Stachyose[b]	
ºC	K_m	K_{cat}	K_m	K_{cat}	K_m	K_{cat}	K_m	K_{cat}
40	0.281	1.7×10^4	2.10	6644	2.10	1328	1.08	1230
45	0.322	3.0×10^4	2.58	8305	2.5	1845	1.21	1661
50	0.384	5.3×10^4	3.07	1.0×10^4	2.96	2657	1.42	2556
55	0.444	1.0×10^5	3.33	1.9×10^4	3.47	3020	1.66	3020
60	0.501	1.3×10^5	3.92	2.3×10^4	4.0	3497	2.0	3691
65	0.606	2.0×10^5	5.0	3.3×10^4	5.0	4152	2.5	4152

[a] Assays were carried out under standard assay conditions as described in methods. Kinetic parameters are expressed as K_m = mM, K_{cat} = min^{-1}. [b] Incubation time was 30'.

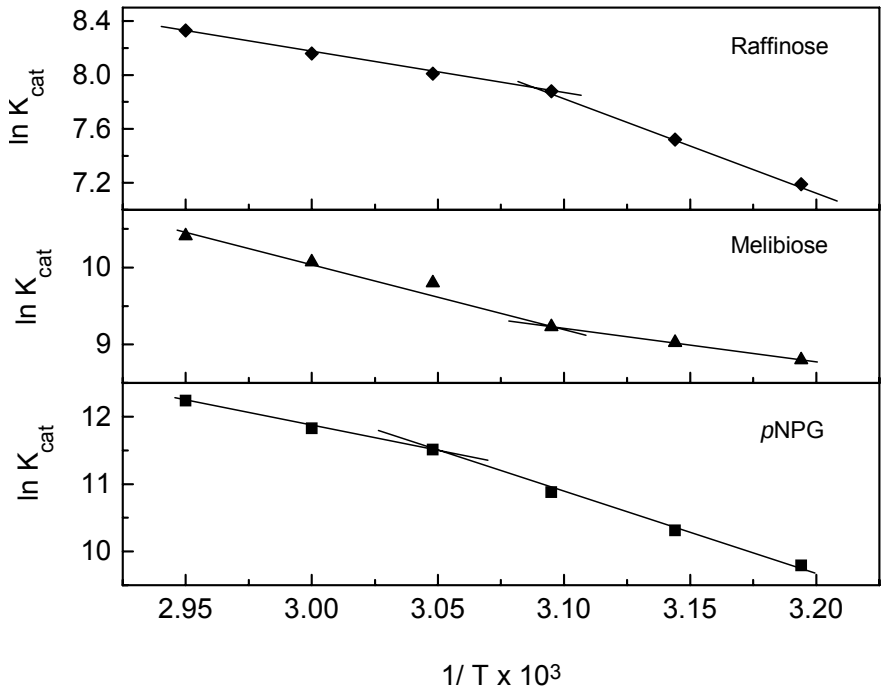


Figure 14. Arrhenius plots showing relationship between rate constant (K_{cat}) and absolute temperature (T) for the hydrolysis of different substrates by a-galactosidase. Activation energies (E_a) were calculated from the slope ($-E_a/R$) of each linear segment.

Table 9. Thermodynamic parameters of activation of different glycosides :

Substrates	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J $mol^{-1}K^{-1}$)	$\Delta G^{\#}_{tb}$ (kJ mol^{-1})
*p*NPG	97.81	95.04	49.0	138.25	-34.66
	59.86	57.09		24.29	
Melibiose.	35.60	35.60	54.0	-55.13	-24.0
	70.58	70.58		49.90	
Raffinose.	61.58	58.81	59.50	-1.59	-18.74
	29.0	26.23		-99.42	
Stachyose.	60.0	57.23	59.11	-5.64	-20.81
	28. 60	25.83		-100	

Activation energy (E_a), change in activation enthalpy ($\Delta H^{\#}$), entropy ($\Delta S^{\#}$) free energy change of activation ($\Delta G^{\#}$) and free energy of transition state binding ($\Delta G^{\#}_{tb}$) are calculated as described in methods, at 60 °C (333 K).

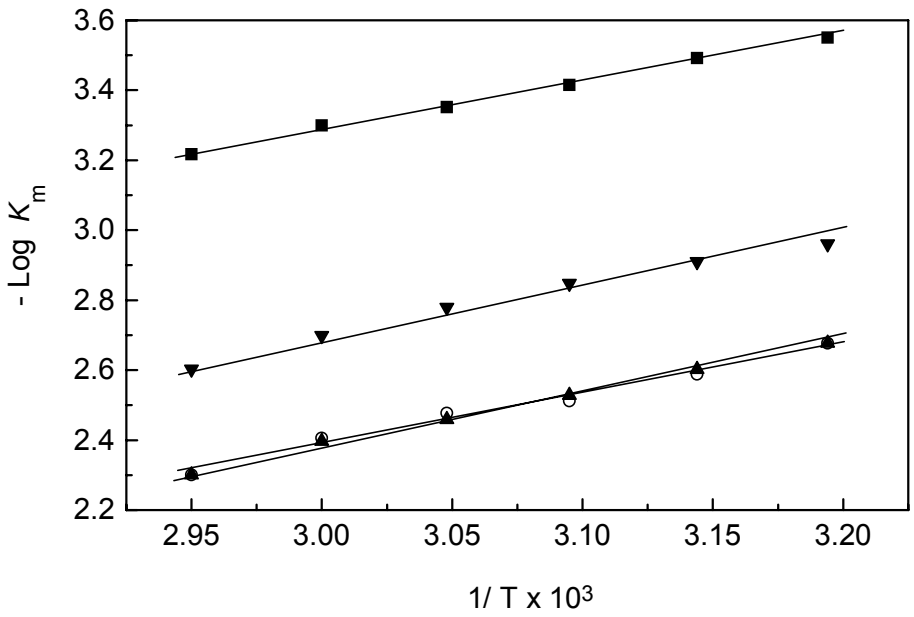


Figure 15. Van't Hoff plots showing the relationship between equilibrium constant, K_m and temperature for the binding of various glycosides to the enzyme. Binding enthalpy values were calculated from the slope (ΔH_b/ 2.303R). Lines represent the best-fit lines to the equation 7.0. *p*NPG (■), Stachyose (▼), Raffinose (▲), Melibise (○).

Table 10. Thermodynamic parameters of binding of different glycosides[a] :

Substrates	K_a (M^{-1})	$-\Delta H_b$ (kJ mol^{-1})	$-\Delta H_{b\ corrt.}$ (kJ mol^{-1})	$-\Delta G_b$ (kJ mol^{-1})	$-\Delta S_b$ (J $mol^{-1}K^{-1}$)
*p*NPG	2000	26.63	27.0	21.0	18
Melibiose.	255	29.38	27.0	15.33	35
Raffinose.	250	27.35	27.88	15.28	37.83
Stachyose.	500	28.08	26.60	17.19	28.0

Association rate constant (K_a), change in binding enthalpy (ΔH_b), entropy (ΔS_b), free energy change of binding (ΔG_b) are calculated as described in methods at 60 °C (333 K). Corrected binding enthalpy values ($-\Delta H_{b\ corrt}$) were calculated from equation 11.0.

Transgalactosylation activity :

Acceptor specificity of α-galactosidase was examined using various monosaccharides and disaccharides, whereas donor specificity was studied by incubating enzyme with different substrates. The products of the reactions were analyzed qualitatively by TLC.

Two transfer products were obtained when raffinose was incubated along with galactose and glucose at 40 ºC for 3 hours (Figure 16A, Lane 4 & 5). Their chromatographic mobility was identical with those of stachyose and melibiose, respectively. Whereas other sugars fructose, sucrose, lactose and cellobiose did not function as galactosyl acceptors at any concentration examined (100-500 mM). Further incubation of various concentrations of raffinose (5-100 mM) in presence of galactose and glucose (100 mM each) showed that 50-100 mM of raffinose concentration was optimum as a galactosyl donor as well as accepter (Figure 16C). However incubation of *p*NPG, melibiose and stachyose in presence of galactosyl accepters (glucose & galactose) did not show any transfer reactions and only hydrolysis products were observed on TLC (Figure 16A, Lane 2 & 3 and Figure 16B, data for *p*NPG hydrolysis are not shown).

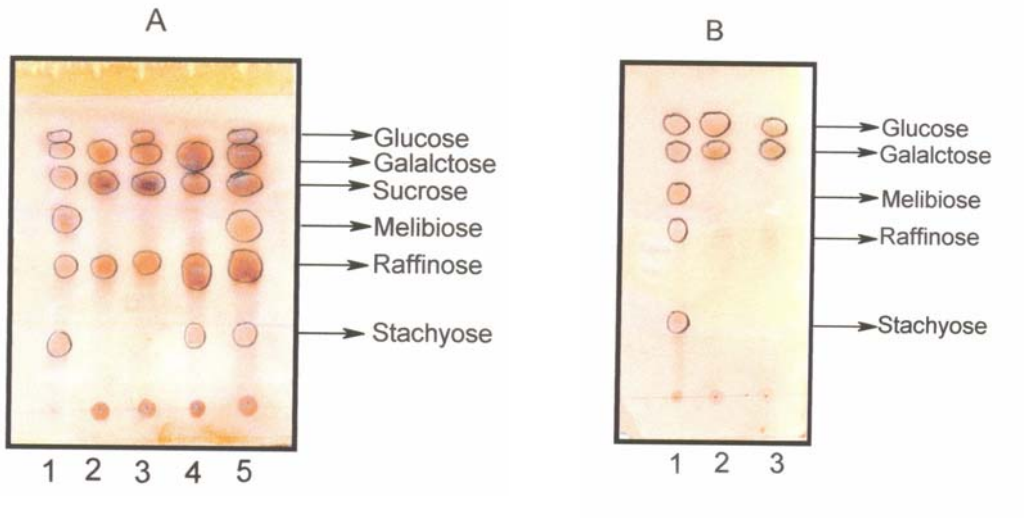


Figure 16. Thin layer chromatography of transfer products to acceptor sugars by a-galactosidase from *Bacillus stearothermophilus* (NCIM-5146): Transfer reactions were carried out as described in methods. **Figure (A):** Incubation of a-galactosidase in presence of 50 mM of raffinose and stachyose along with 100 mM of galactose and glucose. **Lane 1:** standards, **Lane 2:** stachyose + galactose, **Lane 3:** stachyose + glucose, **Lane 4:** raffinose + galactose, **Lane 5:** raffinose + glucose. **Figure (B):** Incubation of a-galactosidase in presence of melibiose, 50 mM and galactose and glucose, 100 mM each. **Lane 1:** standards, **Lane 2:** melibiose + galactose, **Lane 3:** melibiose + glucose.

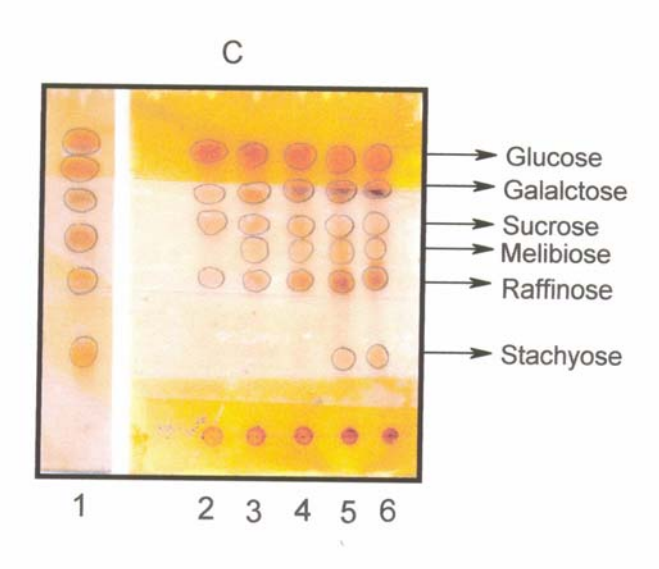


Figure 16C. Effect of raffinose concentrations on the transfer reaction: α-galactosidase was incubated with 5-100 mM of raffinose in presence of 100 mM of galactose and glucose and reactions were carried out at 40 °C as described in methods. **Lane 1:** standards, **Lane 2:** raffinose 5 mM + galactose & glucose, **Lane 3**: raffinose 10 mM + galactose & glucose, **Lane 4:** raffinose 25 mM + galactose & glucose, **Lane 5:** raffinose 50 mM + galactose & glucose, **Lane 6:** raffinose 100 mM + galactose & glucose.

Effect of various metal ions, sugars and reagents :

The sensitivity of purified α-galactosidase to various metal ions, sugars and inhibiting reagents was tested and results are summarized in Table 11. Enzyme was completely inhibited by the Ag^{+}, Hg^{2+} and Cu^{2+} metal ions. Other metal ions Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+} and Zn^{2+} had no influence on enzyme activity. Similarly EDTA, β-mercaptoethanol and Urea did not inhibit the enzyme activity. However enzyme was strongly inhibited by Tris and PCMB. Among various carbohydrates tested, galactose, lactose, melibiose and stachyose also inhibited α-galactosidase activity at 10 mM concentrations, whereas other sugars and sugar alcohols did not inhibit the enzyme activity.

Table 11. Effect of various metal ions, sugars and reagents on α-galactosidase activity[a].

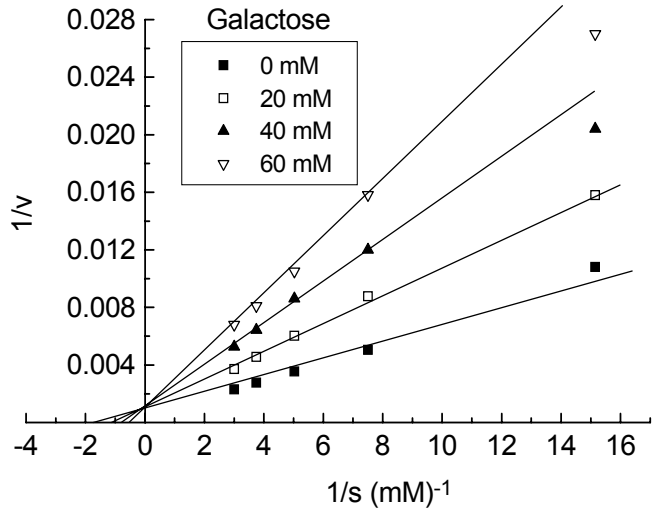
Metal ions/*Reagents	Residual activity (%)	Sugars (10 mM)	Residual activity (%)
None	100	Glucose	100
Ag^{+} (1 mM)	0	Galactose	77
Cu^{2+} (1 mM)	0	Lactose	74
Hg^{2+} (1 mM)	0	Sucrose	105
EDTA (10mM)	100	Melibiose	71
β-Mercaptoethanol (1 mM)	100	Stachyose	90
Urea (1M)	100	Glycerol	100
Tris (10mM)	37	Sorbitol	100
PCMB	00		

[a] A suitably diluted enzyme was pre-incubated with various inhibiting reagents at room temperature for 10 minutes and subsequently residual activity of α-galactosidase was determined as described in methods. Activities are expressed as percentage of activity compare to the control sample.

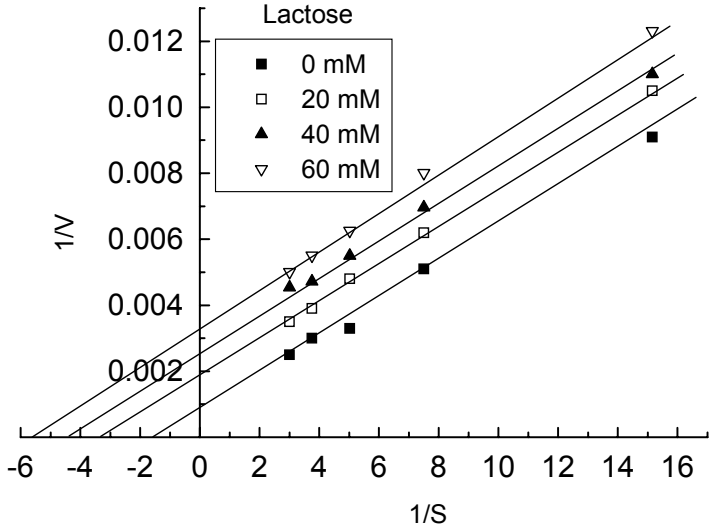
* Enzyme exhibited 100 % activity in presence of Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+} and Zn^{2+} metal ions and was not mentioned in the above table.

Kinetics of α-galactosidase Inhibition :

Lineweaver-Burk plots for the hydrolysis of *p*NPG in the presence of some of the inhibitors are shown in Figure 17 & 19, whereas inhibition constant (K_i) values were determined graphically is shown in Figure 18. α-Galactosidase was competitively inhibited by galactose, melibiose and stachyose with K_i values equal to 16.25 mM for galactose and melibiose and 33.0 mM for stachyose. By contrast Lactose, a β-glycoside inhibits enzyme activity uncompetitively with K_i value equal to 14.5 mM. Inhibition by Ag^{+} Cu^{2+} Hg^{2+} and PCMB seems to be a non-competitive type (Table 12).

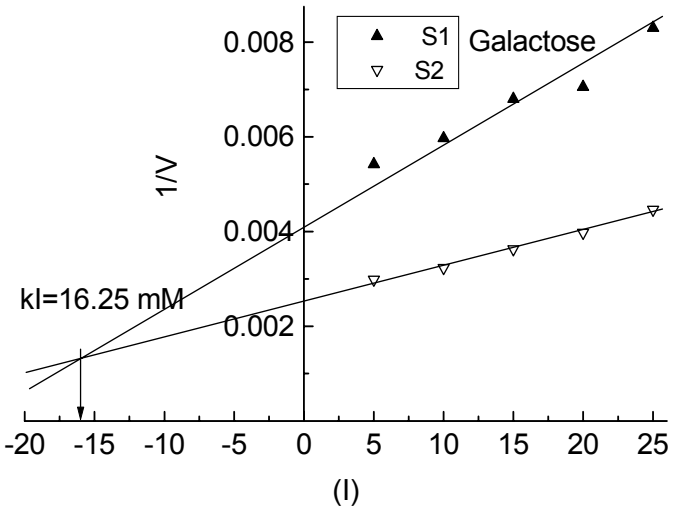


A



B

Figure 17. Lineweaver-Burk plots for the hydrolysis of *p*NPG in the presence of galactose **(A)** and lactose **(B),** showing competitive and uncompetitive inhibition, respectively : α-Galactosidase assays were carried out at different substrate concentrations in the presence of inhibiting sugars under standard assay conditions as described in methods and data was analyzed according to Lineweaver-Burk.



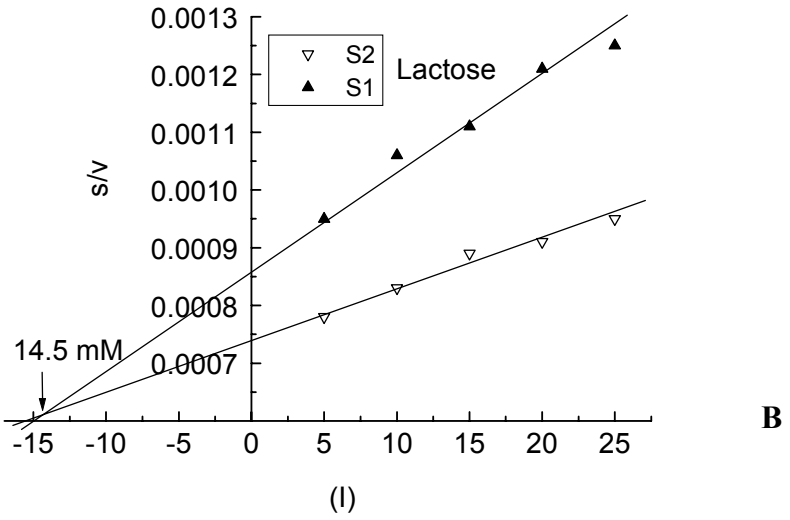


Figure 18. Determination of inhibition constant (K_i) : α-Galactosidase activity was determined at two different substrate concentrations S1 & S2 (S2 > S1) by varying inhibitor concentrations in the range of 5-25 mM. Activities were determined under standard assay conditions as described in methods. K_i value of competitive inhibitor, galactose **(A)** was determined according to Dixion [397] and that of uncompetitive inhibitor, lactose **(B)** was determined by Cornish Bowden method [402].

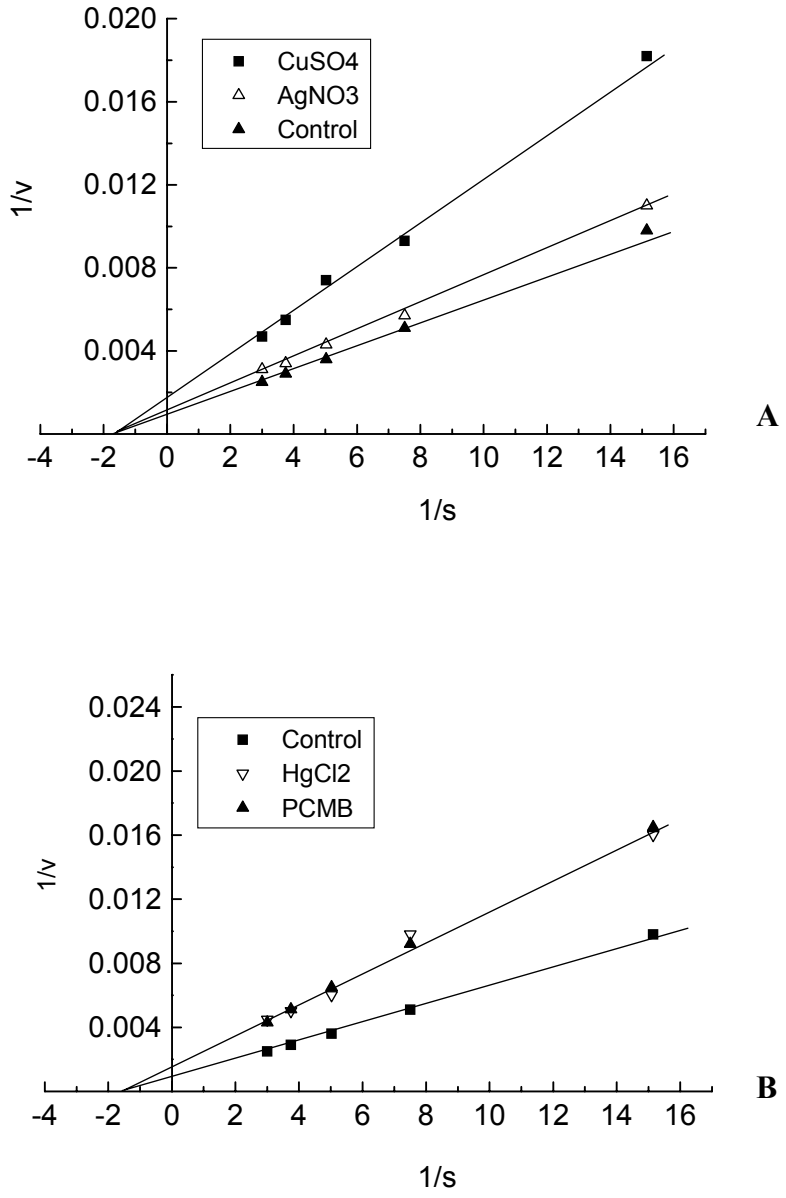


Figure 19. Lineweaver-Burk plots showing non-competitive inhibition of α-galactosidase activity by : **(A)** $AgNO_3$ (0.01 µM) & $CuSO_4$ (20 µM) **(B)** HgCl2 (0.01 µM) & PCMB (0.01 µM). Assays were carried out at different substrate concentrations and in the presence of fixed concentration of inhibitors under the standard assay conditions as described in methods.

Table 12. Determination of type of inhibition and inhibition constant (K_i) of several inhibitors of α-galactosidase[a] :

Inhibitors	Type of Inhibition	K_i (mM)
Galactose	Competitive	16.25
Melibiose	Competitive	16.25
Stachyose	Competitive	33.0
Lactose	Uncompetitive	14.5
Ag^{+}	Non competitive	--
Cu^{2+}	Non competitive	--
Hg^{2+}	Non competitive	--
PCMB	Non competitive	--

[a]Assays were carried out under standard assay conditions in the presence of various inhibitors and nature of inhibition was determined from Lineweaver-Burk plots as described in methods. Ki values for competitive inhibitors were determined from Dixions [397] plot and that for uncompetitive inhibitor was determined from the Cornish Bowden method [402].

