"STUDIES ON α-GALACTOSIDASE FROM THE THERMOPHILIC FUNGUS *HUMICOLA* SP."

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Dedicated to my family

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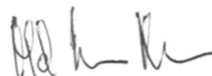
DECLARATION

Certified that the work incorporated in the thesis entitled **"STUDIES ON α-GALACTOSIDASE FROM THE THERMOPHILIC FUNGUS *HUMICOLA* SP."** *submitted by Mr. S.M. Kotwal was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.*



Dr. M. I. Khan
Research Guide

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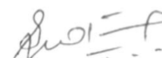

S.M.Kotwal

ABSTRACT

Enzymes, which catalyze the hydrolysis of glycosidic linkages, are widely distributed in nature and include invertase, maltase, α-galactosidase, lactase, β-glucosidase and amylase. Based on the nature of hydrolysis, these glycosidases are classified into exo-glycosidases and endo-glycosidases. Exo-glycosidases (e.g. galactosidases, glucosidases) act on the glycosidic bonds present at the non-reducing end of the saccharide chain whereas endo-glycosidases (e.g. amylases) act on the glycosidic bonds within the saccharide chain. Specific glycosidases are required for hydrolysis of different glycosidic bonds.

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 and of the substrate molecule on which it acts. α-Galactosidase (E.C.3.2.1.22) catalyzes the hydrolysis of α-galactosidic linkages in oligosaccharides, glycoproteins and glycolipids, such as raffinose, melibiose, stachyose, verbascose, galactomannans, ceramide trihexosides and its higher homologues as well as their derivatives. β-Galactosidase (E.C.3.2.1.23), catalyzes the hydrolysis of the β-galactosidic bonds of lactose to glucose and galactose.

α-Galactosidases are ubiquitous in distribution. They are of particular interest in view of their biotechnological applications in processing of foods containing galacto-oligosaccharides such as raffinose and stachyose. Raffinose inhibits sucrose crystallization from beet syrups and hence enzymatic hydrolysis of raffinose increases the yield of sucrose. Moreover, hydrolysis of galactosaccharides alleviates gastric distress caused by fermentation of these carbohydrates in the large intestine thereby improving the nutritional quality of legume based food such as soybean milk and cowpea meal. Production, purification and characterization of α-galactosidases from different sources have been reported. Microorganisms have the advantage of high production yields, and among them fungal α-galactosidases are more suitable for biotechnological applications mainly due to their extracellular nature, acidic pH optima, and broad stability profiles. Among the several fungal isolates screened for their extracellular α-galactosidase producing ability, the thermophilic fungus, *Humicola* sp. (NCIM 1252) secreted large amounts of extracellular α-galactosidase and hence was selected for the present investigation. Hence studies were carried out to (i) optimize the conditions for α-galactosidase production by submerged fermentation as well as solid state fermentation; assess its potential application in the removal of raffinose and stachyose in soymilk (ii)

purify and extensively characterize the enzyme to understand its structure-function correlation. The thesis is divided into five chapters.

Chapter I : General Introduction

This part comprises of literature survey with reference to α-galactosidases, their occurrence, production, properties and industrial applications.

Chapter II : Production of thermostable α-galactosidase from the thermophilic fungus *Humicola* sp. (NCIM 1252) by submerged fermentation (SmF)

The thermophilic fungus *Humicola* sp. isolated from soil, when grown in a medium containing wheat bran and yeast extract secreted α-galactosidase in culture filtrate. The culture filtrate also contained small amount of β-galactosidase activity. α-Galactosidase production was highest in a medium containing 5% wheat bran extract and 5% (w/v) beef extract as a carbon and nitrogen source, respectively. Addition of Zn^{2+} and Mg^{2+} in the fermentation medium stimulated the enzyme production. Secretion of the enzyme was strongly inhibited by the presence of Cu^{2+}, Ni^{2+} and Hg^{2+} in the fermentation medium. The production of enzyme, under stationary conditions, was 10 fold higher than that under shaking conditions. The temperature range for the production of the enzyme was 37-55°C, with maximum enzyme activity (5.54 U ml^{-1}) at 45°C. Optimum pH and temperature of the crude enzyme were 5.0 and 60°C, respectively. *Humicola* α-galactosidase, when incubated 60°C for 60 min retained its full activity. Enzyme activity was strongly inhibited by 5 mM concentration of Hg^{2+}. The K_m and V_{max} values for *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) were 0.6 mM and 33.6 μ mole min^{-1} mg^{-1} and that for raffinose were 10.52 mM and 1.82 μmole min^{-1} mg^{-1}, respectively.