3.5 Discussion :

Bacillus stearothermophilus (NCIM 5146) a thermophile produces extracellular α-galactosidase when grown aerobically in a medium containing soybean meal as a carbon source (Chapter II). However for the purification of α-galactosidase, enzyme was produced using galactose as an inducer instead of soybean meal, in order to reduce the contaminating proteins. Ultra-filtration step with a recovery of 95 % of activity was successfully introduced for the concentration of enzyme activity from the culture filtrate. The ethanol fractionation step increases the specific activity by 19 fold and conditioned the broth for chromatographic step by removing most of the undesirable proteins and colored pigments. The Phenyl Sepharose column chromatography yielded a pure enzyme showing only one band on Native and SDS-PAGE (Figure 3 & 7B). Earlier, purification of α-galactosidases has been achieved by multistep purification procedures, which were tedious and time-consuming [92,100,101,256]. However, we have developed single, relatively simple and highly reproducible chromatographic procedure in which most of the centrifugation, concentration and dialysis steps are omitted (Figure 4).

The existence of multi molecular forms of α-galactosidases have been reported from various prokaryotic and eukaryotic sources [82,212,221,227], however, no multi molecular forms appeared during the purification of α-galactosidases from NCIM 5146, indicated by single protein band on IEF-PAGE (Figure 5B). The isoelectric point of the purified α-galactosidase is 4.9 (Figure 5A), revealing that the enzyme is an acidic protein [115].

The approximate molecular mass of the α-galactosidase estimated by gel filtration and SDS-PAGE (165.9 kDa and 79.9kDa) indicated homodimeric structure. Molecular mass reported here is significantly smaller among the α-galactosidases reported from *Bacillus stearothermophilus* (Table 13, below). The average molecular mass of the α-galactosidases reported from *Bacillus stearothermophilus* has been in the range of 247 kDa to 320 kDa. However, the subunit molecular mass of the α-galactosidases reported from *Bacillus stearothermophilus* is in the range of 80-85 kDa, suggesting that the gene size could be similar in all these stains [100,123,198,212].

The amino acid analysis data revealed protein of 1480 residues with the calculated molecular mass of 162.8 kDa. This agreed with the estimated molecular weight of the α-galactosidase by SDS–PAGE and gel filtration (data above). The higher content of hydrophobic amino acids is in well agreement with the fact that the enzyme is hydrophobic in nature since it binds to Phenyl Sepharose in the absence of ammonium sulfate. The higher content of acidic amino acids is also in agreement with the acidic p*I* of the protein and explained that most of the acidic amino acids are present as aspartic and glutamic acids in enzyme. There is also a high fraction of charged lysine and arginine residues (3.85 and 6.75 %, respectively). The fraction of lysine residues is comparable to that found in moderately thermostable enzyme (3.95) [208] and is larger than corresponding fraction found in mesophilic α-galactosidase (2.52 %) [138]. The high content of proline residues (5.0 %) is explained its propensity to stabilize β-turns and external loops in thermostable enzymes [403]. The present α-galactosidase contains no free cysteine residues in the protein and is surprising in the view of the fact that cysteine residues have been shown to be conserved in family 27 and 36 of glycosyl hydrolases [198,203], however the exact role of these cysteine residues is still unclear.

Unlike eukaryotic α-galactosidases [74,208,219,221], purified α-galactosidase from *Bacillus stearothermophilus* is a non-glycosylated protein.

The partial N-terminal sequence of the first 10 amino acid residues of the α-galactosidase revealed 10-70 % similarity with the earlier reported α-galactosidases from various sources (Table 3). The highest similarity (70 %) was observed with α-galactosidase from *Bacillus stearothermophilus* (NUB 3621) [198]. On the basis of sequence similarity, α-galactosidase from *B stearothermophilus* (NCIM 5146) should belong to the family 36 of glycosyl hydrolases.

The secondary structure of the protein is determined by CD spectroscopy. The far UV CD spectra of α-galactosidase at 50 ºC indicated presence of predominance of α-helical components. The protein type seems to be α/β class because the 208 nm band is of lower intensity than 222 nm band (in α+β proteins the 208 nm band is of higher intensity than 222 nm band [404]. The secondary structure of α-galactosidase from microbial origin has not been reported so far, however the crystal structure of rice α-galactosidase shows $(\beta/\alpha)_8$-barrel structure [289].

Changes in secondary structure of α-galactosidase as a function of temperature were monitored and data was analyzed by prediction method. The amount of α-helix and β-sheets vary with temperature, by contrast amount of β-turns were steadily at 20-25 %. The most stable structural conformation of α-galactosidase was observed at 50 ºC, where enzyme exhibited minimum amount of unordered structure (random coils). α-Galactosidase retains its secondary structure up to 70 ºC; however, it shows small changes in apparent secondary structure (α-helix content) above 50 ºC. The thermal stability of α-galactosidase was decreased above 50 ºC (Figure 11) with concomitant decrease in α-helix content (Table. 4), suggesting that thermostability is associated with the helix content of the enzyme.

α-Galactosidase from *Bacillus stearothermophilus* (NCIM 5146) was most active and stable in the neutral to alkaline pH range (6-9) and exhibited moderate stability towards the acidic side of the pH, similar to the other bacterial α-galactosidases [235,236].

The α-galactosidase described here is moderately thermostable and active only up to 70 ºC and exhibited half-life of 30 minutes at this temperature. The most thermostable α-galactosidase previously reported from *Bacillus stearothermophilus* has half-life of 19 hours at 70 ºC [198]. The relationship between molecular properties and thermal stability in genus *Bacillus* is summarized in Table 13. We hypothesized that the oligomerization of α-galactosidase in genus *Bacillus* could be responsible for its higher temperature stability, as trimeric and tetrameric forms of α-galactosidases have higher temperature optima and stability compare to dimeric form. However thermostability of α-galactosidases in *Bacillus* sp. is not as high as those of *Thermotoga* [115] and *Thermus* enzymes [116].

Table 13. Relationship between molecular properties and thermal stability of α-galactosidase in genus *Bacillus* :

Organism	Mol. mass, kDa.		Temp. (ºC).		Ref
	Native	Subunit	Opt.	Stability.	
Bacillus stearothermophilus (NCIM5146)	165.9 (2)	79.9	65	t/2 at 65°C, 60 mints. t/2 at 70°C, 35 mints	P
Bacillus stearothermophilus (ATCC-266)	247 (3)	82	-	70 % at 65º C, after 24 hrs.	100
Bacillus stearothermophilus (NUB 3621)	320 (4)	83.3	75	t/2 at 70 ºC,19 hrs. t/2 at 75 ºC,3.5 hrs	198
Bacillus stearothermophilus (AT -7) - I	280 (4)	81	73	t/2 at 65 ºC, > 2 hrs.	212
- II	320 (4)	84	69	t/2 at 65 ºC, 3 mints.	
Bacillus stearothermophilus (KVE39) -I	---	80	68	80 % at 65ºC, 4 hrs.	123
-II	---	---	50	-----	

Abbreviations: P-Present strain, ( ) Number of subunits are given in bracket, t/2 –half life, Opt-optimum temperature.

Substrate specificity of the α-galactosidase was determined using various synthetic as well as natural glycosides (Table 5). Purified enzyme exhibited strict specificity towards the α-anomeric carbon atom of the substrate molecule and therefore only hydrolyzes α-galactosidic and not the β-galactosidic linkages [112]. The enzyme also exhibited strict specificity towards the glycone group of the substrate molecule and hence only attacks α–D-galactosides and not the α–D-mannosides and α–D-glucosides, indicating that orientation of hydroxyl groups around C_2 and C_4 of the glycone moiety is important. The structural analogues, α–D-fucoside and β-L-arabinoside were also not hydrolyzed, suggesting that the primary alcohol group of D-galactose moiety is important for both binding and catalysis. Aglycone group of the substrate also plays an important role in substrate specificity of enzyme as the phenyl, methyl and other naturally occurring galactosides were hydrolyzed at different rate. On the other hand α-galactosidase reported from *Vicia faba* seeds seems to be less specific with respect to the glycone and aglycone moiety of the substrate [241].

The α-galactosidase showed highest activity on *p*NP-α-Gal (80 times more than raffinose). The presence of the nitro group at the *ortho* position (*o*NP-α-Gal), reduces 60 % of activity compared to *p*NP-α-Gal. Where as nitro group at meta position did not show any activity indicating that position of substitution in aglycone ring is important for enzyme action. By contrast all the substituents in the aryl moiety of aryl α-galactosides enhances the V_{max} of the sweet almond α-galactosidase [261].

Among the natural glycosides, melibiose a disaccharide of glucose and galactose was the better substrate than raffinose and stachyose. Stachyose, although, a tetrasaccharide had almost double the rate of hydrolysis compare to raffinose. This is surprising in view of the fact that, in a homologous series of α-galactosides an increase in chain length tends to decrease the rate of hydrolysis [56]. Further the TLC analysis of hydrolysis of stachyose indicated accumulation of raffinose during the reaction (Figure 12A). So we can conclude that liberation of first galactose residue from stachyose is the rate-limiting step of the stachyose hydrolysis [263].

The enzyme shows very limited hydrolysis of polymeric substrates as compare to *p*NPG and other oligomeric substrates, indicating that enzyme is specific only for small oligomeric substrates [100]. In contrast, few fungal [82,94,267] and many plant α-galactosides [41,405,406] are known to degrade the polymeric substrates; guar gum and locust bean gum with high efficiency. Such diversity in substrate specificity of α-galactosidases reflects its physiological significance in natural environment.

Apart from α-1-6 galactosidic linkages, present α-galactosidase also cleaves α-1-3 and α-1-4 galactosidic linkages present in disaccharide sugars. Enzyme having α-1-3 type of bond specificity is of great practical interest in blood transfusion since it converts erythrocytes of group 'B' blood cells to universally donor group 'O' type cells. Several α-galactosidases having such specificity are known [79,354,210]. But the successful transfusion of erythrocytes, which were enzymatically transformed, was done only with coffee bean α-galactosidase [407,408].

Kinetic studies of α-galactosidase indicated that substrate dependent K_m and V_{max} are unrelated to each other. A substrate with a lower K_m (except synthetic substrates) is not necessarily rapidly hydrolyzed. Similarly, certain substrates with equal K_m (melibiose and raffinose) were hydrolyzed at different rates. Moreover substrates with different K_m (raffinose and stachyose) were hydrolyzed at similar rate. This suggests that K_m is more or less equal to the dissociation constant of enzyme substrate complex [261].

Thermodynamic parameters of activation and binding of different glycosides were calculated from the Arrhenius and van't Hoff plots, respectively (Table 9 &10). Results indicated that the contributions from the enthalpic components for the binding of galacto-oligosaccharides are moderate and do not vary appreciably with the nature of the substrate. However the relative affinity of the galacto-oligosaccharides towards the enzyme is determined by ΔΔS contributions suggested that binding might be enthalpy as well as entropically driven. The highest catalytic efficiency of *p*NPG and melibiose was due to the highest transition state stabilization ($\Delta G^{\#}_{tb}$ =-34.66, -24.0, respectively) together with the positive entropy of activation suggested these substrates fits properly in the active site cleft and induce less conformational changes during the catalysis. However raffinose and stachyose, shows high losses in activation entropy; indicated strained

substrates and induces high conformational changes. High affinity of tetrasaccharide over trisaccharide may be due to the extra galactose residue in tetrasaccharide, which may form an extra hydrogen bond and or hydrophobic interaction with enzyme. The thermodynamic contributions of binding of the extra galactose residue in tetrasaccharide vs. trisaccharide are estimated to be $\Delta\Delta G_{b=}$ -1.9 kJ, and significant and favorable entropy change $\Delta\Delta S_b$ = 9.83 kJ. However the catalytic efficiency of both the substrates is similar. This may be due to the tetrasaccharide, which couldn't hydrolyze at higher efficiency although; it releases higher energy of transition state stabilization and ground state destabilization over trisaccharide ($\Delta\Delta G^{\#}_{tb}$ -2.07, $\Delta\Delta G_{b=}$ -1.9 kJ, respectively). This can be justified by the observation that, the removal of first galactose residue from stachyose is the rate-limiting step in the stachyose hydrolysis (Figure12A). Hence stachyose may have similar rate of hydrolysis as with raffinose. The calculated ΔH_b and ΔS_b values in this study are negative, hence it could be concluded that the protein-ligand interactions are due to the hydrogen bonding [409]. Such type of temperature dependence studies have been reported earlier for α-galactosidases and values of E_a, ΔH_b, ΔS_b, have been calculated, however significance of these thermodynamic parameters in accordance with the kinetic parameters have not been discussed so far [233,241].

Arrhenius plots of ln K_{cat} versus 1/T shown here is discontinuous or biphasic (Figure 14) with two characteristic activation energies for all the substrates. Such biphasicity in the Arrhenius plot has not been reported earlier in α-galactosidases. However discontinuous Arrhenius plots have been observed in membrane proteins [410] and also in certain enzymes from thermophilic microorganisms [411]. Various reasons for discontinuous Arrhenius plots have been postulated. In the membrane proteins, the inflection temperature has been related to the melting temperature of the lipid fraction and suggested that a phase change is responsible for breaks in the slope of Arrhenius plots of biological systems [410]. Another possible hypothesis is the existence of two conformational states of the enzyme above and below the inflection temperature, each with a different catalytic competence [412]. Latter reason could be true in case of present enzyme since CD analysis of the enzyme at different temperatures showed differences in molar ellipticities above and below the inflection temperature (Table 4).

α-Galactosidases has been generally known to catalyze hydrolytic reactions, however some of the α-galactosidases also found to have galactosyl transfer activity [58,94,112]. The process of hydrolysis will take place when acceptor is a water molecule. However the process of transglycosylation is believed to proceed by transfer of a glycosyl moiety to acceptor molecules, which are generally hydroxylic compounds. The present enzyme also showed weak transferase activity and transfers galactosyl residue to the acceptor sugars and produced two new spots on TLC, tentatively identified as

melibiose and stachyose. Raffinose was the only sugar acting as a galactosyl donor as well as acceptor may be due to it hydrolyzes at slower rate [234].

α-Galactosidase activity was strongly inhibited by Ag^{+}, Hg^{2+}, Cu^{2+} metal ions and thiol modifier, PCMB as several other α-galactosidases [88,132,198,413], this inhibition usually suggests the reaction with thiol groups and/or carboxyl, amino and imidazolium group of histidine at the active site [36]. However amino acid analysis of the present enzyme showed no free cysteine residues. Kinetic study of enzyme inhibition indicated that thiol modifier (PCMB) and other metal ions showed noncompetitive inhibition of enzyme activity. Therefore the inhibition of α-galactosidase activity due to the modification of active site residues is discounted. Hence the reaction of metal ions /reagent with above amino acid residues at centers other than the active site is of course possible [36]. No divalent cation tested stimulated enzyme activity nor was inhibited by EDTA suggesting that enzyme is unlike *E coli* α-galactosidase [251], and did not require any co-factor for activity. Inhibition by Tris is not unusual in α-galactosidases [212] and could be due to the competitive nature of the inhibitor. The purified enzyme also showed substrate/ product inhibition by galactose, melibiose and stachyose as like other α-galactosidases [212]. The nature of inhibition was of course competitive for all the sugars [186]. Lactose inhibition could be due to substrate analogue but reacts uncompetitively with the enzyme.

In conclusion, Table 13 summarizes certain properties of the purified α-galactosidase from *Bacillus stearothermophilus* NCIM-5146 and *Bacillus stearothermophilus* NUB-3621. Although, both the enzyme shares 70 % of N-terminal sequence similarity some of their biochemical and molecular properties are distinct. Therefore, it could be concluded that α-galactosidase isolated and purified from *Bacillus stearothermophilus* NCIM-5146 is a novel enzyme.

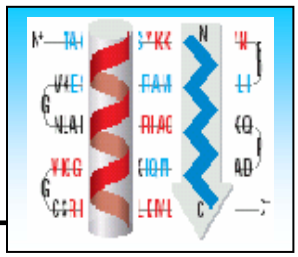
Table 13. Certain characteristics of the α-galactosidase from NCIM-5146 as compared to those of NUB-3621 :

Organism	pH Opt (Stab)	Temp. ºC Opt (stab t1/2)	Molecular mass (kDa.)		No. of Subunit	Rf.
			Native	SDS-PAGE		
B. stearothermophilus (NCIM- 5146)	6.5-7.0 (6-9)	65, (70 ºC, 35 mint.)	165.9	79.9	2	p
B. stearothermophilus (NUB- 3621)	6.5 (ND)	75, (70 ºC, 19 hrs)	320	80.3	4	198

ND - not determined, Ref (P)-Present strain, Opt-Optimum, Stab-Stability.

CHAPTER - 4

Active Site Directed Chemical Modification of α-Galactosidase from *Bacillus stearothermophilus* (NCIM -5146).



4.1 Summary :

The catalytic amino acid residues of α-galactosidase from *Bacillus stearothermophilus* (NCIM -5146) was investigated by the pH dependence of the reaction kinetic parameters and active site directed chemical modification studies. The pH dependence curves gave apparent pK_a value of 6.1 and 8.2 for the free enzyme, while pK_a value of 7.84 was obtained for the enzyme substrate complex using *p*-nitrophenyl-α-D-galactopyranoside as a substrate. These results suggested that an ionized and a protonated group, presumably perturbed carboxylate and a lysine residue took part in catalysis and only lysine residues were essential for substrate binding. Chemical modification studies by amino acid group specific reagents also showed that a carboxylate, tryptophan and amino group of lysine are present at the active site. Further, Carbodiimide modification of enzyme 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 by acetic and citraconic anhydride and sodium borohydride suggested that the protonated lysine residues carrying positive charge on its ε-amino group and exist in solution as NH_3^+ species provides the positive charge density for binding of the substrate and in the stabilization of transition state. We suppose that binding could take place through **H-bonding** between electrostatic hydroxyl oxygen of galactose and protonated amino group of lysine. The kinetic analysis of lysine modified enzyme showed partial increase in the K_m and significant decrease in the $K_{cat,}$ suggest a mechanism in which substrate and enzyme combine irrespective of the protonated state of the groups required for binding and catalysis but only the correctly protonated enzyme substrate complex is catalytically active. Kinetic study suggested that one of lysine residue of the four that are present at the active site must in some manner directly or indirectly implicated in the catalysis. Additionally four tryptophan residues also 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.

4.2 Introduction:

Many α-galactosidases of prokaryotic and eukaryotic sources have been studied extensively for their biochemical and biophysical properties [82,53,100]. Moreover crystallization and preliminary X-ray analysis of some of the α-galactosidases has been reported [414,415]. However structure and a detailed catalytic mechanism of the enzyme have yet to be elucidated at the molecular level. Recently, the crystal structure of rice α-galactosidase has been reported, which throws some light on the mode of substrate binding and catalytic mechanism in family 27 of glycosyl hydrolases [289].

Active site directed chemical modification is an important tool for studying structure-function relationship of enzymes and other biologically active proteins. Usually this procedure is used to identify residues at the active site of an enzyme and to elucidate the chemical mechanism of enzymic catalysis. Site-directed mutagenesis is now widely used for the same purpose, but to use it, one really needs not only the cloned gene but also the three-dimensional structure of the protein. In contrast, active-site-directed chemical modification can be done without knowing anything about the enzyme structure.

Very few studies have been carried out to find out the amino acids present at or near the active site of α-galactosidases. Studies on inactivation of α-galactosidase from *Humicola* sp. by group specific reagents showed the possible involvement of tryptophan, lysine and carboxylate at or near the active site [303]. In contrast, methionine in *Trichoderma reesei* [300] and aspartate-130 in *Phanerochaete chrysosporium* α-galactosidase [302] have been implicated as active site residues. Studies on plant α-galactosidases showed the functionally important imidazole, carboxylic acid and phenolic groups in the catalysis [301,242]. The acid base catalytic mechanism was also proposed for coconut kernel α-galactosidase [242,250]. However no attempt has been made to find out the nature of active site in bacterial α-galactosidases. In the present work, we identified carboxylic acid, amino and indole group of tryptophan at or near the active site of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146).

4.3 Materials and methods :

Materials :

N-ethylmaleimide (NEM), 2,2-dithiobisnitrobenzoic acid (DTNB), Phenylmethyl-sulfonylfluoride (PMSF), *p-C*hloromercuribenzoate (PCMB), Diethylpyrocarbonate (DEPC), 3-Nitro-L-tyrosine ethylester (NTEE), 1-Ethyl-3-(3-dimethylaminoproply) carbodiimide (EDAC), 2,4,6-Trinitrobenzenesulfonic acid (TNBS), N-Bromosuccinimide (NBS), N-Acetylimidazole (NAI), 2,3-Butanedione, Citraconic anhydride, Acetic anhydride, Woodward's reagent K (WRK), HEPES, and MES were purchased from Sigma Chemical Company USA. Analytical grade chemicals and reagents were used.

Organism, enzyme production and purification :

Growth, maintenance of *Bacillus stearothermophilus* (NCIM 5146), production and purification of α-galactosidase was carried out as described in the Chapter-2 and 3. The purified α-galactosidase was used for the modification study.

Methods :

Enzyme assay :

During the course of modification reactions, α-galactosidase activity was estimated by incubating 100 µl of suitably diluted enzyme with 50 µl of 6.66 mM of chromogenic substrate (*p*-nitrophenyl-α-D-galactopyranoside) and 850 µl of 50 mM phosphate buffer (pH 7.0) at 65 °C for 10 minutes. The reaction was terminated by addition of 2 ml of 1M sodium carbonate and *p*-nitrophenol liberated was estimated spectrophotometrically by absorbance at 405 nm. One unit (IU) of α-galactosidase activity was expressed as the amount of enzyme required to liberate 1 µmole of *p*-nitrophenol per minute per ml under the assay conditions. Residual enzyme activity after modification was expressed as % of activity with unmodified sample.

Protein estimation :

Protein concentration in the purified enzyme preparation was determined by method of Lowry *et al.* [374]. The protein concentration in the modified enzyme samples was estimated by dye binding method of Bradford [416]. Every time bovine serum albumin was used as a standard.

pH dependence studies :

The effect of pH on K_m and K_{cat} values of α-galactosidase were determined by varying *p*NPG concentrations in the range of 0.066-0.399 mM, using 50 mM citrate phosphate buffer (pH range, 5.5-6.5) and potassium phosphate buffer (pH range, 7.0-9.0). All the assays were performed by standard assay method. Kinetic constants were calculated using Lineweaver Burk plots and the *p*Ka values of the ionizable groups in the free enzyme and in enzyme substrate complex were determined according to Dixon [417].

Effect of group specific reagents on α-galactosidase activity :

Purified α-galactosidase (25 µg, ~0.150 µM) was incubated with chemical reagents specific to various amino acid functional groups at specific concentration and reaction conditions given in Table 3. After 30 minutes of reaction at room temperature residual activity of enzyme samples was determined by standard assay method.

Modification of histidine :

Purified α-galactosidase (1ml, 100 μg) in 50 mM Sodium phosphate buffer, pH 6.0, was treated with DEPC (100 mM, freshly diluted in absolute ethanol) at 25 ºC. The reagent (5 μl) was added in four installments to obtain an effective concentration of 0.5, 1.0, 1.5, and 2.0 mM, respectively. After each addition the reagent was allowed to react with enzyme for the period of 5 minutes. Subsequently an aliquot 5-μl was removed and assayed for enzyme activity under standard assay conditions. Control sample was treated similarly as above; only DEPC in the reaction mixture was replaced by absolute ethanol. The DEPC mediated modification was also monitored spectrophotometrically by following the increase in absorbance at 240 nm. The total number of histidine residues modified was calculated by considering molar absorption coefficient of 3200 M^{-1} cm^{-1} for carbethoxyhistidine at 240 nm [418]. The side reactions of DEPC with cysteine, lysine and tyrosine residues were also evaluated by calculating number of above residues modified in DEPC treated enzyme samples by their respective modifying agents DTNB, TNBS and NAI.

Modification of cysteine :

Purified α-galactosidase (1ml, 100 μg) in 50 mM Sodium phosphate buffer, pH 8.0, was incubated with DTNB (final concentration 2 mM) at 30 ºC for 30 minutes. Enzyme incubated in absence of DTNB served as control. Aliquots (5 μl) were removed at regular time interval and residual activity was determined under standard assay conditions. The number of cysteine residues modified was determined at 412 nm using a molar absorption coefficient of 13600 M^{-1} cm^{-1} [388].

Modification of tyrosine :

Purified α-galactosidase (1ml, 200 μg) in 50 mM sodium borate buffer, pH 7.5, was incubated with NAI (1mM) at 30 ºC for 30 minutes. Enzyme incubated in absence of NAI served as control. Small aliquots (5 μl) were removed from reaction mixture at regular time interval and residual activity was determined by standard assay method. The number of tyrosine residues modified was calculated by using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm [419].

Modification of lysine :

***Reaction with TNBS* :**

The free amino group of lysine was chemically modified by the TNBS method of Habeeb [420]. Aliquots (1ml) of purified α-galactosidase (200 μg) in 4 % sodium carbonate bicarbonate buffer, pH 8.4, were treated with varying concentration of TNBS (0.1 mM - 1.5 mM) in dark. After 30 minutes of incubation at 30° C, small aliquots (5 μl)

were removed from every reaction mixture and residual activity was determined by standard assay method. Subsequently in the remaining reaction mixture 0.5-ml of 1N HCl and 0.5 ml of 10 % SDS was added to terminate the reaction. The number of amino groups modified was determined spectrophotometrically by using molar absorption coefficient of 9950 M^{-1} cm $^{-1}$ at 335 nm for trinitrophenylated lysine. Enzyme sample incubated in absence of TNBS served as control.