Chapter III : Increased production of α-galactosidase from *Humicola* sp. by solid state fermentation (SSF) and its application in soymilk hydrolysis

Humicola sp. when grown on various agricultural residues in solid state fermentation (SSF), produced α-galactosidase. Maximum enzyme activity (44.6 U/g) was obtained at 45°C when soyflour was used as the carbon source and the moisture content in the substrate was 86%. At 95% humidity, maximum amount of α-galactosidase was secreted irrespective of the substrate used for the enzyme production. The crude enzyme could hydrolyze raffinose and stachyose in soymilk. Scanning electron microscopic (SEM) studies showed high density growth of fungal mycelia on wheat bran particles.

Chapter IV : Purification and characterization of α-galactosidase from *Humicola* sp.

The extracellular invertase free α-galactosidase from the thermophilic fungus *Humicola* sp. was purified to homogeneity by ammonium sulfate precipitation, ethanol precipitation, DEAE cellulose and Sephacryl S-300 chromatography with a 202 fold increase in specific activity and 13.8% recovery. The molecular mass of the enzyme by gel filtration on Sephacryl S-300 was 354.8 kDa and 87.1 kDa by SDS polyacrylamide gel electrophoresis. The enzyme had an optimum pH and temperature of 5.0 and 60°C, respectively. The enzyme was stable in the pH range 4.5 to 6.0. It retained its full activity for 2 h at 55°C and for 50 min at 60°C. *Humicola* α-galactosidase is a glycoprotein with 6.2% carbohydrate content and is acidic in nature with a pI of 4.90. The enzyme activity was strongly inhibited by Hg^{2+}, Mn^{2+} and Cu^{2+}. D-Galactose, D-glucose and D-mannose inhibited α-galactosidase noncompetitively and the inhibition constant (K_i) for galactose was 15.5 mM. The purified enzyme hydrolyzed *p*NPG, *o*NPG, melibiose, raffinose and stachyose exhibiting a K_m value of 0.31, 0.54, 0.70, 3.3 and 7.6 mM, respectively. The energy of activation (E_a) of *Humicola* α-galactosidase was found to be 35.87 Kcal / mole, for *p*NPG. Amino acid composition revealed that the enzyme is rich in glycine and alanine and low in cysteine and methionine.

Chapter V : Active site characterization of α-galactosidase from *Humicola* sp.

Chemical modification studies on the purified α-galactosidase from *Humicola* sp. with group specific modifiers, spectral analysis and kinetic data suggested the involvement of a single tryptophan and lysine and two carboxyl residues in the catalytic activity of the enzyme. The involvement of two carboxyl residues in the catalytic activity of the enzyme indicates that *Humicola* enzyme also follows the classical glycosidase catalytic mechanism. However, active site nature of *Humicola* α-galactosidase is atypical compared to other reported α-galactosidases in that, it shows the involvement of lysine in the catalytic activity of the enzyme.

List of publications

1. Production of thermostable α-galactosidase from thermophilic fungus *Humicola* sp. ***S.M. Kotwal.,*** M.I. Khan and J.M. Khire (1995) *J. Indust. Microbiol.* 15, 116-120.

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

3. Purification and characterization of an extracellular α-galactosidase from the thermophilic fungus *Humicola* sp. ***S.M. Kotwal.***, J.M. Khire and M.I. Khan (1999) *J. Biochem. Mol. Biol. & Biophys.* 3, 9-17.