Reaction with acetic anhydride :

Acetylation of ε-amino group of lysine and N-terminal amino group in α-galactosidase was carried out according to Riordan and Vallee [421]. Aliquots (1ml) of purified α-galactosidase (200 µg) in saturated sodium acetate (pH 8.0) was incubated in ice bath at around 4 °C and titrated with small aliquots (usually 5-10 µl) of acetic anhydride (100 mM stock, in 1,4 dioxane). The final concentration of reagent in the enzyme samples was 1-10 mM. The pH of the reaction was maintained at 8.0 with addition of 2N NaOH. Control sample was treated similarly as above; only acetic anhydride in the reaction mixture was replaced by 1,4 dioxane. After an incubation period of 20 minutes, volume was made up and small aliquots (10 µl) were removed from all the enzyme samples and residual activity was determined by standard assay method. Subsequently, all the modified enzyme samples along with control were dialyzed against carbonate buffer (100 mM), pH 8.0 and lyophilized. Finally the amino group in control and modified enzyme samples was estimated using TNBS method of Habeeb [420].

Reaction with citraconic anhydride :

The ε-amino group of lysine was reversibly blocked with citraconic anhydride according to the method of Dixon and Perham [422]. Aliquots (1ml) of purified α-galactosidase (200 µg) in sodium carbonate bicarbonate buffer (100 mM), pH 8.0, were treated with citraconic anhydride at 25° C. The reagent (200 mM stock, in 1,4 dioxane) was added slowly (5-10 µl aliquots) to obtain an effective concentration in the range of 2.0-20 mM. The pH of the reaction was maintained at 8.0 by the addition of 2N NaOH. Control sample was treated similarly as above; only citraconic anhydride in the reaction mixture was replaced by 1,4 dioxane. After completion of reaction (20 minutes), volume was made up and small aliquots (10 µl) were removed from all the enzyme samples and residual activity was determined by standard assay method. Subsequently, remaining enzyme samples were dialyzed separately against carbonate buffer (100 mM), pH 8.0 and lyophilized. The number of amino groups in control and treated enzyme samples was estimated by TNBS method of Habeeb [420].

Reductive methylation :

The reductive methylation of primary amino group of lysine was carried out according to the Means and Feeney [423]. Purified α-galactosidase 1 ml (200 μg) in sodium borate buffer (100 mM), pH 9.0, containing 0.5 mg/ml sodium borohydride was incubated at 0° C, followed by five aliquots (20 μl each) of formaldehyde solution 0.37 % (v/v) was added at an interval of 5 minutes. After completion of reaction the excess reagent was removed by passing through G-25 column. Control was treated under similar conditions in absence of formaldehyde. Further, the residual activity was determined by standard assay method and number of amino groups in the control and modified enzyme samples were determined by TNBS method of Habeeb [420].

Modification of tryptophan :

Aliquots of purified α-galactosidase 1ml (200 μg) in 100 mM sodium acetate buffer, pH 3.0-5.0, were titrated with 5 μl aliquots of *N*-Bromosuccinimide (NBS) (stock 0.250-2.0 mM) at room temperature. After each addition, an aliquot (5 μl) was removed, quenched with 20 μl of L-tryptophan (50 mM) and the residual activity was determined under standard assay conditions. The NBS mediated inactivation was also monitored spectrophotometrically by following the decrease in absorbance at 280 nm. The number of tryptophan residues modified was calculated by using molar absorption coefficient of 5500 M^{-1} cm^{-1} according to the Spande and Witkop [387]. Structural changes of enzyme after NBS modification were analyzed by SDS-PAGE and CD spectroscopy. The NBS modification was also carried out under denaturing conditions (10-M urea, pH 3.0) and the total number of tryptophan residues present in the enzyme was determined according to Spande and Witkop [387].

Modification of carboxylate :

Aliquots (1ml), of purified α-galactosidase (200 μg) in 50 mM MES/HEPS buffer (75:25), pH 6.0, were incubated with varying concentration of EDAC (10-200 mM) and NTEE (1mM), at 30 °C for 10 minutes. Small aliquots (5 μl) were withdrawn from every reaction mixture and reaction was terminated by addition of 100 μl of 1 M acetate buffer (pH 5.5), and the residual activity was determined by standard assay method. Subsequently, in the remaining reaction mixture 10 % trichloroacetic acid (1 ml) was added and the mixture left at 4 °C for 30 minutes to precipitate EDAC/NTEE enzyme complex. The precipitate was collected by centrifugation (7000 x g 5 minutes), washed extensively with chilled acetone, air dried and reconstituted in 0.5 ml of 0.1 M NaOH. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically at 430 nm using a molar absorption coefficient of 4600 M^{-1} cm^{-1} [424]. Enzyme samples incubated in the absence of EDAC/NTEE served as control.

Reversal of modification :

Decarbethoxylation of histidine and decitraconilation of lysine was carried out according to Miles [418] and Lundblad [425], respectively. The DEPC modified enzyme samples were incubated with 50-mM neutral hydroxylamine at pH 7.0 and 25 °C for a period of 3 hours followed by determination of the enzyme activity. The regenerated histidyl residues after hydroxylamine treatment were calculated from the shift in the absorbance at 240 nm.

Decitraconilation was carried out by incubating modified enzyme samples with 500-mM neutral hydroxylamine at 25 °C for 8 hours. Decitraconilation of lysine was also carried out according to Dixon Perham [422] by incubating modified enzyme samples in citrate phosphate buffer, pH 5.5 (100 mM) at 25 ° C for the period of 3 hours and enzyme activity was determined according to the standard method. For all the treatments native enzyme treated parallel served as control.

Determination of number of essential amino acid residues :

The number of amino acid residues essential for the activity of α-galactosidase was determined by the statistical method of Tsou [426,427]. This method is useful when only one type of essential and nonessential groups are modified at the same rate. The relation- ship between remaining activity against amino acid groups modified is given by the equation.

$$a^{1/i} = x \qquad \text{Eq. (1)}$$

Where, **'a'** is the fraction of activity remaining [usually **(A/A_0)**], **'x'** is the overall fraction of essential groups remaining fully active [**(n-m/n), 'n'** is the total number of residues present and **'m'** is the total number of residues modified] and **'i'** is the number of essential groups (to be presumed, 1,2,3,4…n). The number of essential amino acid residues is that value of **'i'** which gives a straight line when remaining activity **a,** $\sqrt{a}$, $\sqrt[3]{a}$ $\sqrt[4]{a}$ etc is plotted against the **'x'** or simply against the number of residues modified **(m),** since $a^{1/i}$ is proportional to **'m'.**

Substrate protection and kinetic studies :

In all chemical modification reactions the protective effect of substrate or competitive inhibitor on modification was studied by incubating enzyme with excess amount of its substrate/product; raffinose/galactose (10-50 mM) followed by treatment with various modifying reagents. The residual activity of enzyme was assayed periodically by standard assay method.

Kinetic constants K_m and K_{cat} for the native and partially modified α-galactosidase were determined using *p*NPG as a substrate (0.066-0.399 mM.) and the assays were

carried out under standard assay conditions. The values for K_m and K_{cat} were calculated from Lineweaver Burk Plot by fitting the data to the linear regression using Origin 4.0 (MicroCal).

Fluorescence and CD measurements :

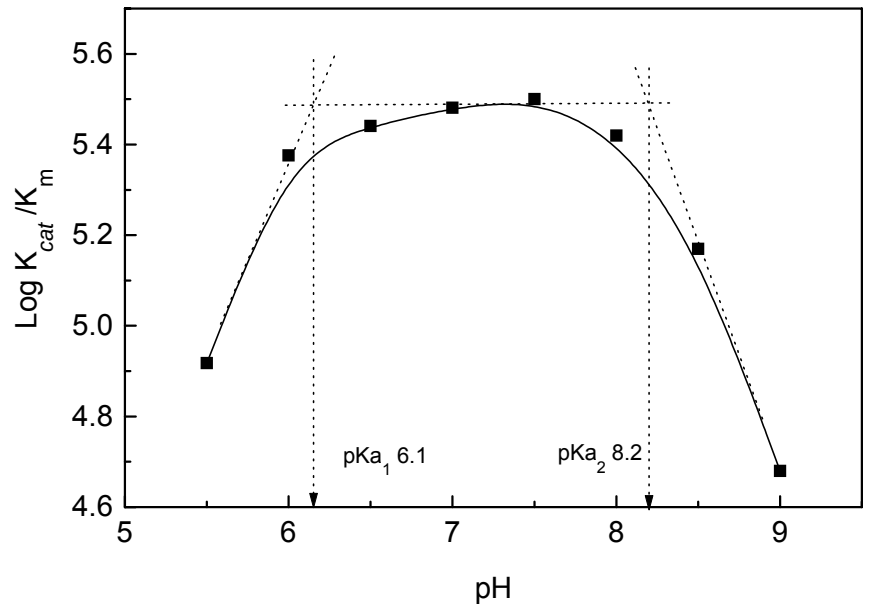
Modification of tryptophan by NBS was also monitored on Spectrofluoremeter (Perkin Elmer LS 5B), at 25 °C using a slit width of 5 nm for both excitation and emission. The enzyme solution (10 µg/2ml) in 50 mM-sodium acetate buffer, pH 3.0, was titrated with 0.2 mM NBS stock solution (5 µl aliquots) and fluorescence spectra were recorded from 300-400 nm using excitation wavelength of 280 nm.

Conformational changes in α-galactosidase after modification were determined by CD Spectroscopy. Spectra were recorded on a Jasco-710 Spectropolarimeter in the range of 190 to 250 nm using a 250-µl-sample holder and 0.1 cm path length, at 25 °C. The concentration of enzyme used was 200 µg/ml.

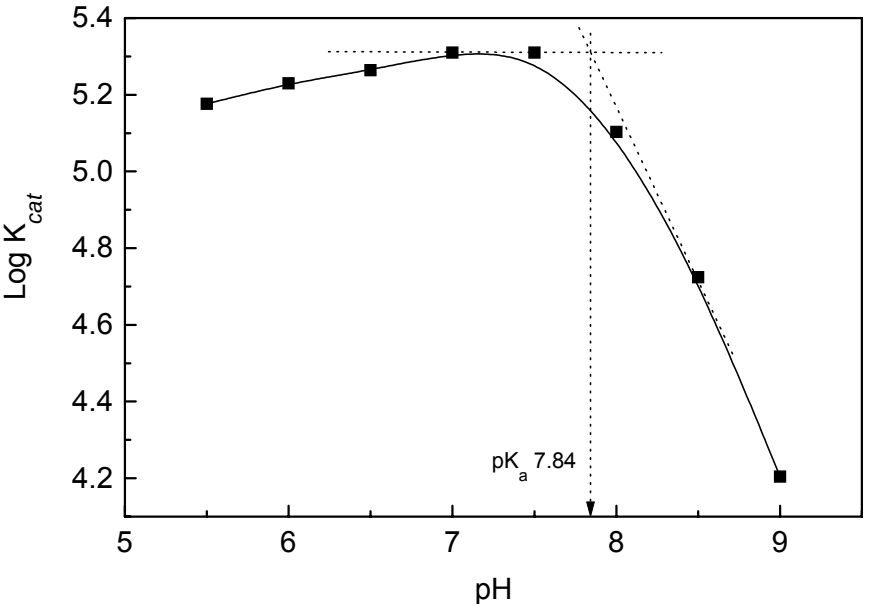
4.4 Results :

The pH dependence of Log (K_{cat}/K_m) and Log K_{cat} for *p*NPG hydrolysis are shown in Figure 1. The enzyme shows a bell shaped pH profile of Log (K_{cat}/K_m) (Figure 1A), indicating involvement of two ionizable amino acid residues in catalysis. One of the two ionizable groups must be ionized while other must be protonated. The protonated form of the group with pK_a of 8.2 is required for catalysis as affinity decreases with increase in pH. On the same basis the ionized form of the group with a pK_a of 6.1 is required as affinity increases with increase in pH. The effect of pH on K_{cat} is constant over the pH range 5.5-7.5 (Figure 1B). The graph indicates only one ionizing group with a pK_a of 7.84 involved in substrate binding. Form these results we can conclude that there could be two protonated groups with pK_a 7.84 and 8.1, presumably perturbed lysine residues in substrate binding and in catalysis. The ionized group with a pK_a of 6.1 has to be a perturbed carboxyl group required as a $-COO^-$ group for catalysis.

pK_as observed for various amino acid groups may vary according to the microenvironment in which these groups are located. Therefore, the value of a pK_a is only a very rough guide to the nature of the groups in the active site. In order to prove this assumption, chemical modification studies were carried out by group specific reagents.



A



B

Figure 1. Determination of pK_a : pH dependence of K_{cat}/K_m (A) and K_{cat} (B) of the hydrolysis of *p*NPG were obtained from the Lineweaver-Burk plot and analyzed according to the Dixion [417].

Active site characterization :

The importance of amino acid functional groups for the activity of α-galactosidase was determined by the use of chemical reagents with restricted amino acid specificity. The results of initial inactivation study are given in Table no 1. Lack of inhibition by NAI and TNM suggested the non-involvement of tyrosine in catalysis. Also inhibition was not observed in presence of PMSF, phenylglyoxal and 2,3 butanedione, indicating that serine and arginine residues do not take part in catalysis. Reaction of α-galactosidase with sulfhydryl reagents showed inhibition only with PCMB and not with NEM, DTNB and Iodoacetate. This inhibition by PCMB may be due to the noncompetitive nature of Hg^{++} ions (Chapter 3, Figure 19B) and not due to the modification of sulfhydryl groups.

α-Galactosidase was inhibited by DEPC, NBS, TNBS and EDAC, indicating presence of histidine, tryptophan, lysine, and carboxylate at or near the active site. In view of these observations the role of above residues at the active site of α-galactosidase was further investigated.

Table 1. Effect of group specific chemical modifiers on α-galactosidase activity :

Chemical Reagents	Conc^{n}.	Possible Reaction site	Buffer	Residual activity (%)
NEM	10 mM	Cys	Sodium phosphate, 50 mM, pH 7.5	100
PCMB	10 µM	Cys	Sodium acetate, 50 mM, pH 5.8	16
DTNB	1 mM	Cys	Sodium phosphate, 50 mM, pH 8.0	100
Iodoacetate	1 mM	Cys	Sodium phosphate, 50 mM, pH 8.0	100
NAI	10 mM	Tyr	Sodium borate, 50 mM, pH 7.5	100
TNM	100 µM	Tyr	Sodium borate, 50 mM, pH 7.5	100
PMSF	2 mM	Ser	Sodium phosphate, 50 mM, pH 7.5	100
Phenylglyoxal	10 mM	Arg	Sodium bicarbonate, 50 mM, pH 8.0	100
2,3 Butanedione	10 mM	Arg	Sodium borate, 50 mM, pH 8.0	100
TNBS	2 mM	Lys	Sodium bicarbonate, 4.0 %, pH 8.4	20
Citraconic anhydride	5 mM	Lys	Sodium bicarbonate, 50 mM, pH 7.8	30
Woodward's Reagent K	5 mM	Asx/Glx	Sodium phosphate, 50 mM, pH 6.0	21

EDAC	100 mM	Asx/Glx	MES /HEPES, 75: 25 mM pH 6.0	31
DEPC	2 mM	His	Sodium phosphate, 50 mM, pH 6.0	00
NBS	20 μM	Try	Sodium acetate, 50 mM, pH 5.0	00

Modification of histidine :

Carbethoxylation of α-galactosidase by DEPC at pH 6.0 resulted in the loss of approximately 67 % of its initial activity. Difference spectroscopy of DEPC mediated inactivation showed increase in absorption around 240 nm (Figure 2) suggesting modification of histidine residues. The inactivation was found to be concentration dependent. No loss of activity was observed in the control samples. Based on a molar absorption coefficient of 3200 M^{-1} cm^{-1}, for carbethoxyhistidine at 240 nm and molecular mass of 165.9 kDa for α-galactosidase, we calculated that 21 histidines (47 %, Table 2) out of 44 (amino acid analysis, Table 2, Chapter 3) were modified by DEPC treatment. The incubation of modified enzyme with 50 mM of hydroxylamine reverses the modification and 100 % of the modified histidines were regenerated (shown by shift in the absorbance at 240 nm) (Figure 3). However, the residual activity of α-galactosidase was not restored. Further activity was not protected by incubation with substrate. Indicating that loss of activity was not due to the modification of histidine residues.

As DEPC also reacts with tyrosine, cysteine and lysine residues the loss of activity was further correlated with the modification of above residues by their respective modifying agents NAI, DTNB and TNBS. Treatment of α-galactosidase with NAI although resulted in the modification of 15 tyrosines, no loss in α-galactosidase activity was detected (data not shown). Further the modification of enzyme with DTNB showed neither loss of activity nor free cysteine residues in α-galactosidase (Table 2, Chapter 3). Quantitation of DEPC modified enzyme by TNBS showed blocking of 11.0 lysine residues (Table 2), suggesting that modification of lysine residues could be responsible for the loss of α-galactosidase activity. Thus the role of lysine at the active site of enzyme was further investigated in this study.

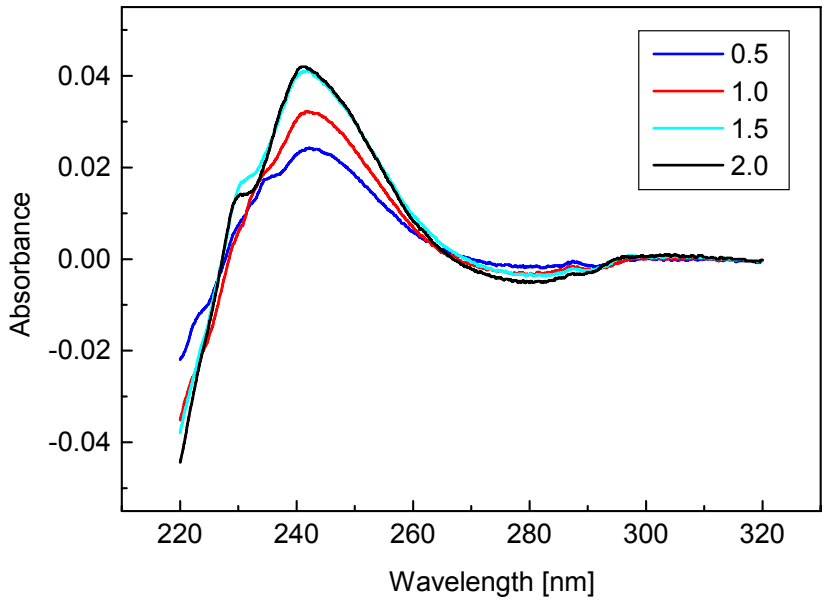


Figure 2. Difference spectra of histidine modification in α-galactosidase. For experimental details refer to methods. Inset shows the effective concentration of DEPC (mM) in reaction mixture.

Table 2. Effect of different concentrations of DEPC on the modification of amino acid groups in α-galactosidase :

DEPC Concentration (mM)	Amino acid residues modified* (moles /mole)		Residual Activity (%)
	Histidine	Lysine	
Control	0	0	100
0.5	11.70	4.0	85
1.0	15.72	6.5	65
1.5	20.72	8.5	50
2.0	21.24	11.0	33
Decarbethoxylation	0.0	11.0	33
Substrate protection [a]	21.24	--	53

*Tyrosine and Cysteine residues were not modified at this reaction conditions.

[a] Protective effect of substrate was monitored by incubating enzyme with 50 mM of raffinose/galactose, followed by treatment with 2.0 mM DEPC.

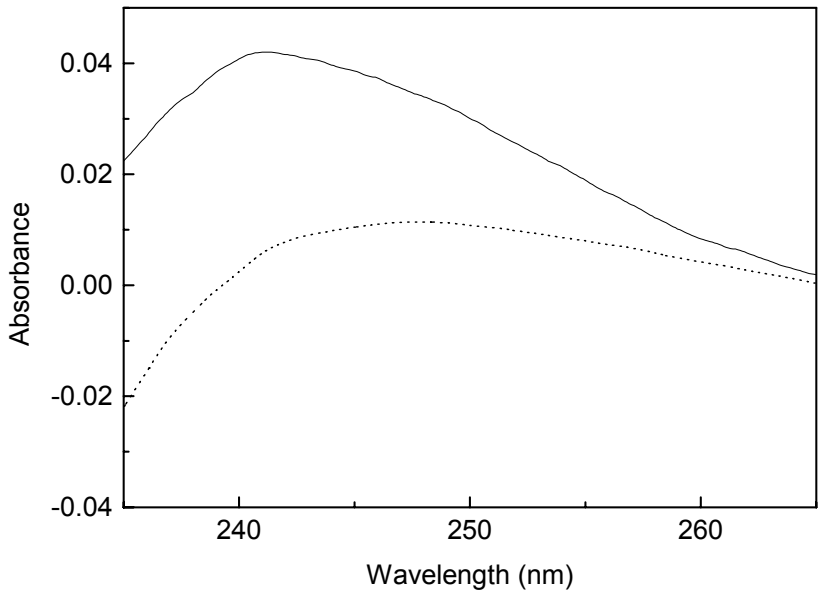


Figure 3. Treatment of DEPC modified α-galactosidase with hydroxylamine. UV-spectra before (doted line) and after (solid line) hydroxylamine treatment are shown. The arrow indicates the decrement in the absorbance at 240 nm. Conditions; 50-mM hydroxylamine, pH 7.0, 3.0 hours.

Modification of amino group :

Reaction of α-galactosidase with TNBS showed 90 % loss of enzyme activity, when as many as 13 lysine residues per mole of enzyme were modified (Figure 4). The inactivation was found to be concentration dependent and did not follow the pseudo first order kinetics. These results indicated that lysine is present at the active of α-galactosidase. Moreover pH dependent study has also indicated that two protonated (positively charged) lysine residues are present in catalysis (Figure 1A & B). To prove this assumption and absolute essentiality of the positive charge on ε-amino group of lysine residues, we produce the charge variants of this residue by acylation and reductive methylation reactions.

The acylation of ε-amino group of lysine with acetic anhydride gives acylated derivative. This reagent suppresses the protonation of ε-amino group of lysine, and thus the derivative carries no charge. This charge neutralization with acetic anhydride also leads to the 62 % loss of α-galactosidase activity concomitant with modification of 10.24

lysine residues (Table 3). Moreover, introduction of an overall negative charge in to the side chain of primary amino group of lysine by means citraconic anhydride resulted in a 68 % loss of α-galactosidase activity (Table 3). The inactivation was readily reversible and decitraconilation of modified enzyme at pH 5.5 and/or by treatment with hydroxylamine restores 85 % of its initial activity. However, reductive methylation of free amino groups did not have any effect on the activity of α-galactosidase; it retained 100 % of its initial activity even when all the 13 amino groups accessible for modification were blocked (Table 3). Reductive methylation maintains the positive charge on lysine residues.

Thus the above findings suggested that net positive charge on amino group of lysine plays an important role in enzyme active site.

The correlation of inactivation of α-galactosidase and number of amino groups modified by acetic anhydride is shown in Figure 5. The number of essential amino groups for the activity of α-galactosidase was further calculated by statistical method of Tsou (Figure 6). The data could be fitted to the straight line when i = 4, suggesting that modification of four lysine residues per monomer are critical for the activity.

Structural changes of enzyme after trinitrobenzylation, citraconilation and acetylation were monitored by CD spectroscopy (Figure 7a) and by electrophoretic mobility under non-denaturing conditions (Figure 7b). Results showed that under strictly controlled reaction conditions loss of enzyme activity was found due to the modification of lysine residues and not due to any structural changes. The analysis of enzyme samples by electrophoresis exhibited higher electrophoretic mobility after trinitrobenzylation and citraconilation, this could be due to the changes in charge property of the enzyme.

Incubation of enzyme with excess amount of its substrate prior to citraconilation and acetylation confers only 12-13 % of protection against inactivation (Table 4). Kinetic study of citraconilated and acetylated enzyme revealed moderately increase in k_m values and significantly decrease in K_{cat} values (Table 5).

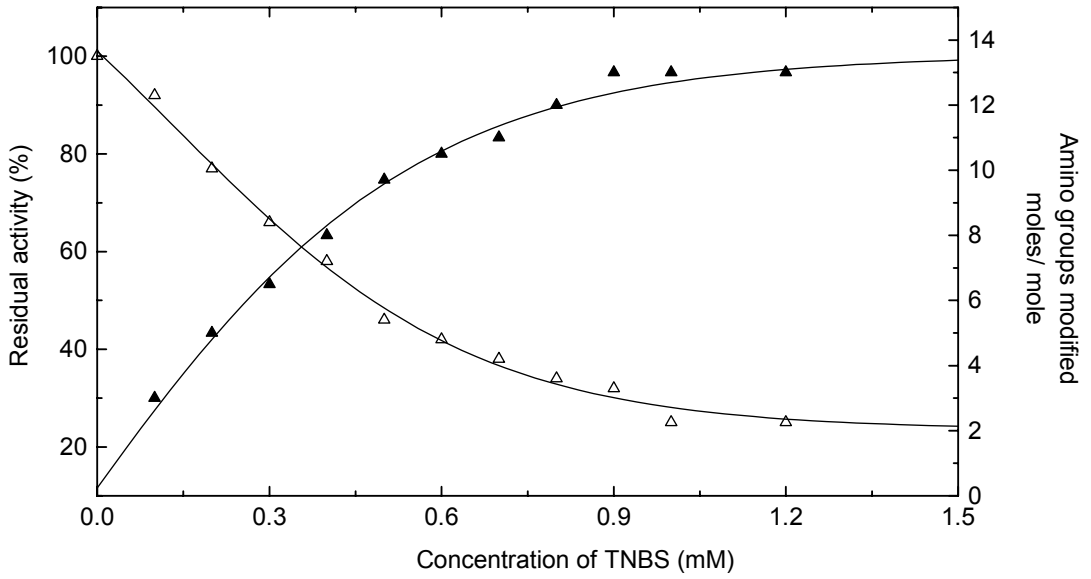


Figure 4. Effect of TNBS on inhibition of α-galactosidase and the modification of amino groups. Residual activity (-Δ-) and amino groups modified (-▲-) was determined as mentioned in methods.

Table 3. Influence of amino group modification on the activity of α-galactosidase :

Modification reactions	Amino groups modified. moles/mole	Residual activity (%)
Enzyme (Control)	0	100
Enzyme (200 μg) + Acetic anhydride (10 mM)	10.24	38
Enzyme (200 μg) + Citraconic anhydride (20 mM)	12	32
Enzyme (200 μg) + Sodium borohydride (0.5 mg/ml) + Formaldehyde (150 μl, 0.37 % v/v)	13	100
Decitraconilation (Hydroxylamine, 500 mM, OR Incubation in pH 5.5 buffer)	0	85

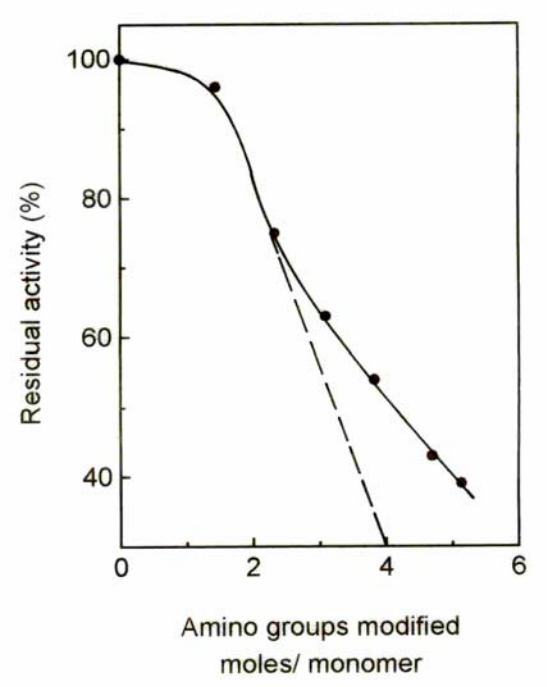


Figure 5. Relationship between residual activity and the number of lysine residues modified by acetic anhydride. The enzyme activity and residues modified was determined as described in the text. Tangent indicates the number of essential amino groups for the activity of a-galactosidase.

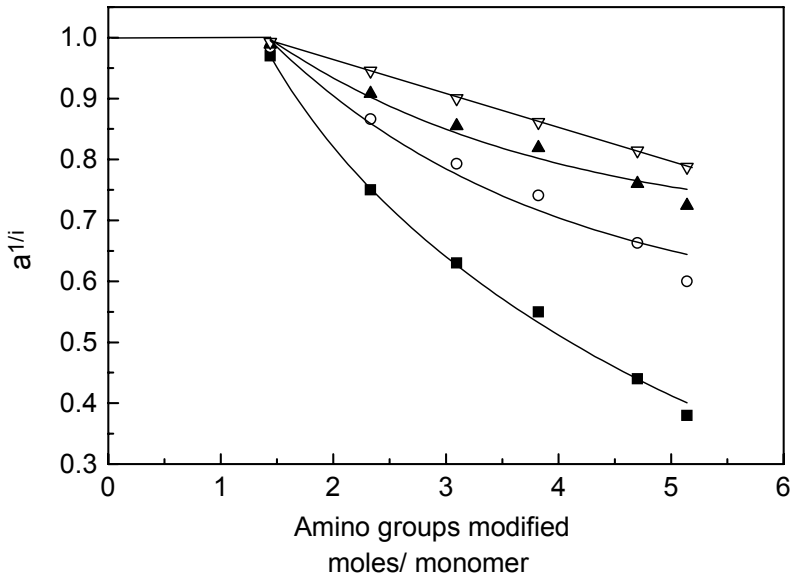


Figure 6. Correlation of inactivation of a-galactosidase with the modification of amino groups by acetic anhydride: Data was analyzed by Tsou's method as described by Paterson A. K. and Knowles J. R. [427] for the determination of essential amino groups present at the active site. Lines were obtained from equation 1 (see methods), when i =1 (-■-), i =2 (-○-), i =3 (-▲-), i = 4(-Δ-).

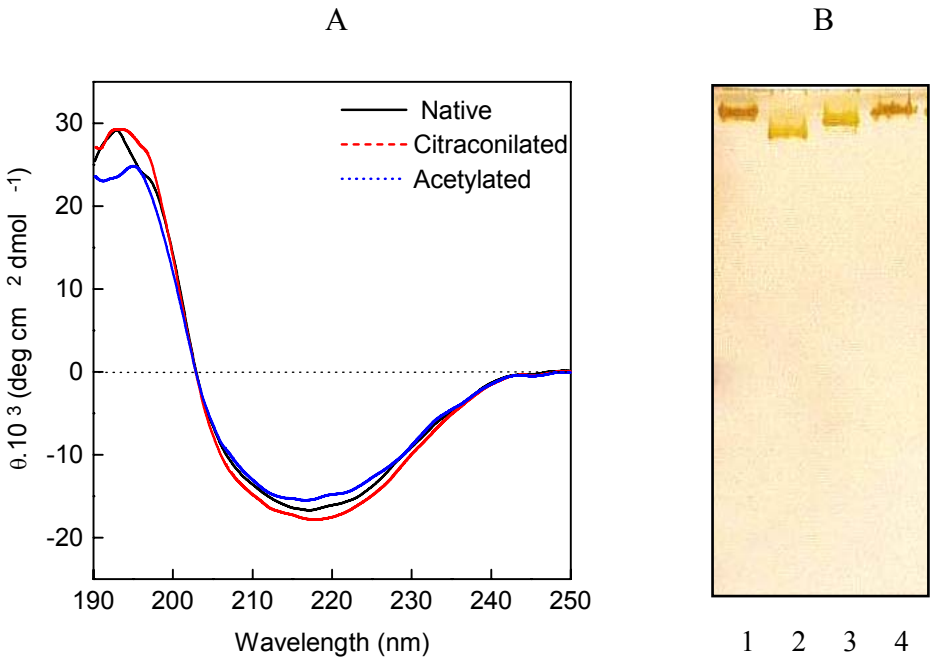


Figure 7. A) CD spectra of the native, acetylated and citraconilated α-galactosidase. B) Analysis of modified enzyme samples by Native-PAGE : (1)-Native enzyme, (2)-Triphenylated, (3)-Citraconilated, (4)-Acetylated, enzyme.

Table 4. Effect of lysine modification on the activity of α-galactosidase: substrate protection studies .

Reaction mixture	R.A. (%)	Inhibition. (%)	Protection (%)
Enzyme (Control)	100	00	100
Enzyme (100 μg) + TNBS (1.5 mM)	25	75	00
Enzyme (100 μg) + 50 mM galactose +TNBS (1.5 mM)	38	62	13
Enzyme (200 μg) + Acetic anhydride (10 mM)	38	62	00
Enzyme (200 μg) + 50 mM galactose +A.A (10 mM)	50	50	12
Enzyme (200 μg) + Citraconic anhydride (20 mM)	32	68	00
Enzyme (200 μg) + 50 mM galactose + C.A (20 mM)	45	55	13

R.A: Residual activity *Similar protection results were obtained when enzyme was preincubated with 50 mM of raffinose before modification.

Table 5. Effect of modification of lysine on K_m and K_{cat} values of α-galactosidase :

Percentage activity	Residue modified	Reagent used*	K_m (mM)	K_{cat} (min^{-1})
100	None	-----	0.606	2.0×10^5
75	Lysine	Acetic anhydride	0.714	1.58×10^5
54			0.833	8.85×10^4
38			1.00	4.42×10^4

* Similar kinetic values were obtained for citraconic anhydride modified enzyme.

Modification of tryptophan :

Oxidation of tryptophan residues in α-galactosidase by reaction with n-bromosuccinimide exhibited pH and concentration dependent inactivation. Difference spectra of modification of α-galactosidase at pH 5.0 indicated decrease in absorbance at 280 nm with no concomitant increase at 250 nm (Figure 8A) and no loss of enzyme activity, suggesting the modification of tyrosine residues. Extensive modification at this pH resulted in the sudden increase in the absorbance at 280 nm, coupled with the loss of isobestic point and total loss of enzyme activity. Further, analysis of NBS treated enzyme samples by SDS-PAGE (Figure 8B) showed the multiple species indicating that the loss of activity is due to structural changes.

In contrast modification of enzyme by NBS at pH 3.0, resulted in the loss of 80 % of the α-galactosidase activity. The difference spectra (Figure 9) of modification at this pH showed decrease in absorbance at 280 nm concomitant with increase at 250 nm. Moreover isobestic points around 260 nm and 305 nm suggest that the loss of activity is due to tryptophan modification rather than structural changes or modification of tyrosine residues. Additionally CD spectroscopic (Figure 10) and SDS-PAGE (data not shown) analysis of modified enzyme showed no structural changes. The plot of percent residual activity versus the number of tryptophan residues modified showed that as many as 9.0 tryptophans per monomer were modified, when 80 % of the initial activity is lost (Figure 11). Analysis of data by Tsou's method indicated loss of activity is due to the modification of four essential tryptophan residues (Figure 12).

Further the role of tryptophan in structure function relationship was investigated by fluorescence spectroscopic, substrate protection, and kinetics and thermal denaturation studies. The fluorescence emission spectra of α-galactosidase before and after modification by NBS at pH 3.0 are shown in Figure 13. The emission maximum at 336 nm of the native enzyme did not change with the modification, but there was a decrease in the relative fluorescence intensity with the progress of modification.

The presence of 50 mM of substrate during modification did not affect the extent of modification (Table 6). Further kinetic analysis of modified enzyme revealed partial decrease in the K_{cat} values and moderate increase in K_m values (Table 7). The thermal stability of modified enzyme was investigated and shown in Figure 14. The stability of enzyme at its optimum temperature (65 ˚C) decreased significantly.

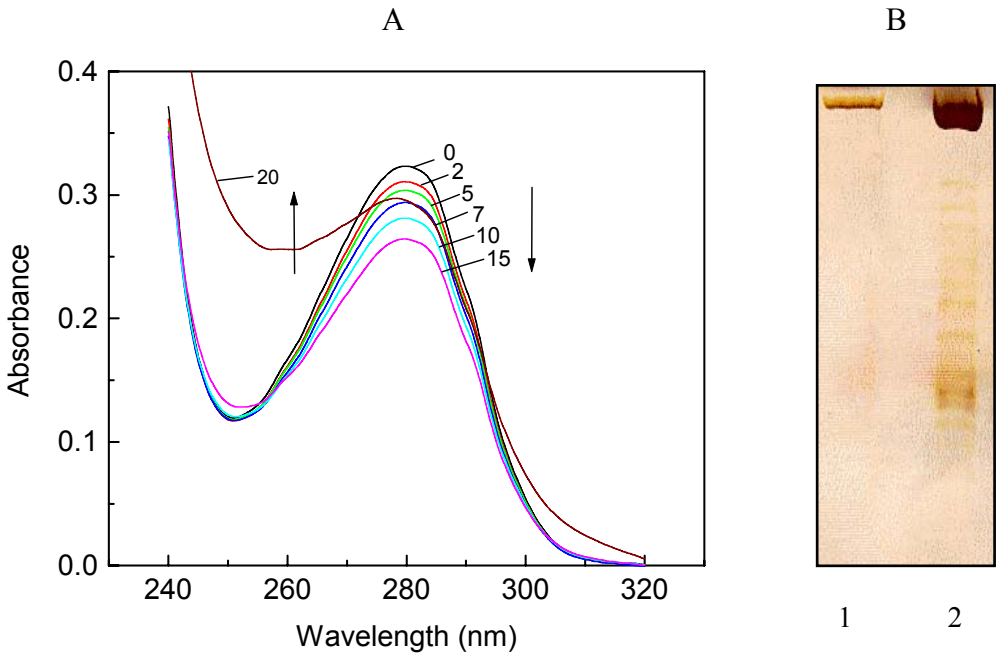


Figure 8. **A)** U.V spectrum of the α-galactosidase modified by NBS at pH 5.0. Number on the spectra indicates the fold excess of NBS added. **B)** Analysis of modified enzyme by SDS-PAGE; 1-Native enzyme, 2-Modified enzyme.

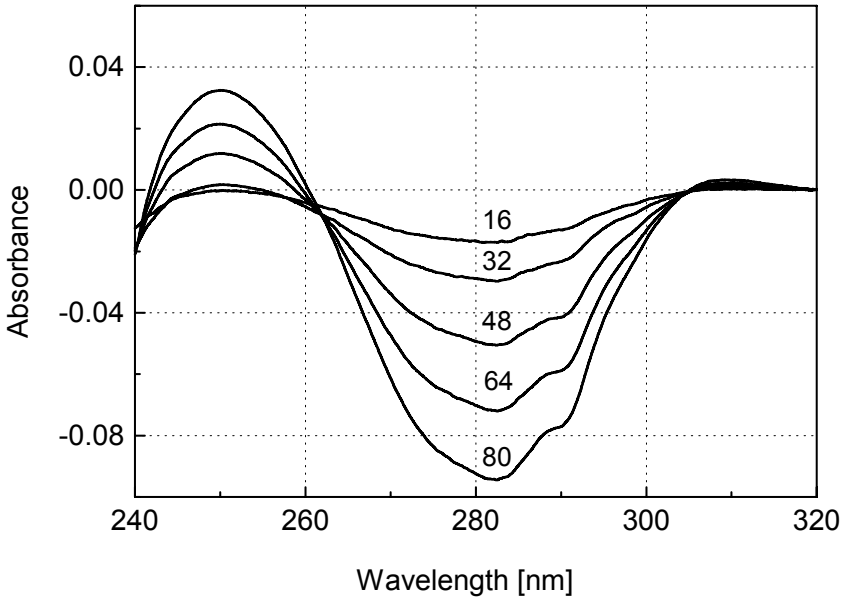


Figure 9. Difference spectra of modification of a-galactosidase by NBS, at pH 3.0.The numbers on spectra indicate the fold excess of NBS added. For experimental details refer to methods.

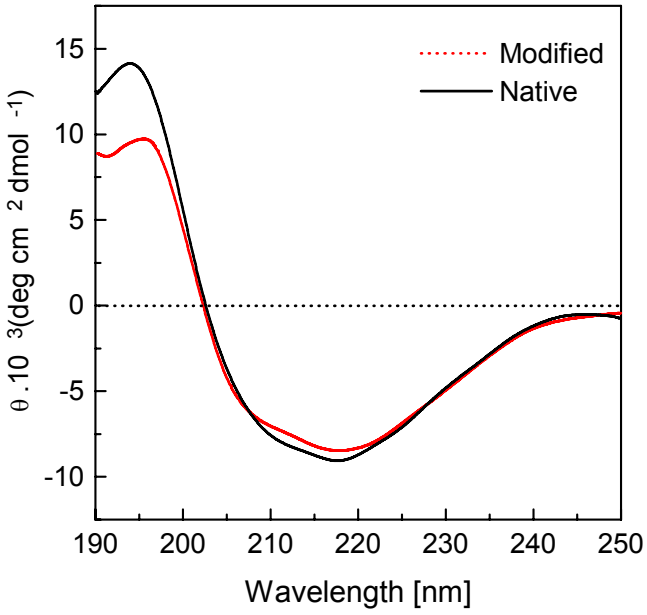


Figure 10. CD spectroscopic analysis of NBS modified a-galactosidase. Spectra : black line indicates enzyme before modification and red line indicates enzyme after modification.

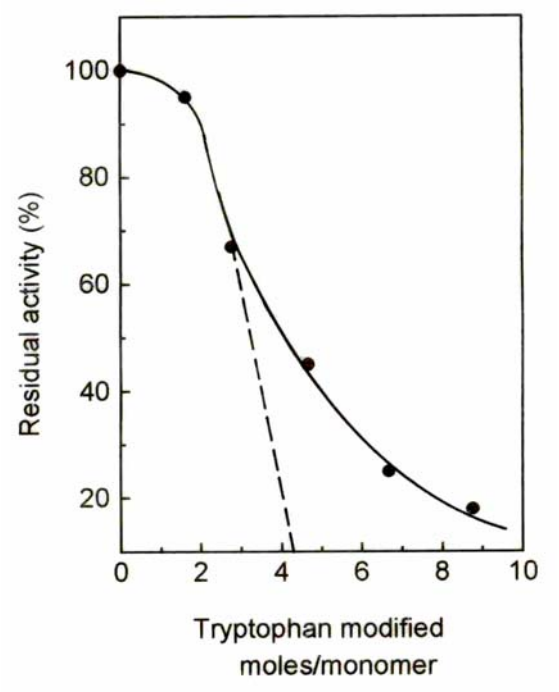


Figure 11. Correlation of inactivation of a-galactosidase with the modification of tryptophan residues. Residual activity and number of tryptophan modified was determined as described in the text. Tangent indicates the number of essential residues present in the active site.

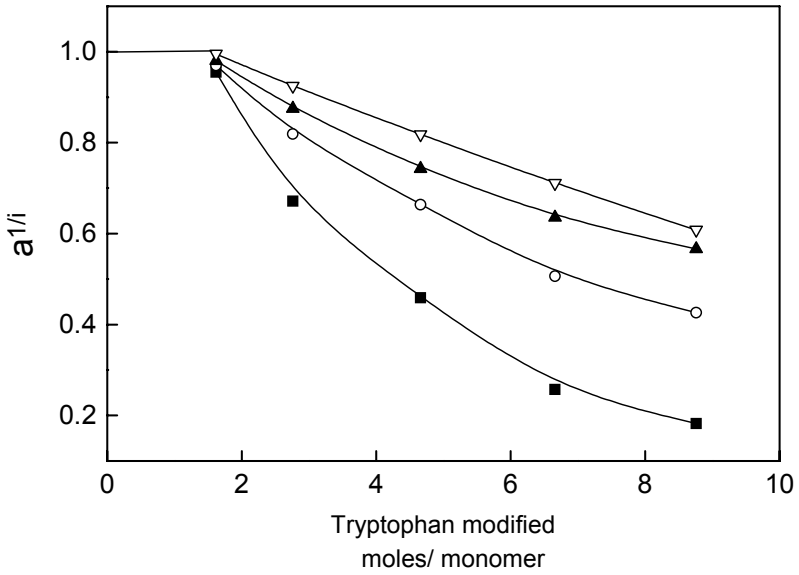


Figure 12. Modification of a-galactosidase by NBS: Data was analyzed by Tsou's plot as described by Paterson A. K. and Knowles J. R. [427] for the determination of essential tryptophan residues present at the active site. Lines were obtained from equation 1 (see methods), when i =1 (-■-), i =2 (-○-), i =3 (-▲-), i = 4(-∆ -).

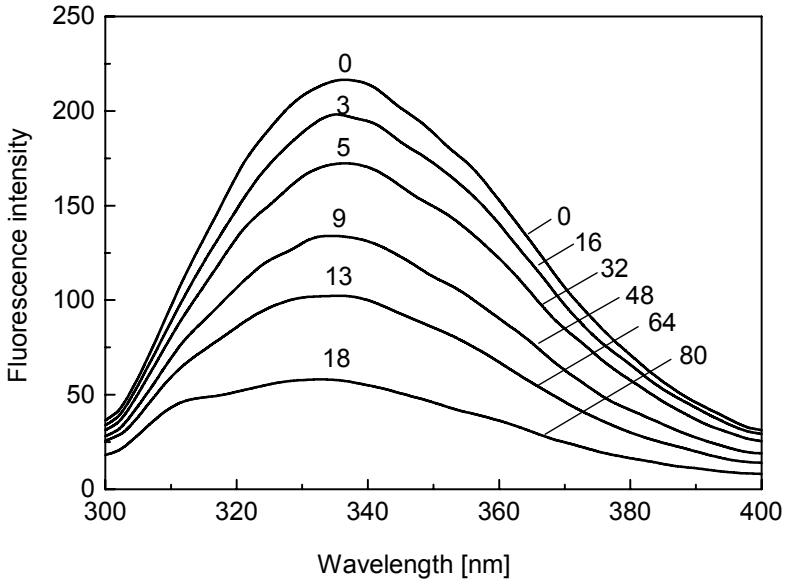


Figure 13. Fluorescence emission spectra of NBS modified α-galactosidase. The numbers on the spectra indicates the number of residues modified, and numbers at right hand side indicates fold excess of NBS added. For experimental details refer to the text.

Table 6. Effect of Tryptophan modification on the activity of α-galactosidase: substrate protection studies.

Reaction mixture	Residual activity. (%)	Inhibition (%)	Protection. (%)
Enzyme (Control)	100	0	100
Enzyme (100 μg) + NBS (50 μM)	20	80	0
Enzyme + raffinose (50 mM) + NBS	20	80	0
Enzyme + galactose (50 mM) +NBS	20	80	0

Table 7. Effect of tryptophan modification on the K_m and K_{cat} values of α-galactosidase :

Percentage activity	Residue modified	Reagent used	K_m	K_{cat}
100	None	----	0.606	2.0×10^5
67	Tryptophan	NBS	0.606	7.8×10^4
25			0.625	6.64×10^4

The kinetic constants were determined from Lineweaver Burk plot.

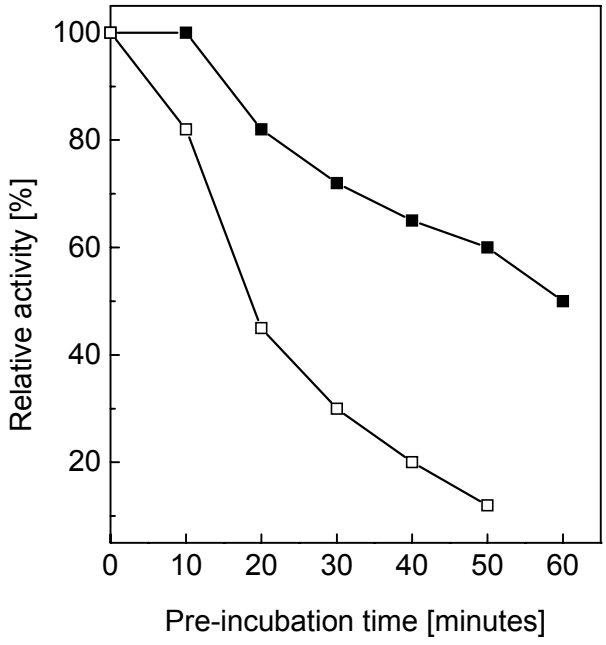


Figure 14. Thermal stability of the native (-■-) and tryptophan modified (-□-) α-galactosidase (45% Residual activity). Samples were incubated at 65 °C for varying time period and residual activity was determined as described in text.

Modification of Carboxylate :

Modification of purified α-galactosidase with 200 mM of EDAC at pH 6.0 and at 30 ˚C, resulted in the loss of 83 % of enzyme activity. The inactivation was concentration dependent and did not follow the pseudo first order kinetics. The loss of activity was further correlated with the number of carboxylic acid groups modified by estimating the number of nitrotyrosyl groups incorporated in the reaction. The plot of percent residual activity versus the number of residues modified showed that loss of 83 % of the activity resulted from the modification of one essential carboxylic acid group per monomer of α-galactosidase (Figure15). The incubation of enzyme with excess of its substrate/product (raffinose/galactose) prior to modification protected 37 % of its initial activity (Table 8). The kinetic constants K_m and K_{cat} (Table 9) of the modified enzyme showed that the K_m values remained unchanged where as K_{cat} values of the modified enzyme decreased considerably from 2.0×10^5 to 1.6×10^4 min^{-1}.