4. Chemical modification of α-galactosidase from the thermophilic fungus *Humicola* sp. ***S.M. Kotwal.***, J.M. Khire and M.I. Khan (1999) *J. Biochem. Mol. Biol. & Biophys.* (In press)

Papers / posters presented at seminars / symposium

1. Studies on thermostable α-galactosidase by thermophilic fungus *Humicola* sp. (NCIM 1252). ***S.M. Kotwal.***, J.M. Khire and M.I. Khan Presented at 64th Annual Meeting of Society of Biological Chemists, held at Lucknow between 6-8 October, 1995.

Patents filed

1. Patent No. 1193/DEL/dtd 23-9-94. A process for the preparation of thermostable α-galactosidase from thermophilic fungus *Humicola* sp. J.M. Khire., ***S.M. Kotwal*** and M.I. Khan (1995)

List of abbreviations

*p*NPG	*p*-nitrophenyl-α-D-galactopyranoside
*o*NPG	*o*-nitrophenyl-α-D-galactopyranoside
YpSs	yeast phosphate soluble starch agar
PDA	potato dextrose agar
DNSA	3,5-dinitrosalicylic acid
SmF	submerged fermentation
SSF	solid state fermentation
SEM	scanning electron microscope
TCA	trichloroacetic acid
NBS	N-bromosuccinimide
TNBS	2,4,6-trinitrobenzenesulfonic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
NTTE	3-nitro-L-tyrosine ethylester hydrochloride
DEPC	diethylpyrocarbonate
TNM	tetranitromethane
NAI	N-acetylimidazole
NEM	N-ethylmaleimide
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
PHMB	*p*-hydroxymercurybenzoate
PCMB	*p*-chloromercurybenzoate
PMSF	phenylmethylsulfonylfluoride
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
MES	2[N-morpholino]ethanesulfonic acid
β-ME	β-mercaptoethanol
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
IEF	isoelectric focusing
DEAE-cellulose	diethylaminoethyl cellulose
BSA	bovine serum albumin
EDTA	ethylene diamine tetraacetic acid
M_r	relative molecular mass
pI	isoelectric point
TEMED	N,N,N',N'-tetramethylethylene diamine

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CHAPTER - I

INTRODUCTION

1.1 GLYCOSIDASES

Enzymes, which catalyze the hydrolysis of glycosidic linkages, are widely distributed in nature and include invertase, maltase, α-galactosidase, lactase, β-glucosidase and amylase [1,2]. Glycosidases are also known as carbohydrases which catalyze the hydrolysis of glycosidic bonds in glycosides, oligosaccharides and polysaccharides as well as in complex carbohydrates such as glycoproteins and glycolipids. The existence of these glycosidases have been known for more than 100 years and they were the very first biological catalysts to be investigated [3]. Based on the nature of hydrolysis, glycosidases are classified into exo-glycosidases and endo-glycosidases. Exo-glycosidases (e.g. galactosidases, glucosidases) act on the glycosidic bonds present at the non-reducing end of the saccharide chain whereas endo-glycosidases (e.g. amylases) act on the glycosidic bonds within the saccharide chain [1]. Specific glycosidases are required for splitting different glycosidic bonds, except in cases where the glycon ring conformation is the same for more than one sugar [2]. Glycosidases have occupied an important place in medicine and food processing. In medicine, glycosidases are useful in enzyme replacement therapy for genetic disorders of carbohydrate metabolism. There is a large demand for glycosidases in food industry for the production of sugars from starch and glucose from sucrose.

1.2 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 [4]. α-D-galactose-galactohydrolase (E.C.3.2.1.22), commonly referred to as α-galactosidase, catalyzes the hydrolysis of α-galactosidic linkages in oligosaccharides, glycoproteins and glycolipids, such as raffinose, melibiose, stachyose, verbascose, galactomannans, ceramide trihexosides, its higher homologues and derivatives [5]. β-D-galactose galactohydrolase (E.C.3.2.1.23), catalyzes the splitting of the β-galactosidic bonds of lactose to glucose and galactose [6].