The CD spectra of the native and modified enzyme were identical, indicating that modification does not lead to any gross changes in the enzyme conformation.

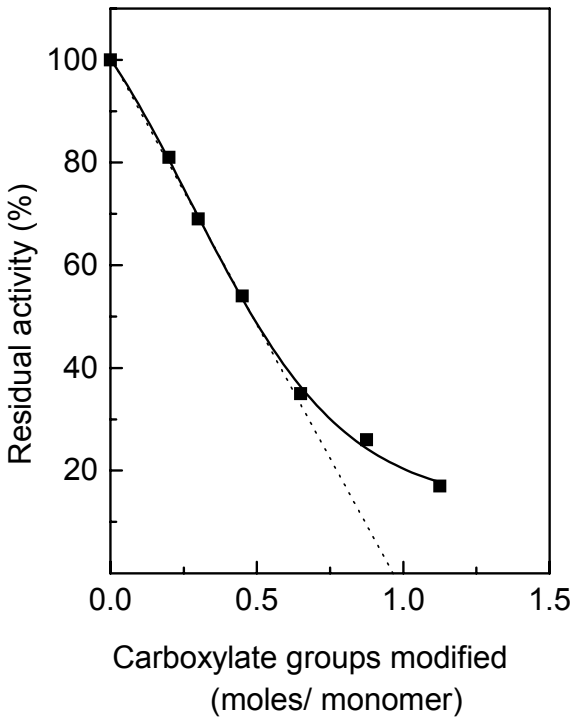


Figure 15. Correlation of inactivation of α-galactosidase with number of essential carboxylate groups modified. Residual activity and number of carboxylate groups modified were determined as described in methods.

Table 8. Effect of carboxylate modification on the activity of α-galactosidase: substrate protection studies.

Reaction mixture	Residual activity. (%)	Inhibition (%)	Protection. (%)
Enzyme Control	100	0	100
Enzyme (200 μg) + EDAC (200 mM)	17	83	0
Enzyme + raffinose (50 mM) + EDAC	54	46	37
Enzyme + galactose (50 mM) +EDAC	54	46	37

Table 9. Effect of carboxylate modification on the K_m and K_{cat} values of α-galactosidase :

Residual activity [%]	Residue modified	Reagent used	K_m [mM]	K_{cat} [min^{-1}]
100	None		0.606	2.0×10^5
69	Carboxylate	EDAC	0.606	7.6×10^4
35			0.606	3.32×10^4
26			0.606	1.6×10^4

K_m and K_{cat} values were determined from Lineweaver-Burk plot.

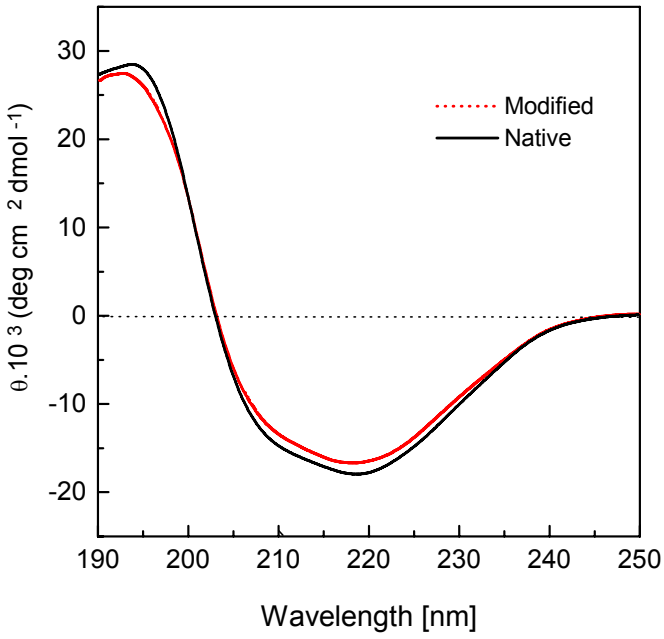


Figure 16. CD spectra of the native and carboxylate modified α-galactosidase. For experimental details refer to the text.

4.5 Discussion :

The pH dependence study and initial inactivation study with site-specific reagents indicated probable role of histidine, lysine, tryptophan and carboxylate at the active site of α-galactosidase.

The modification of α-galactosidase with histidine specific reagent DEPC resulted in the modification of 21 histidine residues and loss of 67 % of its initial activity. Hydroxylamine reverses the modification and 100 % of the modified histidine residues were regenerated (Figure 3), however the residual activity of α-galactosidase was not restored, suggesting that histidine may not be involved in the active site of α-galactosidase. .

Although, DEPC is used for histidine modification, it also reacts with tyrosine, cysteine and lysine residues. Modification of tyrosine was ruled out since there was no decrease in the difference spectra at 278 nm (Figure 2). Moreover the modification of enzyme with NAI (tyrosine specific reagent) did not show any loss of enzyme activity (Table 1). Cysteine can certainly be ruled out as potential site of modification since α-galactosidase contains no free cysteine residues; detected by reaction with DTNB (cysteine specific reagent) (Table 2, Chapter 3). Hence loss of activity could be correlated

to the modification of essential lysine residues. Estimation of lysine residues in DEPC treated enzyme samples showed modification of 11 out of 57 lysine residues present in an enzyme molecule, indicating that possible loss of activity could be due to the modification of critical lysine residues.

Further investigation on modification of lysine with lysine specific reagent; TNBS showed loss of 90 % of enzyme activity concomitant with the modification of 13 lysine residues (Figure 4), this confirms the above observation that lysine residues are present at the active site of α-galactosidase. As the pK_a of an average lysine residue in proteins is around 10.0, it is protonated at all pH's, and carries net positive charge on it. Therefore the absolute essentiality of this positive charge on lysine residues in α-galactosidase was investigated by producing variants of this residue by acylation and reductive methylation. Acetylation neutralizes the positive charge, citraconilation reverses it, and reductive methylation maintains the positive charge on ε-amino group of lysine residues. Modification of α-galactosidase by acetic and citraconic anhydride showed 60-70 % loss in its initial activity (Figure 5 & Table 3). However, reductive methylation did not show any loss in the enzyme activity (Table 3) although 13 lysine residues were modified. Moreover the pH profile of log (K_{cat}/ K_m) and log (K_{cat}) for *p*NPG hydrolysis shows presence of two protonated groups with pK_a = 8.2 & 7.84, respectively in the free enzyme and in ES complex. Furthermore the kinetic analysis of lysine-modified enzyme shows partial increase in K_m values and significant decrease in K_{cat} values. These results altogether led towards the conclusion that protonated lysine residues, carrying positive charge on its ε-amino group are located at the active site of the enzyme and possibly involved in the substrate binding as well as in the catalysis. The presence of lysine residue at the active site of α-galactosidase has been demonstrated earlier [303]. However the exact function of this residue is not elucidated.

α-Galactosidase contains 26 tryptophan residues and most of them are accessible to NBS modification at pH 3.0. Modification was found to be pH dependent and at pH 5.0 only tyrosine residues were modified as reflected in difference spectra (decrease at 280 nm but no increase at 250 nm, which is a characteristic of oxidation of indole to oxindole derivative)(Figure 8). Extensive modification at this pH resulted in the major conformational changes of enzyme, shown by difference spectra (loss of isobestic point) and SDS-PAGE analysis (multiple species) (Figure 8). However modification at pH 3.0 resulted in the modification of only tryptophan residues as shown by difference spectroscopy (decrease at 280 nm with a concomitant increase at 250 nm) (Figure 9), suggesting that the loss of activity could be due to the tryptophan modification rather than cleavage. Additionally, CD spectroscopic analysis of the modified enzyme samples showed no gross structural changes, indicating that loss of activity is due to the modification of tryptophan residues. Further, the study of environment of tryptophan by

fluorescence spectroscopy showed emission maximum at 336 nm. The quenching of tryptophan(s) by NBS retained the fluorescence emission maximum at 336 nm, suggested that the tryptophan(s) are present in a moderately hydrophobic environment and none of the tryptophan from the hydrophobic interior was modified, indicated no structural changes. The statistical analysis of modification data by Tsou's method indicated presence of four critical tryptophan residues at the active site and modification of these residues resulted in the loss of α-galactosidase activity.

Further, the tryptophan modification was not protected by the enzyme substrate. Moreover K_m values were also not affected substantially by modification. However the thermal stability of enzyme at higher temperature (65 °C) was decreased with concomitant decrease in K_{cat} values. Thus the above results suggested that tryptophan may not be directly involved in the substrate binding site but it may probably have a role in the catalytic site as well as in the thermal stabilization of enzyme molecule (active site conformation) at higher temperature, as it is known that hydrophobic interactions are often important contributors to the over all stability of proteins [428]. The tryptophan residue at position 16 of coffee bean α-galactosidase has previously been shown to be essential for enzyme activity [429].

Finally, when carboxyl groups of the enzyme were modified by EDAC, it lost 83 % of the enzyme activity, suggesting that carboxyl groups may have a role in the enzyme active site. Analysis of enzyme for number of essential active site residues showed that loss of activity occurred due to the modification of a single carboxyl group (Figure 15). Substrate protection studies on EDAC modified enzyme showed about 37 % of the protection against modification. Kinetic study of the modified enzyme revealed K_{cat} decreased to 1/10 of that of the unmodified enzyme, whereas K_m remained unchanged. These results clearly indicate that carboxyl group is involved in substrate cleavage and not in binding. This could be the same perturbed carboxyl group appeared in the pH activity profile with pK_a value = 6.1.

Participation of carboxyl groups in the catalytic function has been reported in a variety of glycosyl hydrolases [17,19,20]. Involvement of two carboxyl groups in the catalysis of *Humicola* sp. [303] and Coconut kernel [301] α-galactosidases has already been reported. However we report a single carboxylate group in the catalytic function of α-galactosidase from *Bacillus stearothermophilus* (NCIM 5146). α-Galactosidase from *Vicia faba* also shows the presence of a single carboxylate and a histidine residue in catalysis [241].

Mechanism of enzyme action :

It is known that glycosidases of family 27 and 36 are retaining glycosidases, where the enzymatic product retains the α-anomeric configuration of the substrate. The

mechanism for this class of enzyme has already been proposed and generally accepted. [23,24]. Catalysis in this class of enzymes proceeds via double displacement mechanism, where C_1 of the substrate undergoes two successive nucleophilic attacks [22]. Each attack inverts C_1, and two inversions yield a product with the same anomeric configuration as the substrate.

On the basis of our experimental data and the acid dissociation constant of amino acids obtained, we suppose that a single carboxylate and a protonated group, presumably a lysine residue (pK_a = 8.2) take part in catalysis, but only the protonated lysine residue (pK_a 7.84) is essential for substrate binding.

The carboxylate group (pK_a = 6.1) observed in α-galactosidase is present on the acidic limb of the pH profile hence it could be in the ionized state (COO^- species) and could act as a nucleophile base. Four lysine residues are present immediately around the active site. The lysine residue with pK_a 7.84 may interact with the substrate and that several other lysine residues likely participate in transition state stabilization (positive charge density is provided by four protonated lysine residues). The binding of substrate could take place through **H-bonding** between electrostatic hydroxyl oxygen of galactose and protonated amino group of lysine, which exist in the solution as NH_3^+ species. The possibility of ionic interactions between the substrate and lysine residues can be ruled out since substrate it self is not a charged molecule. The kinetics of the lysine modified enzyme showed partial increase in the K_m and significant decrease in the $K_{cat,}$ suggest a mechanism in which substrate and enzyme combine irrespective of the protonated state of the groups required for binding and catalysis but only the correctly protonated enzyme substrate complex is catalytically active. Although we have no data to interpret the catalytic participation of lysine residues in the active site, it is conceivable that proper binding of substrate to the protonated ε-amino group of lysine must in some manner directly or indirectly implicated in the catalytic step. This assumption can be supported by the evidence that lysine residue is found to be conserved at N-terminal region of α-galactosidases from *Bacillus stearothermophilus* (Chapter 3, Table 3).

Additionally four tryptophan residues were also found in the vicinity of the active site of α-galactosidase. These tryptophan residues may not be directly involved in the substrate binding but may have hydrophobic contacts with the substrate and possibly participate in the transition state stabilization. Additionally these tryptophan residues could be responsible for the stabilization of active site conformation of the enzyme at higher temperature.

Recently crystal structure of rice α-galactosidase [289] and chicken α-N-Acetylgalactosaminidase [290] of family 27 of glycosyl hydrolases has been reported. In rice α-galactosidase Asp-185 works as a catalytic acid/base and Asp-130 act as a nucleophile. Besides the catalytic residues other residues; Arg, Try, Cys, Meth and

Lys were reported to be present in the active site. A total of 11 residues were found to be involved in galactose binding. The ligand galactose was held in the catalytic pocket by hydrogen bonding and hydrophobic contacts with above residues.

In conclusion, the work described here demonstrates the carboxylate as a catalytic nucleophile and lysine may be auxiliary for enzyme catalysis. Thus the above results suggested that the present enzyme is a novel with respect to its structure function relationship and further studies on site directed mutagenesis and crystallographic analysis is required to support our hypothesis.

Conclusions

1. *Bacillus stearothermophilus* (NCIM-5146), a new isolate, produces high amount of an extracellular thermostable α-galactosidase, when grown on cheap agricultural residues under submerged fermentation conditions. The enzyme exhibited broad pH stability and optimum activity in the neutral to alkaline pH range.

2. The α-galactosidase preparation has been purified to homogeneity by single chromatographic step; using Phenyl Sepharose CL4B, with final yield of protein is about 44 %. The purification procedure developed contains minimum centrifugation and dialysis steps.

3. The purified α-galactosidase is a non-glycosylated protein. The molecular mass reported here is smallest among the α-galactosidases reported from *Bacillus stearothermophilus.*

4. The partial N-terminal sequence of the α-galactosidase from NCIM-5146 exhibited remarkable homology with the earlier reported α-galactosidase from *Bacillus stearothermophilus* (NUB 3621) of family 36 of glycosyl hydrolases, hence the present enzyme could be assigned to the similar family of glycosyl hydrolases. The secondary structure of the enzyme determined by CD spectroscopy and analyzed by prediction method, exhibited α/β class of protein.

5. The purified enzyme exhibited strict anomer specificity towards the substrates and hydrolyzes only the α-galactosidic linkages and not the β-galactosidic linkages. The enzyme also hydrolyzes α-1-3 and α-1-4 galactosidic linkages from disaccharide sugars and suggests its potential application in the blood group transformation.

6. The kinetics of hydrolysis of number of natural and synthetic substrates showed aryl glycosides having higher affinity and higher catalytic efficiency than the alkyl glycosides.

7. Temperature dependence of rate constant, $K_{cat,}$ exhibited discontinuous or biphasic Arrhenius plots for all the substrates, with two characteristic activation energies below and above the transition temperature. This together with the observations of changes in secondary structure of α-galactosidase as a function of temperature suggested that there could be two conformational states of the enzyme above and below the inflection temperature, each with a different catalytic competence

8. Thermodynamic parameters of binding and hydrolysis of different glycosides calculated from the temperature dependence studies, indicated that binding might be enthalpy as well as entropy driven process, suggests that hydrophobic interactions as well as hydrogen bonds play a predominant role in substrate binding. The high catalytic efficiency of *p*NPG and melibiose is due to the highest transition state stabilization and ground state destabilization together with the positive entropy of activation, suggested that these substrates fit properly in the active site cleft. By contrast high entropy loss in binding and activation of raffinose and stachyose indicated strained substrates and induce the conformational changes in enzyme.

9. Active site characterization by pH dependence studies and chemical modification studies indicated participation of carboxylate (pKa = 6.1) and lysine (pKa = 8.2) in enzyme catalysis.

10. Lysine and tryptophans are also involved in substrate binding and stabilization of active site conformation, respectively.

11. Finally, since *Bacillus stearothermophilus* is generally recognized as a safe (GRAS) for food applications. Moreover the present enzyme preparation is free from invertase and protease activities, it has potential applications in the soymilk hydrolysis and other related food industries (publication No 1. 2003).

References :

1. Miquel J. C., Cited in web site, URL: www.weblife.org/humanure/chapter3_8.html

2. Sunna A., Moracci M., Rossi M., Antranikian G., Glycosyl hydrolases from hyperthermophiles. Extremophiles, 1996a; 1 : 2 –13.

3. Sundaram T.K., Thermostable enzymes for biotechnology. J. Chem. Technol. Biotechnol., 1988; 42: 308.

4. Cowan D. A., Enzymes from thermophilic archaebacteria: current and future applications in biotechnology., Biochem. Soc. Symp., 1992; 58; 149-169.

5. Crout D.H.G., Gabin Vic, Glycosidases and glycosyl transferases in glycoside and oligo-saccharides synthesis., Current Opinion in Chemical Biology, 1998; 2:98-111.

6. Diane W., Stephan R., Wolfgang, Z., Application of thermostable enzymes for carbohydrate modification. In: Contribution of the Fourth International Workshop on Carbohydrate as Organic Raw Materials, 1997. WUV-Universitatverlag, Vienna.

7. Coutinho, P. M., & Henrissat, B., (1999) Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html

8. Boel E., Brady L., Brzozowski A. M., Derewenda Z., Dodson G. G., Jensen V. J., Petersen S. B., Swift H., Thim L., and Woldike, H. F., Calcium binding in a-amylases: an X-ray diffraction study at 2.1 Å resolution of two enzymes from *Aspergillus*. Biochem., 1990; 29: 6244-6249.

9. Mikami B., Sato M., Shibata T., Hirose M., Aibara S., Katsube Y., and Morita Y., Three-dimensional structure of soybean a-amylase determined at 3.0 Å resolution - Preliminary chain tracing of the complex with a-cyclodextrin. J. Biochem. Tokyo., 1992; 112: 541-546.

10. Lawson C. L., van Montfort R., Strokopytov B., Rozeboom H. J., Kalk K.H., de Vries G. E., Penninga D., Dijkhuizen L., and Dijkstra B. W., Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. J. Mol. Biol. , 1994; 236: 590-600.

11. Kuriki T., Okada S., and Imanaka T., New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. J. Bacteriol., 1988; 170: 1554-1559.

12. Svensson B., Regional distant sequence homology between amylases, a-glucosidases and transglucanosylases. FEBS letters., 1988; 230: 72-76.

13. Kizaki H., Hata Y., Watanabe K., Katsube Y., and Suzuki Y., Polypeptide folding of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase revealed by 3.0 Å resolution X-ray analysis. J. Biochem. Tokyo., 1993; 113: 646-649.

14. Amemura A., Chakraborty R., Fujita M., Noumi T., and Futai M., Cloning and nucleotide sequence of the isoamylase gene from *Pseudomonas amyloderamosa* SB-15. J. Biol. Chem., 1988; 263: 9271-9275.

15. Withers S. G., Warren R. A. J., Street I. P., Rupitz K., Kempton J. B., and Aebersold R., Unequivocal demonstration of the involvement of a glutamate residue as a nucleophile in the mechanism of a retaining glycosidase. J. Am. Chem. Soc., 1990; 112: 5887-5889.

16. Overbeeke N., Termorshuizen G .H., Giuseppin M. L., Underwood D., and Verrips C., Secretion of the a-galactosidase from *Cyamopsis tetragonoloba (guar)* by *Bacillus subtilis*. Appl. Environ. Microbiol. 1990; 1429-1434.

17. Jenkins J., Leggio L. L., Harris G., and Pickersgill R., β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold β/a architecture and with two conserved glutamates near the carboxy-terminal. FEBS Lett., 1995; 362; 281-182.

18. DePinto J. A. and Campbell L.L. Purification and properties of the cyclodextrinase of *Bacillus macerans*. Biochem., 1968; 7: 121-125.

19. Blake C. C. F., Johnson L. N., Mair G. A., North A. C. T., Phillips D. C., and Sarma V. R. Crystallographic studies of the activity of hen egg-white lysozyme. *Proc. R. Soc. Ser. B.,* 1967; 167: 378-388.

20. Campbell R., Rose D., Wakarchuk W., To R., Sung W., and Yaguchi M., A comparison of the structures of the 20 kD xylanase from *Trichoderma harzianum* and *Bacillus circulans*. In: Edited by Suominen, P. and Reinikeinen, T. Helsinki, Foundation for Biotechnical and Industrial Fermentation Research. 1993; 63-72.

21. Divne C., Stahlberg J., Reinikainen T., Ruohonen L., Petterson G., Knowles J. K. C., Teeri T., and Jones T. A., The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesi*. Science., 1994; 265: 524-528.

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

23. Koshland D. E. J., Stereochemistry and the mechanism of enzymatic reactions. Biol. Rev., 1953; 28: 416-436.

24. McCarter J. D., Withers S. G., Mechanisms of enzymatic glycoside hydrolysis., Curr. Opin. Struct. Biol., 1994; 4:885-92.

25. Sinnott M. L., Catalytic mechanisms of enzymic glycosyl transfer., Chem. Rev., 1990; 90: 1171-1202.

26. Vernon C.A., The mechanisms of hydrolysis of glycosides and their relevance to enzyme-catalyzed reactions., Proc. R Soc. Ser B. 1967; 167 :389-401.

27. Henrissat B. A., classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem. J., 1991; 280: 309-316.

28. Henrissat B., Bairoch A., New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem. J., 1993; 293: 781-788.

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

30. Dey P. M., Biochemistry of a-galactosidic linkages in the plant kingdom. Adv. Carbohydr. Chem. Biochem., 1980; 37: 283-371.

31. French D., The raffinose family of oligo-saccharides., In: Adv. Carbohydr. Chem. 9: 149., Academic Press New York (1954).

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

33. Fischer, E. and Lindner, P. "Uber die Enzyme einigen Hefen," Chem. Ber., 1895; 28, 3034

34. Helferich B., and Appel H., Emulsin-X., Z. Physiol. Chem., 1932; 237: 254.

35. Thomas B., Webb J. A., Multiple forms of a-galactosidase in mature leaves of *Cucurbita pepo.*, Phytochem., 1977; 16: 203-206.

36. Chinen I., Nakamura T., Fukuda N., Purification and properties of α-galactosidase from immature stalks of *Saccharum officinarum* (sugar cane). J. Biochem., 1981; 90: 1453-61.

37. Balasubramaniam, K., Mathew, D., Purification of a-galactosidase from Coconut., Phytochem., 1986; 25: 1817.

38. Feurtado J. A., Banik M., Bewley J. D., The cloning and characterization of α-galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seed. J. Exp. Bot., 2001; 52: 1239-49.

39. Kang H. C., Lee S. H., Characteristics of an α-galactosidase associated with grape flesh. Phytochem., 2001; 58: 213.

40. Kim, W. D,, Kobayashi O., Kaneko S., Sakakibara Y., Park G. G., Kusakabe, I., Tanaka, H., Kobayashi H., α-Galactosidase from cultured rice (*Oryza sativa* L. var. Nipponbare) cells., Phytochem., 2002; 61:621-30.

41. Kim W. D., Kaneko S., Park G. G., Tanaka H., Kusakabe I., Kobayashi H., Purification and characterization of a-galactosidase from sunflower seeds. Biotechnol. Lett., 2003; 25:353-8.

42. Sugawara S, Nakagawa R, Urashima T, Sato T, Muratubaki T, Sayama K. Transgalactosylation products from melibose by the α-galactosidase of *Absidia corymbifera*. Agric. Biol. Chem., 1990 ; 54: 211-3.

43. Barham, D., Dey, P., M., Griffith, D., and Pridham, J.,B., Studies on the distribution of a-galactosidase in seeds., Phytochemistry, 1971; 10: 1759.

44. Dey P. M., Dixon M. Separation and properties of α-galactosidase and β-galactosidase from *Cajanus indicus*. Biochim. Biophys. Acta., 1974; 370: 269-75.

45. Haibach F., Hata J., Mitra M., Dhar M., Harmata M., Sun P., Smith D., Purification and characterization of a *Coffea canephora* a-D-galactosidase isozyme. Biochem. Biophys. Res. Commun., 1991; 181: 1564-71.

46. Overbeeke N., Fellinger A. J., Toonen M. Y, van Wassenaar D, Verrips C. T., Cloning and nucleotide sequence of the α-galactosidase cDNA from *Cyamopsis tetragonoloba* (guar). Plant. Mol. Biol., 1989; 13: 541-50.

47. Guimaraes V. M., de Rezende S. T., Moreira M., A., de Barros E., G., Felix C., R., Characterization of a-galactosidases from germinating soybean seed and their use for hydrolysis of oligosaccharides. Phytochem., 2001; 58: 67-73.

48. Dey P. M., Wallenfels K. Isolation and characterization of a-galactosidase from *Lens esculanta*. Eur. J. Biochem., 1974; 50: 106-12.

49. Dey P. M., Khaleque A, Pridham J. B., Further observations on the α-galactosidase activity of *Vicia faba* seeds. Biochem. J., 1971; 124: 27P.

50. Hadacova, V., and Benes, K., The differentiation of a and β glucosidase and a and β galactosidase isoenzymes from Maize and Broad Bean root tips using disc electrophoresis in polyacrylamide gels. Biol. Plant., 1977; 19: 436.

51. Dhar M., Mitra, M., Hata, J., Butnariu, O., Smith, D., Purification and characterization of *Phaseolus vulgaris* α-D-galactosidase isozymes. Biochem. Mol. Biol. Int. 1994; 34:1055-62.

52. Williams J., Villarroya H., Petek F., Purification and properties of an α-D-galactoside galactohydrolase from the seeds of *Trifolium repens* (white clover). Biochem. J., 1977; 161: 509-15.

53. Malhotra O. P, Dey P. M., Purification and physical properties of sweet-almond α-galactosidase. Biochem. J. 1967; 103: 508-13.

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

55. Sastry P. S., and Kates M., Lipid components of leaves-V., Galactolipids, Cerebrosides and Lecithin of runner beans leaves., Biochemistry, 1964; 3: 1271.

56. Dey, P. M., and Pridham J.,B., Biochemistry of α-galactosidases., Adv. Enzymol., 1972; 36 : 91.

57. McCleary , Galactomannan structure and β-mannanase and β-mannosidase activity in germinating legume seeds., Phytochem., 1975; 14 : 1187.

58. Dey, P. M. Transgalactosylation activity of sweet almond α-galactosidase: synthesis of saccharides., Phytochemistry, 1979; 18: 35.

59. Strobel G.A., toxin binding protein of sugar cane, its role in the plant., Proc., Natl., Acad. Sci., U.S.A., 1974; 71: 4234.

60. Plant A. R., Moore K.G., a-D-mannosidase and a-D-galactosidase from protein bodies of Lupinus *angustifolius cotyledons*. Phytochem., 1982; 21: 985-989.

61. Bierry H., Diastatic cleavage of glucosides and galactosides., Compt. Rend. Soc. Biol., 1913; 156:265.

62. Bishop DF, Desnick RJ. 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., 1981; 256: 1307-16.

63. Dean K. J., Sweeley C. C., Studies on human liver α-galactosidases. III. Partial characterization of carbohydrate-binding specificities. J. Biol. Chem., 1979; 254: 10006-10.

64. Suzuki I., Kushida H., and Shida H., Seikagaku, 1969; 41: 333. [Cited in Adv. Enzymol. 1972; 36: 91-130].

65. Monis B. and Wasserkrug, H. Histochemistry of glycosidases of Megakaryocytes and Platelets, A comparative study. Histochemie., 1967; 10: 363-368.

66. Szmigielski S., An improved method for histochemical demonstration of β-glucuronidase and α-galactosidase activity in bone marrow and blood cells., J. Lab. Clin. Med., 1966; 67: 709

67. Bajwa, S. S. and Sastry. P. S., Degradation of Monogalactosyl diglyceride and Digalactosyl diglyceride by sheep pancreatic enzymes., Biochem. J., 1974; 144: 177.

68. Chang A. Y., Difference in renal α-galactosidase levels in male and female Chinese hamsters (*Cricetulus griseus*). Compt. Biochem. Physiol. B, 1978; 61B, 133.

69. Chapman V. M., West, J. D. and Adler, D. A., Bimodal distribution of α-galactosidase activities in mouse embryos. Basic Life Sci., 1978; 12: 227.

70. Heurtz S. and Hars-Cayla, M. C. Annu. Genet., 1978; 21: 197. [Cited in the ph. D. thesis of S. M. Kotwal., 'Studies on α-galactosidase from the thermophilic fungus *Humicola* sp.' 1999].

71. Shepard D., Donovan M., Raghupathy E., Yeung K. K., Owen A. J., and Dain J. A., Effect of immobilization on the stability and substrate specificity of α-galactosidase from the invertebrate *Turbo cornutus*., Carbohydr. Res., 1983; 118: 239.

72. Feldt-Rasmussen U, Rasmussen AK, Mersebach H, Rosenberg KM, Hasholt L, Sorensen SA., Fabry disease a metabolic disorder with a challenge for endocrinologists ?, Horm Res., 2002;58:259-65. (Review).

73. Subba Rao K. and Pieringer R. A., Metabolism of glyceride glycolipids-IV : Enzymatic hydrolysis of monogalactosyl and digalactosyl diglycerides in rat brain., J. Neurochem., 1970; 17: 483.

74. Lazo P. S., Ochoa A. G., Gascon S., α-Galactosidase from *Saccharomyces carlsbergensis*. Cellular localization, and purification of the external enzyme. Eur. J. Biochem., 1977; 77: 375-82.

75. Church F. C., Jr.; Meyers, S. P.; Srinivasan, V. R. Isolation and characterization of α-galactosidase from *Pichia guilliermondii*. Dev. Ind. Microbiol.,1980; 21: 339-48.

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

77. Kremnicky L., Biely P., β-Mannanolytic system of *Aureobasidium pullulans.,* Arch. Microbiol., 1997; 165: 350-355.

78. Gomes J., Gomes I. I., Terler K., Gubala N., Ditzelmuller G., Steiner W., Optimisation of culture medium and conditions for alpha-l-Arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*., Enzyme Microb. Technol., 2000; 27:414-422.

79. Bakunina I. Y., Sova V. V., Nedashkovskaya O. I., Kuhlmann R. A., Likhosherstov L. M., Martynova M. D., Mihailov V. V., Elyakova L. A., Alpha-galactosidase of the marine bacterium *Pseudoalteromonas sp*. KMM 701. Biochem., (Mosco), 1998; 63: 1209-15.

80. Coombs J., Brenchley J. E., Characterization of two new glycosyl hydrolases from the lactic acid bacterium *Carnobacterium piscicola* strain BA., Appl. Environ. Microbiol., 2001; 67: 5094-9.

81. Somiari R.I, Balogh E., Properties of an extracellular glycosidase of *Aspergillus niger* suitable for removal of oligo-saccharides from cow pea meal., Enzyme. Microb. Technol., 1995; 17: 311-316.

82. Ademark P., Larsson M., Tjerneld F., and Stalbrand H. Multiple α-galactosidases from *Aspergillus niger* : Purification, Characterization and Substrate specificity. Enzyme. Microb. Technol., 2001;29: 441-448.

83. Zaprometova, O. M., and Ulezo I. V., Studies on the optimal cultivation conditions of *Cephalosporium* sp., a producer of a-galactosidase., Mikrobiol. Promst., 1981;4:20.

84. Kaji, A., Yoshihara, O., Purification and properties of α-galactosidase produced by *Corticium rolfsii*. Agr. Biol. Chem., 1972; 36: 1335-1342.

85. Mulimani, V.,H., and Ramlingam., Enzymic hyrolysis of Raffinose and Stachyose in soymilk by α-galactosidase from *Gibberella fujikuroi.* Biochem. Mol. Biol. Intl., 1995; 36: 897-905.

86. Galas E, Miszkiewicz H, Purification and some properties of a-galactosidase from *Mortierella vinacea* IBT-3. Acta Microbiologica polonica,, 1996; 45: 143-154.

87. Luonteri E, Alatalo E, Siika-Aho M, Penttila M, Tenkanen M., α-Galactosidases of *Penicillium simplicissimum*: production, purification and characterization of the gene encoding AGLI. Biotechnol. Appl. Biochem., 1998; 28: 179-88.

88. Wong H., Hu C., Yeh H., Su W., Lu H. and Lin C. Production, Purification and Characterization of α-galactosidase from *Monascus pilosus*. Appl. Environ. Microbiol., 1986; 52: 1147-1152.

89. Ohtakara Akira., Mitsutomi M., Uchida, Y., Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus*., Agr. Bio. Chem., 1984; 48: 1319-1327.

90. Bei-Zhong, H., Yong M., Frans M.R., Robert, N., M., J., Effects of temperature and relative humidity on growth and enzyme production by Actinomucor elegans and *Rhizopus oligosporus* during sufu pehtze preparation., Food Chemistry, 2003 81: 27-34.

91. McKay, A., M., Production of extracelluar β-glucosidase and α-galactosidase during fungal growth on polygalacturonate., J. Food Sci., 1991; 56: 1749.

92. Zeilinger S, Kristufek D, Arisan-Atac I, Hodits R, Kubicek CP. Conditions of formation, purification, and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. Appl. Environ. Microbiol. 1993; 59: 1347-53.

93. Kotwal S. M., Gote M. M., Khan M. I., Khire J. M., Production, purification and characterization of constitutive intracellular a-galactosidase from the thermophilic fungus *Humicola* sp. J. Ind. Microbiol & Biotechnol., 1999; 23: 661-667.

94. Puchart V., Vrsanska M., Bhat M. K., Biely P., Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. Biochim. Biophys. Acta, 2001; 1524:27-37.

95. Zaprometova, O. M.; Ulezlo, I., V. Isolation and purification of a mold a-galactosidase. Biotechnol. Appl. Biochem., 1988; 10: 232-41.

96. Li Y., T., and Shetlar M., R., Occurrence of α-galactosidase in higher fungi: Isolation of α-galactosidase from *Calvatia cyathiformis.,* Arch., Biochem., Biophys., 1964; 108: 523.

97. Hogness D., S., Battley E.,H., The α-galactosidase of *Aerobacter aerogenes*, its characteristics and induce synthesis., Fed. Proc., 1957; 16:197.

98. Wong T. Y., Melibiose is hydrolyzed exocellularly by an inducible exo-α-galactosidase in *Azotobacter vinelandii.*, Appl. Environ. Microbiol., 1990; 56: 2271-3.

99. Delente J., Johnson J. H., Kuo M. J., O'Connor R. J., Weeks L. E., Production of a new thermostable neutral α-galactosidase from a strain of *Bacillus stearothermophilus*. Biotechnol. Bioeng. 1974; 16: 1227-43.

100. Talbot G., Sygusch J., Purification and characterization of thermostable β-mannanase and a-galactosidase from *Bacillus stearothermophilus.*, Appl. Environ. Microbiol., 1990; 56: 3505-10.

101. Gherardini F., Babcock M., Salyers A. A., Purification and characterization of two α-galactosidases associated with catabolism of guar gum and other alpha-galactosides by *Bacteroides ovatus.*, J. Bacteriol. 1985; 161: 500-6.

102. Berg J. O., Lindqvist L., Nord C. E., Purification of glycoside hydrolases from *Bacteroides fragilis.* Appl. Environ. Microbiol., 1980; 40: 40-7.

103. Leder S., Hartmeier W., Marx S. P., α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083., Curr. Microbiol., 1999; 38: 101-6.

104. Xiao M., Tanaka K., Qian X. M., Yamamoto K., & Kumagai H., High yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose., Biotechnol. Lett., 2000; 22: 747-751.

105. Durance T., and Skura B., Purification and characterization of a *Clostridium perfringens* a-galactosidase., J. Food Sci., 1985; 50 : 518.

106. Li Y.T., Li S., C., and Shetlar M. R., α-Galactosidase from *Diplococcus pneumoniae*. Arch. Biochem. Biophys., 1963; 103: 436-442.

107. Sheinin R., Crocker B. F., The induced concurrent formation of α-galactosidase and β-galactosidase in *E. coli* B^1., Can. J. Biochem. Physiol., 1961; 39: 63-71.

108. Garro M. S., de Valdez G. F., Oliver G., de Giori G. S., Influence of Carbohydrates on the α-Galactosidase Activity of *Lactobacillus fermentum.*, Curr. Microbiol., 1996; 33: 302-5.

109. Schuler R., Kinetic properties of α-galactosidase from *Lactobacillus fermenti.*, Enz. Microbial Technol., 1985; 7: 207-211.

110. Mital B. K., Shallenberger R. S., Steinkraus K. H., α-Galactosidase activity of *Lactobacilli.*, Appl. Microbiol., 1973; 26: 783-788.

111. Akiba T., Horikoshi K., Localization of α-galactosidase in an Alkalophilic strain of *Micrococcus.* Agri. Biol. Chem., 1978; 42: 2091-2094.

112. Hashimoto H., Goto M., Katayama C., Kitahata S., Purification and some properties of α-Galactosidase from *Pseudomonas fluorescens* H-601., Agr. Biol. Chem., 1991; 55: 2831-2838.

113. Bailey R . W., The intracellular alpha- galactosidase of a rumen strain *streptococcus bovis*. Part-IV. Characteristics of α-galactosidase and estimation of raffinose by the enzyme preparation., Biochem J., 1963; 86: 509.

114. Aduse-Opoku J., Tao L., Ferretti J. J., Russell R. R. B., Biochemical and genetic analysis of *Streptococcus mutans* α-galactosidase."; J. Gen. Microbiol., 1991; 137: 757-764.

115. Duffaud G. D., McCutchen C. M., Leduc P., Parker K. N., Kelly R. M., Purification and characterization of extremely thermostable beta-mannanase, β-mannosidase, and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. Appl. Environ. Microbiol., 1997 ; 63: 169-77.

116. Ishiguro M., Kaneko S., Kuno A., Koyama Y., Yoshida S., Park G. G., Sakakibara Y., Kusakabe I., Kobayashi H., Purification and characterization of the recombinant *Thermus* sp. strain T2 α-galactosidase expressed in *Escherichia coli*. Appl. Environ. Microbiol., 2001; 67:1601-6.

117. Suzuki H., Ozawa Y., and Tanabe O., Studies on the decomposition of raffinose by α-galactosidase of Actinomycetes. Part IV, characterization of α-galactosidase and estimation of raffinose by enzyme preparation., Agri. Biol. Chem., 1966; 30: 1039.

118. Segel I. H., In Biochemical calculations 2nd ed, Wiley and Sons, New York, 1976; p. 346.

119. Reynolds J. H., An immobilized α-galactosidase continuous flow reactor., Biotechnol. Bioeng., 1974; 16: 135-147.

120. Oishi K., Aida K., Blood group substance degrading enzymes obtained from *Streptomyces sp.* Part-I. Screening test and culture conditions for best enzyme production. Agr. Biol. Chem., 1971; 35: 1101-1108.

121. Folin O., Denis W., A qualitative (reduction) test for sugar in normal urine., J. Bio. Chem., 1915; 22: 305.

122. Rios S, Pedregosa AM, Fernandez Monistrol I, Laborda F. Purification and molecular properties of an α-galactosidase synthesized and secreted by *Aspergillus nidulans*. FEMS Microbiol. Lett., 1993; 112: 35-41.

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

124. Nelson N. A., Photometric adaptation of the Somogi method for the determination of glucose., J. Biol. Chem., 1944; 153: 375-378.

125. Dygert S., Li L. H., Florida D., and Thoma J. A., Determination of reducing sugar with improved precision. Anal. Biochem., 1965; 13: 367-374.

126. Miller G. L., Use of Dinitrosalicyclic acid reagent for determination of reducing sugar. Anal. Chem., 1959; 31 : 426.

127. Sinner H., and Puls J., Non corrosive dye reagent for detection of reducing sugar in borate complex ion exchange chromatography. J. Chromat., 1978; 156: 197-204.

128. Moppar K., Gindler J. M., A new noncorrosive dye reagent for automatic sugar chromatography., Anal. Biochem., 1973; 56: 440-442.

129. Dahlqvist A., Determination of maltase and isomaltase activities with a glucose oxidase reagent. Biochem. J., 1961; 80: 547-551.

130. Kurz G., and Wallenfels K., D-galactose. UV-test kit galactose dehyrogenase. In: H. U. Bergmeyer (Ed.), Methoden der enzymatischen analyse II, Verlag Chemie GmbH, Weinheim., 1970.

131. Bruss M. L., Black A. L., Enzymatic microdetermination of glycogen. Anal. Biochem., 1978; 84: 309-12.

132. Fridjonsson O., Watzlawick H., Gehweiler A., Rohrhirsch T., Mattes R., Cloning of the gene encoding a novel thermostable α-galactosidase from *Thermus brockianus* ITI360., Appl. Environ. Microbiol., 1999; 65: 3955-63.

133. Kilpatrick D. C., Stirling J. L., Glycosidases from the culture medium of *Physarum polycephalum.*, Biochem. J., 1977; 161: 149.

134. Dey P. M., Kauss H., a-Galactosidase of *Poterioochromonas malhamensis.*, Phytochem., 1981; 20: 45-8.

135. Yosida S., Sako Y., Uchida A., Purification, properties and N-terminal amino acid sequences of Guar Gum degrading enzyme from *Bacillus circulans* K-1. Biosci. Biotech. Biochem. 1997; 61: 251-255.

136. Kabat E. A., In blood group substances, Academic press., New York, 1956, PP 56-58.

137. Hankins C. N., Shannon L. M., The physical and enzymatic properties of a phytohemagglutinin from Mung Beans., J. Biol. Chem., 1978; 253; 7791-97.

138. Schmid K., Schmitt R., Raffinose metabolism in *E. coli* K_{12} . Eur. J. Biochem., 1976; 67: 95-104.

139. Bom I., Wassenaar D., Boot J., Hybrid affinity chromatography of a-galactosidase from *Verbascum thapsus* ., J. Chromato. A., 1998; 808: 133-139.

140. Müller-Hill, B. 1996. *In* The *lac* operon: a short history of a genetic paradigm. Walter de Gruyter, Berlin, Germany.

141. Russell, R. R., Aduse-Opoku, B., J., Sutcliffe, I. C., Tao, L., and., Ferretti J. J., A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolisms. J. Biol. Chem. 1992; 267: 4631-4637.

142. Saier, M. H., Jr., Chauvaux, S. Cook, G. M. Deutscher, J. Paulsen, I. T. Reizer, J. and Ye,J. J., Catabolite repression and inducer control in Gram-positive bacteria. Microbiology, 1996; 142:217-230.

143. Bernlohr, R. W., Maddock, M. K., and Goldberg, N. D., Cyclic guanosine 3':5'-monophosphate in *Escherichia coli* and *Bacillus licheniformis*. J. Biol. Chem., 1974; 249: 4329-4331.

144. Yeung, K. H., Larsson, G. C., and Yamazaki, H., Evidence against the involvement of adenosine 3', 5'-cyclic monophosphate in glucose inhibition of β-galactosidase induction in *Bacillus megaterium*. Can. J. Biochem., 1976; 54: 854-865.

145. Hueck, C. J., and W. Hillen. 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? ., Mol. Microbiol. 15:395-401.

146. Weickert, M. J., and Chambliss, G. H., Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA., 1990; 87: 6238-6242.

147. Hueck, C.J., W. Hillen, and J.M.H. Saier. Analysis of a *cis*-active sequence mediating catabolite repression in Gram-positive bacteria. Res. Microbiol. 1994; 145: 503-518.

148. Weickert, M. J., and S. Adhya. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. 1992; 267:15869-15874.

149. McLaughlin, R. E., and Ferretti, J. J., The multiple-sugar metabolism (*msm*) gene cluster of *Streptococcus mutans* is transcribed as a single operon. FEMS Microbiol. Lett. 1996; 140: 261-264.

150. Rosenow C, Maniar M, Trias J. Regulation of the α-galactosidase activity in *Streptococcus pneumoniae*: characterization of the raffinose utilization system. Genome Res., 1999; 9: 1189-97.

151. Pardee, A., An inducible mechanism for accumulation of melibiose in *E. coli*. J. Bacteriol., 1957; 73: 376-385.

152. Orskov I., Orskov F., Plasmid determined H_2S character in E. coli and its relation to plasmid carried raffinose fermentation and tetracycline resistance characters. J. Gen. Microbiol., 1973; 77: 487-499.

153. Prestidge, L. S., and Pardee A. B., A second permease for methyl-thio-β-D-galactoside in *E. coli* . Biochim. Biophys. Acta, 1965; 100: 591-593.

154. Muiznieks I., Rostoks N., Schmitt R., Efficient control of raf gene expression by CAP and two Raf repressors that bend DNA in opposite directions. Biol. Chem., 1999; 380: 19-29.

155. Stassi, D. L., J. J. Dunn, and S.A. Lacks. Nucleotide sequence of DNA controlling expression of genes for maltosaccharide utilization in *Streptococcus pneumoniae.* Gene, 1982; 20:359-366.

156. Vadeboncoeur, C. and M. Pelletier. 1997. The phosphoenolpyruvate: sugar phosphotransferase system of oral *streptococci* and its role in the control of sugar metabolism. FEMS Microbiol. Rev. 19: 187-207.

157. Cvitkovitch, D.C., D.A. Boyd, and I.R. Hamilton. 1995. Regulation of sugar transport via the multiple sugar metabolism operon of *Streptococcus mutans* by the phosphoenolpyruvate phosphotransferase system. J. Bacteriol. 177: 5704-5706.

158. Fridjonsson O., Watzlawick H., Mattes R., The structure of the α-galactosidase gene loci in *Thermus brockianus* ITI 360 and *Thermus thermophilus* TH125. Extremophiles, 2000; 4: 23-33.

159. Silvestroni A., Connes C., Sesma F., De Giori G., S., Piard J., C**.,** Characterization of the *melA* locus for α-galactosidase in *Lactobacillus plantarum.*, Appl. Environ. Microbiol., 2002;8: 5464-71.

160. Boucher I, Vadeboncoeur C, Moineau S. Characterization of genes involved in the metabolism of alpha-galactosides by *Lactococcus raffinolactis*., Appl. Environ. Microbiol., 2003; 69: 4049-56.

161. Hama H., Wilson T., H. Primary structure and characteristics of the melibiose carrier of *Klebsiella pneumoniae*. J. Biol. Chem., 1992; 267: 18371-6.

162. Sumner-Smith M., Bozzato R., P., Skipper N., Davies R., W., and Hopper J. E., Analysis of the inducible *MEL1* gene of *Saccharomyces carlsbergensis* and its secreted product, alpha-galactosidase (melibiase). Gene, 1985; 36: 333-340.

163. Post-Beittenmiller, M A., Hamilton R., W., Hopper J., E., Regulation of basal and induced levels of the MEL1 transcript in *Saccharomyces cerevisiae*. Mol. Cellular Biol., 1984; 4: 1238.

164. Narita S., Naganishi H., Yokouchi A., and Kagaya I., Method for manufacture of α-galactosidase by microorganisms. US Patent, 3,867,256 (1975).

165. Narita S., Naganishi H., Yokouchi A., Kagaya I., Method for manufacture of α-galactosidase by micro-organism. U.S Patent, 3,957,578; 1976 (Assignee: Hokkaido Sugar Co., Ltd. (Tokyo, JA).

166. Suzuki H., Kobayashi H., Ozawa Y., and Kamibayashi A., Method for manufacture of α-galactosidase by microorganisms. U.S. Patent, 3,795,585; (1974).

167. Delente J., Johnson J.H., Kuo M.J., O'Connor R.J., Process for the preparation of heat resistant α-galactosidase enzyme., U.S. Patent, 3,846,239; (1974).

168. Knap I. H., Hjort C., M., Halkier T., Kofod L., V., α.-Galactosidase enzyme., U. S Patent, 6,197, 566; 2001. (Assignee: Novo Nordisk A/S (Bagsvaerd, DK).

169. Olivieri R., Pansolli P., Fascetti E., and Cuiffiolotti P., Process for the production of α-galactosidase and uses of the enzyme thus obtained. US Patent 4,431,737 (1984).

170. Kotwal S. M., Gote M. M., Sainkar S. R., Khan M. I., and Khire J. M., Production of α-galactosidase by thermophilic fungus *Humicola* sp. in solid-state fermentation and its application in soya milk hydrolysis. Process Biochem., 1998; 33: 337-343.

171. Annunziato M., E., Mahoney R., R., and Mudgett R., E., Production of α-galactosidase from *Aspergillus oryzae* grown in solid state culture. J. Food Sci., 1986; 51: 1370.

172. Frost G. M., Moss D. M., Production of enzymes by fermentation, Biotechnology Vol. 7a. Ed. John F. Kennedy, 1987.pp 65.

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

174. Bridson E. Y., and Brecker A., "Design and formulation of microbial culture medium " In " Methods in Microbiology Microbiology " , Ed, J. R. Norris and D.W. Ribbons, 1970; vol. 3A. PP 229-295. Academic Press London.

175. Roy D., Chevalier P., Ward P., Savoie L., Sugar fermented by Bifidobacterium infants ATCC 27920 in relation to growth and α-galactosidase activity. Appl. Microbiol. Biotechnol. 1991; 34: 653-655.

176. Ikura Y., Horikoshi K., Isolation and some properties of α-galactosidase producing Bacteria., Agric. Bio. Chem., 1987; 51: 243-245.

177. Suzuki H., Kobayashi H., Ozawa Y., and Kamibayashi A., Method for manufacture of α-galactosidase by microorganisms. U.S. Patent, 1974; 3,832,284.

178. Kotwal S. M., Khan M. I., Khire J. M., Production of thermostable a-galactosidase from thermophilic fungus *Humicola* sp., J. Industrial. Microbiol., 1995; 15: 116-120.