1.3 α-GALACTOSIDASES - OCCURRENCE AND DISTRIBUTION

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) catalyzes the hydrolysis of α-galactosidic linkages present in oligosaccharides, galactomannans and

glycoproteins and is found in many plant seeds, animal tissues and microorganisms [5,7,8]. In plant kingdom, galactose containing oligo- and polysaccharides and lipids are ubiquitous by its presence in the tissues which contain α-galactosidase. The importance of glycosidases in the metabolism of carbohydrates in plants, particularly in storage organs is well recognized [5]. In many maturing seeds, there is a concomitant synthesis of galactosyl sucrose derivatives during germination and α-galactosidase is involved in the hydrolysis of these oligosaccharides, which serve as a readily metabolizable energy reserve [5,9]. Few reviews are available on plant α-galactosidases, which mainly cover the occurrence, isolation, physico-chemical and kinetic properties, specificity, mechanism of action and physiological significance [5,10]

The role of α-galactosidase in animal kingdom is very limited. The enzyme present in brain tissues is responsible for the hydrolysis of digalactyl diglycerides [11]. Number of microorganisms are known to produce α-galactosidase in appreciable quantity and the microbial production of α-galactosidase has been reviewed [4,5,12-14]. SmF technique is employed for the industrial production of α-galactosidase [12]. Research and developmental efforts on enzyme production by fermentation started in late 1960's. A critical analysis of all fermentative aspects to understand various physico-chemical parameters, process optimization strategies for the production of α-galactosidase in high yield has not been dealt systematically at one place. SSF has also been employed for the production of α-galactosidases [8].

1.4 HISTORICAL HIGHLIGHTS

It is interesting from biotechnological point of view, to study the chronological and historical developments with respect to the occurrence, specificity, mode of action, industrial production and applications of α-galactosidase.

Bau [15] and Fischer and Lindner [16] in 1895, for the first time, isolated α-galactosidase from bottom yeast and due to its action on melibiose named it as melibiase. The presence of α-galactosidase in the digestive juice of *Helix promatia* was then noticed by Bierry [17]. The first report of α-galactosidase from plant i.e. sweet almond emulsin came from Helferich and Appel in 1932 [18]. Subsequently its presence in alfalfa seeds and coffee emulsin was also discovered [19,20]. The α-and β-galactosidase activities in alfalfa seeds were separated by adsorption on alumina wherein, β-Galactosidase was strongly adsorbed on alumina while the unadsorbed fraction contained α-galactosidase [19]. At the same time, microorganisms such as *Aspergillus niger* [21] and *Aspergillus oryzae* [22] were also reported to produce α-galactosidase constitutively. The enzyme

from sweet almond emulsin was best separated by chromatography on bauxite [23]. The typical substrate for α-galactosidase is melibiose and galactose-α-1-6-glucose. Weidenhagen [24] in 1928, noted that α-galactosidase can hydrolyze α-D-galactosides as well as oligo- and polysaccharides of α-galactosides. Pigman [25] in 1944, slightly modified the theory of Weidenhagen [24] and stated that α-galactosidases are responsible for the hydrolysis of α-D-galactoside, β-L-arabinoside, α-D-fucoside, melibiose, α-D-and L-glycero-D-galacto-aldoheptoside. Thereafter, substrate specificity of α-galactosidases from plants, animals and microorganisms using a variety of sugars possessing non-reducing terminal α-D-galactosyl residues have been reported [26-33].

The production of α-galactosidase from *Mortierella vinacea* var raffinoseutilizer by SmF was reported for the first time by Suzuki *et al* [34]. A continuous process for hydrolyzing raffinose in beet sugar syrup by using α-galactosidase pellets from *Mortierella vinacea* var raffinoseutilizer [34] was developed by Hokkaido Sugar Co. Ltd, Japan in 1969. Subsequently, development of several processes for the production of this enzyme have been reported [35-37]. All the commercial developments, for the production of α-galactosidase are based on SmF. However, very little information is available on the production of α-galactosidase by SSF, although many commercial advantages are offered by this technique [38].