179. Suzuki H., Ozawa Y., Tanabe O., Method of reducing raffinose content of beet molasses., US Patent, 1972; 3,647,625.

180. Arnaud N., Bush D., α-Galactosidase production., US Patent, 1976; 3,966,555.

181. Wong-Leung Y. L., Fong W., F., and Lam W., L., Production of α-galactosidase by Monascus grown on soybean and sugarcane wastes., World J. Microbiol. Biotechnol., 1993; 9 : 529-533.

182. Dey P. M., Patel S., Brownleader MD. Induction of α-galactosidase in *Penicillium ochrochl*oron by guar (*Cyamopsis tetragonobola*) gum. Biotechnol. Appl. Biochem., 1993; 17: 361-71.

183. Kaji A., Ichimi T., Production and properties of galactosides from *Corticium rolfsii*., Appl. Microbiol., 1969; 18: 1036-40.

184. Miller E. S., Jr, Kimberley, Parker N., Liebl W., Lam D, Callen W., Snead M. A., Mathur E. J., Short J. M., Kelly RM. Alpha-D-galactosidases from *Thermotoga* species. Methods. Enzymol., 2001; 330: 246-60.

185. Brown D. E., "Aeration in the submerged culture of microorganisms" In Methods in Microbiology (J.R. Norris and D.W. Ribbons, eds) ,1970; Vol. 2, pp 137-174. Academic press London.

186. Zapater I. G., Ullah A. H. J., and Wodzinski R. J., Extracellular α-galactosidase from *Aspergillus ficuum* NRRL3135 purification and characterization. Preparative Biochem., 1990; 20: 263-296.

187. Mitchell D. A. and Lonsane, B. K. (1992). Definition, Characterstics and Potential In Solid Substrate Cultivation (Eds) Doelle, H.W., Mitchell, D.A. and Rolz, C. E. Elsevier Science Publishers, London. pp 1.

188. Lonsane B. K., Ghildyal N. P., Budiatman S., and Ramkrishna S. V., Engineering aspects of solid state fermentation., Enzy. Microbial. Technol., 1985; 7: 258-265.

189. Lonsane B., K., Saucedo-Castaneda G., Raimbault M., Roussos S., Viniegra-Gonzalez G., Ghildyal N., P., Ramakrishna M., Krishnaiah M., M., Scale-up strategies for solid-state fermentation systems. Process Biochem., 1992; 27: 259-73.

190. Srinivas M. R. S., Padmanabhan S., Lonsane B., K., Growth kinetics, α-galactosidase biosynthesis, and concomitant invertase production by *Aspergillus niger* NCIM 839 in a solid-state fermentation system. Chem. Mikrobiol. Technol. Lebensm., 1993; 15 : 41-6.

191. Suzuki H., Ozawa Y., Oota H., and Yoshida H., Stidies on the decomposition of raffinose by α-galactosidase of Mold. Part-I α-Galactosidase formation and hydrolysis of raffinose by the enzyme preparation., Agri. Biol. Chem., 1969; 33: 506.

192. Silman R., W., Black L., T., McGhee J., E., Bagley E., B., Hydrolysis of raffinose in a hollow-fiber reactor using an unrefined mixture of a-galactosidase and invertase. Biotechnol. Bioeng., 1980; 22 : 533-41.

193. Somiari R., I., Balogh E., Hydrolysis of raffinose and stachyose in cowpea (*Vigna unguiculata*) flour, using a-galactosidase from *Aspergillus niger*., World J. Microbiol. Biotechnol., 1992;8: 564-6.

194. Cruz R., and Park Y.K., Production of fungal α-galactosidase and its application to the hydrolysis of galacto-oligosccharides in soybean milk. J. Food. Sci. 1982; 47: 1973-1975.

195. Srinivas M., R., S., Chand N., Lonsane B. K. 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., 1994; 10 : 139-44.

196. Liljestrom P., L., Tubb R., S., Korhola M., P., Construction of new α-galactosidase producing yeast strains and the industrial application of these strains. U.S Patent, 5, 055, 401 ; 1991 (Assignee: Alko Ltd. (Helsinki, FI).

197. Halstead J. R., Fransen M. P., Eberhart R. Y., Park A. J., Gilbert H. J., Hazlewood G. P., α-Galactosidase A from *Pseudomonas fluorescens* subsp. cellulosa: cloning, high level expression and its role in galactomannan hydrolysis. FEMS Microbiol. Lett., 2000; 192:197-203.

198. Fridjonsson O, Watzlawick H, Gehweiler A, Mattes R. Thermostable α-galactosidase from *Bacillus stearothermophilus* NUB 3621: cloning, sequencing and characterization. FEMS Microbiol. Lett., 1999; 176: 147-53.

199. Shibuya H, Kobayashi H, Yoshida S, Kaneko S, Park GG, Kusakabe I. Purification and characterization of recombinant Mortierella vinacea α-galactosidases I and II expressed in *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem., 1999; 63: 1096-9.

200. King MR, Yernool DA, Eveleigh DE, Chassy BM. Thermostable a-galactosidase from *Thermotoga neapolitana* cloning, sequencing and expression. FEMS Microbiol. Lett., 1998 ; 163: 37-42.

201. Liljestrom-Suominen P. L., Joutsjoki V., Korhola M., Construction of a stable α-galactosidase producing baker's yeast strain. Appl. Environ. Microbiol.,1988; 54: 245-249.

202. Giuseppin M. L., Almkerk J. W, Heistek J. C, Verrips CT. Comparative study on the production of guar α-galactosidase by *Saccharomyces cerevisiae* SU50B and *Hansenula polymorpha* 8/2 in continuous cultures. Appl. Environ. Microbiol., 1993 ; 59: 52-9.

203. Shibuya H, Nagasaki H, Kaneko S, Yoshida S, Park GG, Kusakabe I, Kobayashi H. Cloning and high-level expression of α-galactosidase cDNA from *Penicillium purpurogenum*. Appl. Environ. Microbiol., 1998; 64: 4489-94.

204. de Vries RP, van den Broeck HC, Dekkers E, Manzanares P, de Graaff LH, Visser J., Differential expression of three α-galactosidase genes and a single beta-galactosidase gene from *Aspergillus niger*. Appl. Environ. Microbiol., 1999; 65: 2453-60.

205. Kocabas E E, Dizbay M. Production of α-galactosidase Using *Bacillus stearothermophilus* Var. Calidolactis C953., Tr. J. of Biology, 1999; 23: 47-54.

206. Robert K. S., Protein purification, principles and practice. In Springer Advanced Text in Chemistry, Ed., Charles R. Cantor (1982), Springer Verlag, New York.

207. Shibuya H., Kobayashi H., Park G. G., Komatsu Y., Sato T., Kaneko R., Nagasaki H., Yoshida S., Kasamo K., Kusakabe I., Purification and some properties of α-galactosidase from *Penicillium purpurogenum*. Biosci. Biotechnol. Biochem., 1995;59: 2333-5.

208. Kotwal S. M., Khire J. M., Khan M. I., purification and characterization of an extracellular α-galactosidase from the thermophilic fungus *Humicola* SP. J. Biochem. Mol. Biol & Biophys.,1999; 3: 9-17.

209. Garro M. S., de Valdez G. F., Oliver G., de Giori G. S., Purification of α-Galactosidase from *Lactobacillus fermentum*. J. Biotechnol., 1996; 33: 302-5.

210. Varbanets D. L., Malanchuk V. M., Buglova T. T., and Kuhlmann R. A., *Penicillium* sp. 23 a-galactosidase: purification and substrate specificity., Carbohydr. Poly., 2001; 44: 357-363.

211. King M. R., White B. A., Blaschek H. P., Chassy B. M., Mackie R. I., Cann I. K., Purification and characterization of a thermostable α-galactosidase from *Thermoanaerobacterium polysaccharolyticum.*, J. Agric. Food Chem., 2002; 50: 5676-82.

212. Pederson D. M., Goodman R. E., Isozymes of α-galactosidase from *Bacillus stearothermophilus*. Can. J. Microbiol, 1980; 26: 978-84.

213. Mapes C., Sweeley C. C, Preparation and properties of an affinity column adsorbent for differentiation of multiple forms of α-galactosidase activity., J. Bio. Chem., 1973; 248: 2461-2470.

214. Berry M. J., Immunoaffinity purification of industrially relevant enzymes. Anal. Process., 1991; 28: 141-142.

215. Porter E. J. and Ladisch M., R., Ion exchange and affinity chromatography in the scaleup of the purification of α-galactosidase from soybean seeds. Biotechnol. Bioeng. 1991; 37: 356-363.

216. Del Campillo., and Shannon L., M., An a-galactosidase with hemagglutinin properties from soybean seeds. Plant Physiol., 1982; 69: 628-631.

217. Nadkarni M., A., Nair C., K., K., Pandey V., N., Pradhan D., S., Characterization of α-galactosidase from *Corynebacterium murisepticum* and mechanism of its induction. J. Gen. Appl. Microbiol., 1992; 38: 23-34.

218. Shibuya H, Kobayashi H, Sato T, Kim WS, Yoshida S, Kaneko S, Kasamo K, Kusakabe I. Purification, characterization, and cDNA cloning of a novel α-galactosidase from *Mortierella vinacea.* Biosci. Biotechnol. Biochem., 1997; 61: 592-8.

219. Dey PM, Del Campillo E. Biochemistry of the multiple forms of glycosidases in plants. Adv. Enzymol. Relat. Areas. Mol. Biol., 1984; 56: 141-249. Review.

220. Pridham J.B., Dey P. M., The nature and function of higher plant α-galactosidases. Plant. Carbohydr. Biochem., 1974; 83-89. Pridham J.B ED, Academic Press New York.

221. Kusiak J. W., Quirk J. M., Brady R. O., Purification and properties of the two major isozymes of α-galactosidase from human placenta. J. Biol. Chem., 1978; 253: 184-90.

222. Leenhouts K. K. J., Bolhuis A. A., Kok J. J., Venema G. G., Nucleic acid sequence of α-galactosidase from *Pediococcus pentosaceus.*, 1994; Submited to EMBL/GenBank/ DDBJ database.

223. Nagao Y., Nakada T., Imoto M., Shimamoto T., Sakai S., Tsuda M., Tsuchiya T., Purification and analysis of the structure of α-galactosidase from *Escherichia coli.*, Biochem. Biophys. Res. Commun., 1988; 151: 236-41.

224. Liebl W., Wagner B., and Schellhase J., Properties of an α-galactosidase, and structure of its gene GalA within an α and β-galactosidase utilization gene cluster of the hyperthermophilic bacterium *Thermotoga maritima*. Syst. Appl. Microbiol., 1998; 21: 1-11.

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

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

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

228. Zaprometova O. M., Ulezlo IV, Lakhtin VM. Structure and properties of a *Cephalosporium acremonium* α-galactosidase. Glycoconj. J. 1990; 7: 287-300.

229. Lazo P. S., Ochoa AG, Gascon S. α-Galactosidase (melibiase) from *Saccharomyces carlsbergensis*: structrual and kinetic properties. Arch. Biochem. Biophys., 1978; 191: 316-24.

230. Baumann, C. M. Rudiger, H. and Strosberg, A.D. (1981) A Comparison of two lectins from *Vicia cracea*. In: Lectins Biol. Biochem. Clin. Biochem. (Ed.) Bag. Hansen, T.C. Vol. 1. Walter de Gruyter and Co. New York pp 93.

231. Shannon, L. M., Hankins, C. N. and Strosberg, A. D. (1981) Enzyme Phytohemagglutinins: Their Reaction to Classic Legume Phytohemagglutins, In Lectins-biology, Biochemistry, Clinical Biochemistry, Vol. 1, Walter de Gruyter and Co., NewYork, pp 81.

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

233. Dey P. M., Del Campillo E. M., Lezica R., P., Characterization of a glycoprotein α-galactosidase from lentil seeds (*Lens culinaris*). J. Biol. Chem., 1983; 258: 923-9.

234. Wallenfels K, Malhotra P, Galactosidases., Adv. Carbohydr. Chem., 1961; 16: 238-298.

235. Ulezlo I. V., Zaprometova OM. Microbial alpha-galactosidase (a review)., Prikl. Biokhim. Mikrobiol., 1982; 18: 3-15.

236. Akiba T., Horikoshi K., Properties of α-galactosidase of alkalophilic Bacteria. Agr. Biol. Chem., 1976; 40: 1851-55.

237. Lee Y. C., Wacek V., Galactosidases from *Aspergillus niger*. Arch. Biochem. Biophys., 1970; 138 : 264-271.

238. Arnaud N., Bush D., and Horisberger M., Study of an Intracellular α-galactosidase from the thermophilic fungus *Penicillium duponti.* Biotech. Bioeng., 1976; 18 : 581.

239. Porter J. E., Sarikaya A., Herrmann K. M., Ladisch M.R., Effect of pH on subunit association and heat protection of soybean α-galactosidase. Enzyme Microb. Technol., 1992; 14: 609-13.

240. Shivanna B. D., Ramakrishna M., Prakash V., Ramadoss C.S., Influence of detergents and pH on the isolation, purification and molecular properties of α-galactosidase-C2 from germinating guar (*Cyamopsis tetragonolobus*). Indian J. Biochem. Biophys., 1989 Aug;26(4):234-42.

241. Dey P. M., Pridham J. B. Substrate specificity and kinetic properties of α-galactosidase from *Vicia faba*. Biochem. J. 1969; 115: 47P.

242. Mathew C. D., Balasubramaniam K., Mechanism of action of alpha-galactosidase. Phytochem., 1987; 26: 1299-1300.

243. Dey P. M., Malhotra O. P., Kinetic behavior of sweet almond α-galactosidase. Biochim. Biophys. Acta., 1969;185: 402-8.

244. Schmitt R., Rotman B., α-galactosidase activity in cell free extract of E. Coli., Biochem., Biophys. Res. Commun., 1966; 22 : 473.

245. Miller E. S. Jr, Kimberley, Parker N., Liebl W., Lam D., Callen W., Snead M. A., Mathur E. J., Short J. M., Kelly R. M. α-D-Galactosidases from *Thermotoga* species. Methods. Enzymol., 2001; 330: 246-60.

246. Sharma C. B., Sharma T. N., Multiple forms of α-galactosidase in chick pea seedlings., Phytochem., 1977; 16: 1053.

247. Agrawal K.M.L., Bahl O.P., Glycosidases of *Phaseolus vulgaris*; II-Isolation and general properties. J. Biol. Chem., 1968; 243: 103.

248. Dey P. M., Pridham J. B., Purification and properties of α-galactosidase from *Vicia faba* seeds. Biochem. J., 1969; 113: 49.

249. Brumer H., 3rd, Sims P. F., Sinnott ML. Lignocellulose degradation by *Phanerochaete chrysosporium*: purification and characterization of the main α-galactosidase. Biochem. J., 1999; 339: 43-53.

250. Dey P. M., Inhibition, transgalactosylation and mechanism of action of sweet almond α-galactosidase. Biochim. Biophys. Acta., 1969; 191: 644-52.

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

252. Elshafei A. M., Foda M. S., Aboul-Enein A., Afify A. S., Ali N. H., Purification and enzymic properties of a-galactosidase from *Penicillium janthinellum.*, Acta Biotechnol., 1993; 13 : 351-9.

253. Manzanares P., de Graaff L, H., Visser J. Characterization of galactosidases from *Aspergillus niger:* purification of a novel α-galactosidase activity. Enzyme Microb. Technol., 1998; 22: 383-90.

254. Kelemen M. V., Whelan W.J., Inhibition of glucosidases and galactosidases by polyols., Arch. Biochem. Biophys., 1966; 117: 423-428.

255. Gao Z., Schaffer A., A novel alkaline a-galactosidase from melon fruit with a substrate preference for raffinose., Plant Physiol., 1999; 119 : 979-987.

256. Agnantiari G., Christakopoulos P., Kekos D., Macris B. J., A purified a-galactosidase from *Aspergillus niger* with enhanced kinetic characteristics. Acta Biotechnol, 1991; 11: 479-484.

257. Suzuki H., Li, S.C. and Li Y.T., α-Galactosidase from *Mortierella vinacea*: Crystalization and properties., J. Biol. Chem. 1970; 245: 781.

258. Pigman W. W., Specificity, Classification and Mechanism of action of glycosidases., Adv. Enzymol., 1946; 14: 41-74.

259. Kato K., Ikami T., Kono H., Yamauchi R., Ueno Y., Transferase action of a-galactosidase from tubers of *Stachys affinis.*, Agric. Boil. Chem., 1982; 46: 1089-1090.

260. Shannon L. M., Hankins C.N., Enzymatic properties of phytohemagglutinins., Recent. Adv. Phytochem., 1981; 15: 93-110.

261. Malhotra O. P., Dey P. M., Specificity of sweet almond α-galactosidase., Biochem J., 1967; 103: 739.

262. Adams M., Richtmyer N., K., Hudson C., S., Some enzymes present in highly purified invertase preparations; a contribution to the study of fructofuranosidases, galactosidases and mannosidases., J. Am. Chem. Soc., 1943; 65: 1369.

263. Shabalin K. A., Kulminskaya A.A., SaVEL' ev, Shishlyannikov S. M., Neustroev K. N., Enzymatic properties of α-galactosidase from *Trichoderma reesei* in the hydrolysis of galacto-oligosaccharides. Enzy. Microbial. Technol., 2002; 30: 231-239.

264. Savel'eV A. N., Eneyskaya E. V., Isaeva-Ivanova L. S., Shabalin K. A., Golubev A. M., Neustroev K. N., Enzymatic properties of α-galactosidase from *Trichoderma reesei*. Carbohyd. Res., 1996; 296: 261-273.

265. Dey P. M., Biochemistry of plant Galactomannan., Adv. Carbohydr. Chem. Biochem., 1978; 35: 341-376.

266. McCutchen C. M., Duffaud G. D., Leduc P., Petersen A.R.H., Tayal A., Khan S.A., Kelly R. M., Characterization of extremely thermostable enzymatic brekers (α-1,6-galactosidase and β-1,4-mannanase) from the hyperthermophilic bacterium Thermotoga neapolitana 5068 for hydrolysis of guar gum. Biotechnol. Bioeng., 1996; 52 : 332-339.

267. Luonteri E., Tenkanen M., Viikari L., Substrate specificities of *Penicillium simplicissimum* alpha-galactosidases. Enzyme Microb. Technol., 1998; 22: 192-8.

268. Li Y.T., Shetlar M. R., Galactosyl transfer reactions catalyzed by *Pneumococcal* α-galactosidase. Arch. Biochem. Biophys., 1964; 108: 301-313.

269. Mitsutomi M., Ohtakara A., Isolation and identification of oligo-saccharides produced from raffinose by transgalactosylation reaction of thermostable alpha-galactosidase from *Pycnoporus cinnabarinus*. Agri. Biol.Chem., 1988; 52: 2305-2311.

270. Sugawara S., Nakagawa R., Urashima T., Sato T., Muratubaki T., Sayama K., Transgalactosylation products from melibose by the alpha-galactosidase of *Absidia corymbifera*. Agric. Biol. Chem., 1990 ; 54: 211-3.

271. Scigelova M., Crout D. H. G., Purification of alpha-galactosidase from *Aspergillus niger* for application in the synthesis of complex oligo-saccharides. J. Mol. Catalys. B; Enzymatic., 2000, 8: 175-181.

272. Clancy M.J., Whelan W.J., Enzymatic polymerization of monosaccharides., Arch. Biochem. Biophys., 1967; 118: 730-735.

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

274. Spangenberg P, Andre C, Dion M, Rabiller C, Mattes R. Comparative study of new α-galactosidases in transglycosylation reactions. Carbohydr. Res., 2000; 329: 65-73.

275. Goldstein I.J., and Hayes C. E., The lectins: Carbohydrate binding proteins of plants and animals, Adv. Cabohydr. Chem. Biochem., 1978; 35: 127-340.

276. Tenno M., Kézdy F. J., Elhammer A.P., and Kurosaka A., Function of the lectin domain of polypeptide *N*-acetylgalactosaminyltransferase 1., Biochem. Biophys. Res. Comm., 2002; 298: 755.

277. Hankins C. N., Kindinger J. I., Shannon L. M., Legume α-galactosidases which have hemagglutinin properties., Plant Physiol., 1980; 65: 618-622.

278. Del Campillo E., Shannon L. M., Hankins C. N., Molecular properties of the enzymic phytohemagglutinin of mung bean., J. Biol. Chem., 1981; 256: 7177-80.

279. Hankins C. N., Kindinger J. I., Shannon L. M., Legume: a-galactosidase form devoid of hemagglutinin activity. Plant Physiol., 1980; 66: 375.

280. Overbeeke N, Fellinger AJ, Toonen MY, van Wassenaar D, Verrips CT. Cloning and nucleotide sequence of the alpha-galactosidase cDNA from *Cyamopsis tetragonoloba* (guar). Plant. Mol. Biol., 1989; 13: 541-50.

281. Gotlib R.W., Bishop D F., Wang A. M., Zeidner K. M., Ioannou Y. A., Adler D. A., Disteche C. M., Desnik R. J., The entire genomic sequence and cDNA expression of mouse a-galactosidase A. Biochem. Mol. Med., 1996; 57: 139-148.

282. Liljestrom P. L., and Liljestrom P., Nucleotide sequence of the mel-A gene coding for α-galactosidase in E. coli K_{12}. Nucl. Acids Res., 1987; 15: 2213-2220.

283. Zverlov V., Nucleic acid sequence of α-galactosidase from *Thermoanaerobacter ethanolicus*., 1998; Submited to EMBL/GenBank/ DDBJ database.

284. Jindou S, Karita S, Fujino E, Fujino T, Hayashi H, Kimura T, Sakka K, Ohmiya K., α-Galactosidase Aga27A, an enzymatic component of the *Clostridium josui* cellulosome. J. Bacteriol., 2002; 184: 600-4.

285. Margolles-Clark E., Tenkanen M., Luonteri E., Penttila M., Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. Eur J Biochem. 1996; 240: 104-11.

286. Liebl W, Wagner B, Schellhase J. Properties of a α-galactosidase, and structure of its gene galA, within an α-and β-galactoside utilization gene cluster of the hyperthermophilic bacterium *Thermotoga maritim*a. Syst. Appl. Microbiol. 1998; 21: 1-11.

287. Shibuya H., Kobayashi H., Kasamo K, Kusakabe I. Nucleotide sequence of α-galactosidase cDNA from *Mortierella vinacea*. Biosci. Biotechnol. Biochem., 1995; 59: 1345-8.

288. Bishop DF, Calhoun DH, Bernstein HS, Hantzopoulos P, Quinn M, and Desnick RJ. Human α-galactosidase A: nucleotide sequence of a cDNA clone encoding the mature enzyme. Proc. Natl. Acad. Sci. U S A., 1986; 83: 4859-63.

289. Fujimoto Z., Kaneko S., Momma M., Kobayashi H., Mizuno H., Crystal structure of rice α-galactosidase complexed with D-Galactose. J. Biol. Chem., 2003; 278: 30.

290. Garman S. C., Hannick L., Zhu A., Garboczi D. N., The 1.9 Å structure of α-N-Acetylgalactosaminidase: Molecular basis of Glycosidase deficiency diseases. Structure; 2002; 10: 425.

291. Grace DeSantis, J. Bryan Jones., Chemical modification of enzymes for enhanced functionality Current Opinion in Biotechnol., 1999; 10: 324-330.

292. Profy A.T., Schimmel, P. P., Complementary use of chemical modification and site-directed mutagenesis to probe structure-activity relationships in enzymes. Nucleic Acid Res. Mol. Biol., 1988; 35: 1-26.

293. Stemmer W. P., DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution., Proc. Natl. Acad. Sci. USA., 1994; 91: 10747-10751.

294. Adair, John R., Protein engineering., Molecular Biology and Biotechnology (4th Edition), 379-404. Edited by: Walker, John M.; Rapley, Ralph. Royal Society of Chemistry: Cambridge, UK. 2000.

295. Chirumamilla R. R., Muralidhar R., Marchant, R., Nigam P., Improving the quality of industrially important enzymes by directed evolution. Molecular and Cellular Biochemistry, 2001; 224: 159-168.

296. Gerlt J. A., Relationships between enzymatic catalysis and active site structure revealed by applications of site-directed mutagenesis. Chem. Rev., 1987; 87:1079-105.

297. Means G. E., Feeney R. E., Chemical modification of proteins. 1971; Holden-Day, Inc. California.

298. Zhu A., Monahan C., Wang Z.K., Trp-16 is essential for the activity of α-galactosidase and α-N-acetylgalactosaminidase. Biochim. Biophys. Acta., 1996; 1297: 99-104.

299. Maranville E, Zhu A. Assessment of amino-acid substitutions at tryptophan 16 in α-galactosidase. Eur. J. Biochem., 2000; 267: 1495-501.