Malhotra and Dey [32], for the first time, purified α-galactosidase from sweet almonds by CM-cellulose chromatography followed by size exclusion chromatography. The crystallization of α-galactosidase from *Mortierella vinacea* was reported by Suzuki *et al* [39]. The mechanism of action of α-galactosidase, for the first time was proposed by Dey *et al* [40] wherein the concerted action of carboxylate and histidine residue were implicated.

The existence of multimolecular forms of α-galactosidase from the seeds of coffee and *Planteago ovato* was, for the first time, reported by Petec and Dong [41], by chromatography on alumina. Multimolecular forms of α-galactosidase have been reported from several plants, animals and microorganisms [5,42-46]. Civas *et al* [44,45] reported three forms of α-galactosidase from *Aspergillus tamarii* where α-galactosidase form I and II were intracellular and form III was extracellular. They observed that α-galactosidase form III differed markedly from forms I and II. Rios *et al* [47] have also reported intra and extracellular α-galactosidase from *Aspergillus nidulans*. From enzymatic characteristics and the cross reactivity of the antibodies, they showed that both α-galactosidases were the same.

1.5 ENZYME ASSAYS

A number of methods have been described in literature for the determination of α-galactosidase activity. Melibiose and raffinose are the natural substrates for plant α-galactosidases and hence most commonly used for determining the enzyme activity [32,34,48]. The assay is based on the determination of liberated glucose by glucose-oxidase method when melibiose is used as substrate [49]. Alternatively, when raffinose is used as substrate, the activity can be measured by monitoring the increase in reducing sugar [50], or by the coupled enzymatic method, using galactose dehydrogenase [51]. Synthetic substrates such as oNPG and *p*NPG are commonly used for the routine analysis of enzyme activity and the o-and *p*-nitrophenol is estimated by measuring the absorbance at 400-420 nm under alkaline conditions [52]. Hydrolysis of substrates involving phenol as the aglycon moiety is estimated by Folin-reagent [53].

Other substrates used for the assay of α-galactosidase include, 4-methyl umbelliferyl-α-D-galactoside, 1-naphthyl-α-D-galactoside, and 6-bromo-2--naphthyl-α-D-galactoside [50,54,55]. When 4-methyl umbelliferyl-α-D-galactoside is used as substrate, the liberated aglycon can be measured fluorimetrically and this is probably the most sensitive of all the methods described in literature [55]. When 6-bromo-2-naphthyl-α-D-galactoside is used as a substrate, the liberated water insoluble aglycon portion is coupled with Fast Blue BB, and the coloured complex formed is measured [54,56,57]. Galactomannans, which have a basic structure consisting of a backbone of β-1,4-linked D-mannosyl residue to which D-galactosyl residues are attached by α-1,6 linkages, have also been used as substrates for a few plant and microbial α-galactosidases [30,31,33]. The terminal galactose residue liberated following the action of α-galactosidase is estimated by galactose dehydrogenase method [58].

1.6 SOURCES OF α-GALACTOSIDASES

1.6.1 Plants

The presence of α-galactosidases in plants was first reported in sweet almond emulsin [18] and subsequently α-galactosidases from many plants have been reported [Table 1.1]. The levels of α-galactosidase in most plants are generally low [58]. But, in the case of germinating leguminous seeds, the enzyme levels are higher in the later part of germination [10]. In general, α-galactosidases occur in the cell in association with various other glycosidases.