300. Kachurin A. M., Golubev A. M., Geisow M. M., Veselkina O. S., Isaeva-Ivanova L. S., Neustroev K. N., Role of methionine in the active site of α-galactosidase from *Trichoderma reesei*. Biochem. J., 1995; 308: 955-64.

301. Mathew C. D., Balasubramaniam K., Chemical modification of α-galactosidase from coconut. Phytochem., 1986; 25: 2439-2443.

302. Hart D. O., He S, Chany C. J. 2nd, Withers S. G., Sims P. F., Sinnott M. L., Brumer H., 3rd. Identification of Asp-130 as the catalytic nucleophile in the main α-galactosidase from *Phanerochaete chrysosporium*, a family 27 glycosyl hydrolase. Biochemistry, 2000; 39: 9826-36.

303. Kotwal S. M., Khire J. M., Khan M. I., Chemical modification of a-galactosidase from the thermophilic fungus *Humicola* sp. J. Biochem. Mol. Biol. & Biophys., 2000; 4: 66-72.

304. Koide T., Ishiura M., Iwai K., Inoue M., Kaneda Y., Okada Y., Uchida T., A case of Fabry's disease in a patient with no α-galactosidase-A activity caused by single amino acid substitution of Pro-40 by Ser., FEBS Lett., 1990; 259: 353-356.

305. Pluckthun A., Studying protein structure and function by directed evolution., Protein Dynamics, Function and Design, edited by Jardetzky *et al*. Plenum Press, New York, 1998.

306. Wang Y., Evolving enzymes., Caltech Undergraduate Res. J., 2001; 1: 32. [URL.-http:/www.curj.caltech. edu/archives/vol1/1001.pdf].

307. Dion M., Nisole A., Spangenberg P., Andre C., Glottin-Fleury A., Mattes R., Tellier C., Rabiller C., Modulation of the regioselectivity of a Bacillus a-galactosidase by directed evolution., Glycoconjugate J., 2001; 18: 215.

308. Ohtakara A., Mitsutomi M., Immobilization of thermostable a-galactosidase from *Pycnoporus cinnabarinus* on chitosan beads and its application to the hydrolysis of raffinose in beet sugar molasses. J. Ferment. Technol., 1987; 65: 493-498.

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

310. Messina M. J., Legumes and Soybeans overview of their Nutritional Profiles and Health Effects. Am. J. Clin. Nutr., 1999; 70: 439S-450S.

311. Steggerda F. R., Richard E. A., Rackis J. J., Effect of various Soybean products on Flatulence in the adult Man. Proc. Soc. Exp. Biol. Med., 1966; 121: 1235-1239.

312. Singh U., Antinutritional factors of Chick pea and Pigeonpea and their removal by Processing. Plant Foods. Hum. Nutr., 1988; 38: 251-261.

313. Gitzelmann R. and Auricchio S., The handling of soy α-galactosidase by a normal and galactosemic child. Pediatrics. 1965; 36: 231-235.

314. Cristofaro F. M., Wuhrmann J. J., Involvement of the raffinose family of oligo-saccharides in Flatulence. In: Sipple HL and Mcnutt KW, editor. Sugar in Nutrition Academic Press New York. 1974; 313-336.

315. Suarez F. L., Springfield J. F., Julie K., Lohrmann T. T., Kerr P. S., Levitt M. D., Gas production in humans ingesting a soybean flour derived from beans naturally low in oligo-saccharides. Am. J. Clin. Nutr., 1999; 69: 135-139.

316. Akinyele, I.O., Effects of traditional methods of processing on the nutrient content and some anti-nutritional factors in cowpeas *(Vigna unguiculata).* Food Chem., 1989; 33: 291-299.

317. Scalabrini P., Rossi M., Spettoli P., Matteuzzi D., Characterization of Bifidobacterium strains for use in soymilk fermentation., Int. J. Food Microbiol., 1998; 39: 213-219.

318. Thananunkul D., Tanaka M., Chichester C. O., Lee T. C., Degradation of raffinose and stachyose in soymilk by α-galactosidase from *Moretierella vinacea*. Entrapment of α-galactosidase within polyacrylamide gel. J. Food. Sci., 1976; 41: 173-75.

319. Thippeswamy S. Mulimani V. H., Enzymatic degradation of Raffinose family oligo-saccharides in Soyamilk by immobilized α-galactosidase from *Gibberella fujikuroi*., Process Biochem., 2002; 38: 635-640.

320. Yamane T., Decomposition of raffinose by α-galactosidase. An enzymatic reaction applied in the factory-process in Japanese beet sugar factories. Sucr. Belge/Sugar Ind. Abstr. 1971; 90: 345-348.

321. Linden, J. C., Immobilized a-D-galactosidase in the sugar beet industry. Enzyme Microb. Technol., 1982; 4: 130-6.

322. Buckeridge M. S, Panegassi V. R., Rocha D. C. and Dietrich S. M. C., Seed galactomannan in the classification and evolution of the leguminosae., Phytochem., 1995; 38: 871-875.

323. Fox J. E., In A. Imeson, Thickening and gelling agents for food., (1992) pp 153-171. Blackie Academics and Professional, London.

324. Whistler R. L., Bushway A. A., Singh P. P, Naxahara W., Tokuzen R., Non cytotoxic, antitumor polysaccharides., Adv. Carbohydr. Chem. Biochem., 1975; 32: 235-275.

325. Dea I. C. M., Morrison A., Chemistry and interaction of seed galactomannan., Adv., Carbohydr. Chem. Biochem., 1975; 31: 241-245.

326. Pai V.B., Khan S.A., Gelation and rheology of xanthan /enzyme modified guar blends., Carbohydr. Poly., 2002; 49: 207-216.

327. Cronin, C. E., Giannouli P., McCleary B. V., Brooks M., Morris E. R. 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), 2002; 278: 289-296.

328. Joersbo, Morten, Marcussen Jan, Brunstedt Janne., In vivo modification of the cell wall polysaccharide galactomannan of guar transformed with a a-galactosidase gene cloned from senna. Molecular Breeding, 2001; 7: 211-219.

329. Ademark P., De Vries R. P., Hagglund P., Stalbrand H., Visser J., Cloning and characterization of Aspergillus niger genes encoding an a-galactosidase and a b-mannosidase involved in galactomannan degradation. European Journal of Biochemistry,2001; 268 : 2982-2990.

330. Bulpin P. V., Gidley M.J., Jeffcoat R., and Underwood D. R., Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase, Carbohydr. Poly., 1990; 12: 155-168.

331. Barry V., McCleary R. A., Robert W., and Hans N., Effect of galactose content on the solution and interaction properties of guar and carob galactomannans. Carbohydrate Research, Volume 92, Issue 2, 1 June 1981, Pages 269-285.

332. Kuo T. M., Van Middlesworth J. F., and Wolf W. J., Content of raffinose oligo-saccharides and sucrose in various plant seeds. J. Agri. Food Chem., 1988; 36: 32-36.

333. Gdala J., Johansen H. N., Bach K. E., Knap I. H., Wagner P. and Jorgensen O.R., The digestibility of carbohydrates, protein and fat in the small and large intestine of piglets fed non-supplemented and enzyme supplemented diets. Animal Feed Science Technology., 1997; 65:15-33.

334. Marquardt R. R., Brenes A., Zhang Z., and Boros D., Use of enzymes to improve nutrient availability in poultry feedstuffs, Animal Feed Science and Technology, 1996; 60: 321-330.

335. Kidd M. T., Morgan G. W., Jr., Zumwalt C. D., Price C. J., Welch P. A., Brinkhaus F. L., Fontana E. A., a-Galactosidase enzyme supplementation to corn and soybean meal broiler diets. Journal of Applied Poultry Research, 2001; 10: 186-193.

336. Pan B., Li D., Piao, X., Zhang L., Guo L., Effect of dietary supplementation with a-galactosidase preparation and stachyose on growth performance, nutrient digestibility and intestinal bacterial populations of piglets. Archives of Animal Nutrition, 2002; 56: 327-337.

337. Desnick R. J., Ioannou Y. A. , Eng C. M., α-Galactosidase A deficiency: Fabry disease. In: The Metabolic and Molecular Bases of Inherited Disease. New York, NY: McGraw Hill, 2001; 3733-3774.

338. Gillard, B. K., Thurmon, L.T., Marcus, D. M., Variable Subcelluler Localization of Glycosphingolipids. Glycobiology 3, 57-67, 1993

339. Hakomori S., Structure, organization, and function of glycosphingolipids in membrane. Curr. Opin. Hematol. 2003 Jan;10(1):16-24.

340. Peters F. P. J., Kho T. L. and Vermeulen A., Anderson-Fabry's disease: a-galactosidase deficiency, The Lancet, 2001; 357: 138-140.

341. Ashley G. A., Shabbeer J., Yasuda M., Eng C. M., Desnick R. J., Fabry disease: twenty novel α-galactosidase A mutations causing the classical phenotype. J Hum. Genet., 2001; 46: 192-6.

342. Breunig F., Weidemann F., Beer M., Eggert A., Krane V., Spindler M., Sandstede J., Strotmann J., Wanner C., Fabry disease: diagnosis and treatment. Kidney Int. Suppl. 2003; 84: S181-5.

343. Breunig F., Knoll A., Wanner C., Enzyme replacement therapy in Fabry disease: clinical implications. Curr. Opin. Nephrol. Hypertens.2003; 12: 491-5.

344. Ye Y., Niekrasz M., Kosanke S., et al. The pig as a potential organ donor for man. Transplantation 1994; 57:694-703.

345. Moses R. D., Auchincloss H. Mechanisms of Cellular Xenograft Rejection. Xenotransplantation. The transplantation of organs and tissues between species. Cooper DKC, Kemp E, Platt JL, White D. J. G., Springer. Berlin. Chapter 12 p. 140-174. 1997.

346. Galili U., Clark M. R., Shohet SB, Buehler J, Macher BA. Evolutionary relationship between the natural anti-Gal antibody and the alpha 1-3 Gal epitope in primates. Proc. Natl. Acad. Sci. USA., 1987; 84: 1369-1373.

347. LaVecchio, J. A., Dunne, A. D. & Edge, A. S. Enzymatic removal of α-galactosyl epitopes from porcine endothelial cells diminishes the cytotoxic effect of natural antibodies. Transplantation, 1995; 60: 841–847.

348. Yan J. L., Yu L. Y., Zhu L. H., Guo L. H, Expression of human α-galactosidase leads to reduction of major xenoepitope Gal-alpha (1,3) Gal in NIH 3T3 cell. Acta Pharmacol Sin. 2003; 24: 985-90.

349. Stone K. R., Ayala G., Goldstein J., Hurst R., Walgenbach A. & Galili U. Porcine cartilage transplants in the cynomolgus monkey. III. Transplantation of α-galactosidase-treated porcine cartilage. Transplantation, 1998; 65: 1577–1583.

350. K. Landsteiner, Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. Zbl Bakt, 1900; 27: 357–362.

351. Goldstein j., Conversion of ABO blood groups. Transfus. Med. Rev., 1989; 3: 206–212.

352. Harpaz, N., Flowers, H. M., and Sharon, N. studies on B antigenic sites of human erythrocytes by use of Coffee bean α-galactosidase. Arch. Biochem. Biophys., 1975; 170: 676–683.

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

354. Vosnidou N. C., Johnson S. A., Mitra M. M., Wells D. C., Li C. Q., Evans M, L., Harmata M. A., Walker J. C., Smith D. S., Seroconversion of type B to O erythrocytes using recombinant Glycine max a-D-galactosidase. Biochem. Mol. Biol. Int., 1998; 46: 175-186.

355. Oishi K., Aida K., Blood group substance degrading enzymes obtained from *Streptomyces sp.* Part-II. Purification and characterization of a-D-galactosidase from *Sterptomyces* 9917S_2.. Agr. Biol. Chem., 1971; 35: 1101-1108.

356. Kusakabe I., Kaneko R., Takada N., Zamora A., and et al. A simple method for elucidating structures of galactomanno-oligosaccharides by sequential action of β-mannosidase and α-galactosidase. Agri. Biol. Chem., 1990; 54: 1081-1083.

357. Ajisaka K., and Fujimoto H., Regioselective synthesis of trisaccharides by use of a reverse hydrolysis activity of α-and β-galactosidase., Carbohydr. Res., 1989; 185: 139-146.

358. Clarke J. H., Davidson K., Rixon J. E., and et al., A comparison of enzyme aided bleaching of soft wood paper pulp using combination of xylanase, mannanase and α-galactosidase., Appl. Microbiol. Biotechnol., 2000; 53: 661-667.

359. Coolber, T., Daniel, R. M., Morgan H. W., The Enzymes from extreme thermophiles Bacterial sources: Thermostability and industrial Relevance., Adva. Biochem. Eng / Biotechnol., 1992; 45: 57-97.

360. Shekhar Sharma, S. H., Economic importance of thermophilic fungi., Appl. Microbiol. Biotechnol.,1989; 31: 1-10.

361. Bylina E. J., Swanson R.V., Mathur E. J., Lam D. E., Thermostable glycosidases, their production with recombinant cells, and their use in hydrolysis of cello-oligosaccharides. US patent; 6,368,844 (1997).

362. Scigelova M., Singh S., Crout D., Glycosidases-a great synthetic tool. J. Mol. Catal. B: Enzym., 1999; 6: 483-494.

363. Buchanan R. E. and Gibbons N. E. editor. Bergey's Manual of Determinative Bacteriology (8 th Ed.). The Williams and Wilkins Company, Baltimore, USA. 1974.

364. Sneath Peter H. A., editor, Bergey's Manual of Systemic Bacteriology Vol. 2. The Williams and Wilkins Company, Baltimore, USA. 1986.

365. Berkeley R. C. W., Logan N. A., Shute L. A., and Capey A. G., Identification of *Bacillus* species. In Bergan T., editor, Methods in Microbiology Vol.16. Academic Press London. 1984; 291-328.

366. Lachake A.H., 1,4,β-D-xylan xylohydrolase of *Scerotium rolfsii*. In: Wood W.A., and Kellogg S.T., editor, Methods In Enzymology[, Vol. 160, . Academic Press, Inc. New York, 1988; p 679.

367. Somogyi M., Notes on sugar determination., J. Bio. Chem., 1952; 195: 19-23.

368. Tinder P., Determination of blood glucose using an oxidase peroxidase system with a non carcinogenic chromogen., J. Clin. Pathol., 1969; 22: 158-161.

369. A. Goldstein and O. Lampen, Methods Enzymol., 42: 504. Academic Press, New York, 1975, p. 504.

370. P. Bernfeld, in: S.I. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, vol. 1, Academic Press, New York, 1955, pp. 149–158.

371. Kunitz M., Crystaline soybean trypsin inhibitor. II. General properties., J. Gen. Physiol., 1947; 30: 291-310.

372. Laskowski M., Enzymes of protein metabolism: Trypsinogen and trypsin. In: Colowick S. P. and Kaplan N. O., editor, Methods In Enzymology, Vol.2. Academic Press, Inc. New York, 1955; 26-36.

373. Bernfeld p., in: S.I. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, vol. 1, Academic Press, New York, 1955, pp. 155.

374. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. L., Protein measurement with the Folin Phenol reagent., J. Biol. Chem., 1951; 193: 265-275.

375. Lineweaver H., and Burk D., Determination of enzyme dissociation constants., J. Amer. Chem. Soc.,1934; 56: 658.

376. Sebastiao T., de Rezende and Felix C. R., Raffinose hydrolyzing activity of *Aspergillus fumigatus* Biotechnol. Lett. 1997; 19: 217-220.

377. Schaffer A., Zhifang G., Alkaline α-galactosidase., United States Patents., 6,607,901; 2003.

378. Janz L., Ganter C., Stezowski J., Mattes R., Elucidation of functional domain in thermostable isoenzymes of alpha-galactosidase in *Bacillus stearothermophilus*. Enzyme properties are encoded

in a genetically exchangeable domain. Biochem. Eng. -Stuttgart, [Proc. Int. Symp.], 2nd. Meeting, Edited by: Reuss Matthias. Fischer. 1990: 170-173.

379. Watzlawick H., Ganter C., Stezowski J., Mattes R., Biochem. Eng.--Stuttgart, [Proc. Int. Symp.], 2nd, Meeting Date 1990, 174-6. Edited by: Reuss, Matthias. Fischer: Stuttgart, Fed. Rep. Ger. (English) 1991. Comparison of alpha-galactosidase genes homologous domains in thermostable and thermolabile enzymes.

380. Gurjar M.M., Khan M.I. Gaikwad S.M., Alpha-Galactoside binding lectin from Artocarpus hirsuta: characterization of the sugar specificity and binding site. Biochim Biophys Acta. 1998;1381:256-64.
381. Laemmli U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, Nature (London). 1970; 227: 680-685.

382. Deutscher M. P., Guide to protein purification., Methods. Enzymol., 1990; 182, pp430. academic press, Toronto.

383. Bollag D. M., Rozycki M. D., Edelstein S. J., Isoelectric focusing and two dimensional electrophoresis., In: Protein methods, 2nd Edition. (1996) A John Wiley & Sons, Inc., Publication.

384. Chinnatambi S., Bodhe A. M., and Lachke A. S., Fabrication of improved mini scale eletrofocusing unit. Biotechnol. Techniques, 1994; 8: 681-682.

385. Andrews P. Estimation of the molecular weights of proteins by sephadex gel filtration. Biochem. J., 1964; 91: 222-233.

386. Weber K. and Osborn M. The Reliability of Molecular Weight Determination by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J. Bio. Chem., 1969; 244: 4406-4412.

387. Spande T. F., & Witkop B., Determination of Tryptophan content of protein with N-bromosuccinimide., Methods In Enzymol. 1967; 11: 498-506.

388. Habeeb A. F. S. A., Reaction of protein sulfhydryl groups with Ellman's reagent. Methods In Enzymol. 1972; 25: 457-464.

389. Dubois M., Gilles J.K., Hamilton P.A., and et al., Colorimetric method for determination of sugars and related substances., Anal. Chem., 1956; 28: 350.

390. Clustal W, multiple sequence alignment program., URL: http://www.ebi.ac.uk/clustalw/.

391. Adler A. J., Greenfield N. J., Fasman G. D., Circular dichroism and optical rotatory dispersion of proteins and polypeptides. Methods In Enzymology, Vol., 27, pp 675-735.

392. Provencher, S. W. and Glockner, J., Estimation of globular protein secondary structure from circular dichroism. Biochem.,1981; 20: 33. (URL: http://lamar.colostate.edu/~sreeram/CDPro/).

393. Segel I. H., Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady state Enzyme Systems, New York, Wiley 1975.

394. Siddiqui K. S., Saqib A. A. N., Rashid M. H., Rajoka M. I., Carboxyl group modification significantly altered the kinetic properties of purified carboxylmethylcellulase from *Aspergillus niger*. Enzyme Microb. Techno., 2000; 27 : 467-474.

395. Dann W.J., The temperature coefficients and energy exchanges of the citric acid dehydrogenase of Cucumber seeds., Biochem, 1931, 25: 12.

396. Albon N and Gross D., The chromatographic determination of Raffinose in raw sugars. Analyst, 1950; 75: 454-457.

397. Dixion M., Determination of enzyme inhibition constant., Biochem., J., 1952; 55 : 170-171.

398. Kornreich R., Desnick R.J., Bishop D.F., Nucleotide sequence of the human alpha-galactosidase A gene., Nucleic Acids Res., 1989;17:3301-3302.

399. Liljestroem P.L."The nucleotide sequence of the yeast MEL1 gene. Nucleic Acids Res., 1985; 13:7257-7268.

400. Watzlawick H., Ganter C., Mattes R. Nucleic acid sequence of alpha-galactosidase from *Bacillus stearothermophilus* strain KVE39; gene AGAA Submitted to the EMBL/GenBank/DDBJ databases. Nov-2000.

401. Ethier N., Talbot G., Sygusch J., Cloning, sequencing and expression of a *Bacillus stearothermophilus* alpha-galactosidase gene clustered with a beta-mannanase gene., Submitted to the EMBL/GenBank/DDBJ databases. , Apr-1999.

402. Cornish-Bowden, A., A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem. J. 1974; 137 143–144.

403. Watanabe K, Chishiro K, Kitamura K, Suzuki Y., Proline residues responsible for thermostability occur with high frequency in the loop regions of an extremely thermostable oligo-1,6-glucosidase from *Bacillus thermoglucosidasius* KP1006. J Biol Chem., 1991; 266 :24287-94.

404. Pelton J.T. and McLean L.R., Spectroscopic Methods for Analysis of Protein Secondary Structure. (Review)., Analytical Biochem., 2000; 277: 167-176.

405. McCleary B. V., Enzyme interactions in the hydrolysis of galactomannan in germinating guar: The role of Exo-β-mannanase., Phytochem., 1983; 22: 649-658.

406. McCleary B. V., Enzyme hydrolysis, fine structure and gelling interaction of legume seed D-galacto-D-mannans., Carbohydr. Res., 1979; 71 : 205-230.

407. Lenny LL, Hurst R, Zhu A, Goldstein J, Galbraith RA. Multiple-unit and second transfusions of red cells enzymatically converted from group B to group O: report on the end of phase 1 trials. Transfusion.,1995; 35 : 899-902.

408. Kruskall MS, AuBuchon JP, Anthony KY, Herschel L, Pickard C, Biehl R, Horowitz M, Brambilla DJ, Popovsky MA. Transfusion to blood group A and O patients of group B RBCs that have been enzymatically converted to group O. Transfusion., 2000; 40: 1290-8.

409. Ross P. D., Subramanium S., thermodynamics of protein association reactions: forces contributing to stability, Biochem., 1981; 20: 3096-3102.

410. Kawada N., Nosoh Y., Relation of Arrhenius discontinuities of NADH dehydrogenase to change in membrane lipid fluidity of *Bacillus caldotenax*. FEBS Lett., 1981; 124 :15-8.

411. Douglas S. B., Vieille C* and Zeikus J. G., Cloning and expression of the gene encoding the *Thermoanaerobacter ethanolicus* 39E secondary-alcohol dehydrogenase and biochemical characterization of the enzyme., Biochem. J., 1996; 316, 115-122.

412. Dixon M., Webb E. C., EnzymeS., Longman group Ltd., 1979. pp 174-176.

413. Gaudreault P., Webb A., Partial purification and properties of an alkaline alpha-galactosidase from mature leaves of *Cucrbita pepo.* Plant. Phyiol., 1983; 71: 662-668.

414. Murali R., Ioannou Y. A., Desnick R. J., Burnett R. M., Crystallization and preliminary X-ray analysis of human alpha-galactosidase A complex. J. Mol. Biol., 1994; 239: 578-80.

415. Golubev A. M., Neustroev K. N., Crystallization of alpha-galactosidase from *Trichoderma reesei*. J. Mol. Biol., 1993; 231: 933-4.

416. Bradford M. M., A rapid and sensitive method for the Quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 1976; 72 : 248-254.

417. Dixion M., The effect of pH on the affinities of enzymes for substrates and inhibitiors. Biochem., 1953; 55: 161.

418. Miles W., Modification of histidyl residues in proteins by Diethylpyrocarbonate. Methods Enzymol., 1977; 47: 431-441. . Ed. C.H.W. Hris, Academic press, New York and London.

419. Riordan vallee., O-Acetyltyrosine., Methods Enzymol., 1972; 25, Part B., Ed. Hirs C. H. W., Academic Press.

420. Habeeb A. F. J. A., Determination of free amino groups in proteins by Trinitrobenzenesulfonic acid. Anal. Biochem. 1966; 14, 328-336.

421. Riordan J. F. and Vallee B. L., Modification reactions; Acetylation., Methods in Enzymol., 1967; Vol. 11: 565-570. Ed. C.H.W. Hris, Academic press, New York and London.

422. Dixon H. B. and Perham R. N.,Reversible blocking of amino groups with citraconic anhydride., Biochem. J., 1968; 109: 312-315.

423. Means G. E., Feeney R. E., Reductive alkylation of amino groups in proteins., Biochem., 1968; 7: 2192-2201.

424. Pho D.B., Roustan C., Tot A. N. T. and Pradel L. A., Evidence for an essential glutamyl residue in yeast hexokinase. Biochemistry. 1977; 16: 4533-4537.

425. Lundblad R. L., Chemical reagents for protein modification. 2nd ed.1991; pp. 105-128, CRC Press. Inc. Florida.

426. Tsou C. L., Relation between modification of functional groups of proteins and their biological activity. Scientia Sinica., 1962; 11: 1535-1558.

427. Paterson A. K., Knowles J. R., The number of catalytically essential carboxyl groups in pepsin., Eur. J. Biochem., 1972; 31: 510-535.

428. Bund R. K., Singhal R. S., An alkali stable cellulase by chemical modification using maleic anhydride. Carbohydr. Poly. 2002; 47: 137-141.

429. Zhu A., Monahan C., Wang Z., Trp-16 is essential for the activity of a-galactosidase and a-N-acetylgalactosiminidase. Biochim. Biophys. Acta, 1996; 1297: 99-104.