Table 1.1 : α-Galactosidase producing plants

Source		Reference
Acer pseudoplatanus	(seeds)	59
Arachis hypocea	(seeds)	60
Cajanus indicus	(seeds)	59, 61
Cassia sericea	(seeds)	62, 63
Ceratonia siliqua	(seeds)	64, 65
Cicer arietinum	(seeds)	66
Cocos mucifera	(various)	67-70
Coffee sps	(seeds)	20,41,71-77
Cucurbita sps	(various)	79,80
Cyamopsis tetragonolobus	(seeds)	64, 78, 81, 82
Glycine max sps	(various)	64, 77, 78, 83, 84
Lens culinaris	(seeds)	85-87
Lens esculanta	(seeds)	88
Maize	(roots)	56,89
Medicago sps	(seeds)	19,59, 64, 90
Ochronomas sps	-	51
Phaseolus vulgaris	(seeds)	91,92
Planteago ovata	(seeds)	41
Runner bean	(leaves)	93
Saccharum officinarum sps	(various)	94, 95
Sweet almond	(seeds)	96
Trifolium repens	(seeds)	97
Vicia faba sps	(seeds)	98, 99
Vigna radiata	(seeds)	59, 100, 101
Vicia sativa	(seeds)	102

Adapted from Shivanna [2]

1.6.2 Animals

Bierry in 1913 [17], first reported the presence of α-galactosidase in *Helix pomatia*. Subsequently, α-galactosidase activity from a number of animals have been reported [Table 1.2]. The presence of α-galactosidase in rat was found in higher titres in the cytoplasm of epithelial cells of Brunner's glands in the intestine. The distal segment of the proximal tubes of the kidney, thyroid and parathyroid glands also possess high activity. Blood cells and bone marrow of some animals were found to contain α-galactosidase [103-105]. In most cases α-Galactosidase is present in the soluble fraction in cells. However, Debris *et al* [106] isolated a particulate renal enzyme from pig, which could be solubilized using trypsin. The α-galactosidase from human [107-110] has also been reported and their involvement in Fabry's disease studied [111-113].

Table 1.2 : α-Galactosidase producing animals

Source	Reference
Bovine	114, 115
Chineese hamsters	116
Helix pomatia	17
Human	42, 109, 111, 117-120
Mouse	121
Sheep	122
Turbo cornutus	123

Adapted from Srinivas [8]

1.6.3 Microorganisms

A number of microorganisms have been reported to produce α-galactosidase [Table 1.3 a,b,c]. Microorganisms have the advantage of high production yields, and among them fungal cultures are able to produce the enzyme in high levels [34,146,217]. In addition, few actinomycetes [7], yeast [37] and bacteria [172, 176, 177, 183, 189, 198, 202,] are also known to produce α-galactosidase. The production of α-galactosidase by higher fungi has also been reported by Li and Shetlar [31].

Table 1.3a : α-Galactosidase producing fungi

Organism	Reference
Absidia corymbifera	124
Agaricus bisporus	125
Aureobasidium pullulans	126, 127
Aspergillus awamori	128-131
Aspergillus ficcum NRRL 3135	132
Aspergillus fumigatus	133
Aspergillus nidulans	47
Aspergillus niger	21, 33, 46, 134 - 141
Aspergillus oryzae	22, 126, 142-144
Aspergillus saitoi	145
Aspergillus tamarii	44,45
Calvatia cyathiformis	31
Cephalosporium acremonium	146 - 148
Cladosporium cladosporides	149
Corticium rolfsii	150, 151
Fusarium sp.	152
Gibberella fujikuroi	153, 154
Monascus anka	155
Monasus pilosus	156
Mortierella vinacea	34, 39, 157 - 160
Penicillium brevicompactum	126
Penicillium dupontii	161
Penicillium janthinellum	162
Penicillium ochrochloron	58
Penicillium purpurogenum	163
Penicillium simplicissimum	164
Pycnoporous cinnabarinus	165, 166
Rhizopus	167
Scopulariopsis sp.	126
Sporotrichum cellulophilum	168
Trichoderma reesei	48,169 - 171

Table 1.3b : α-Galactosidase producing bacteria

Organism	Reference
Actinomycetes sp.	172, 173
Aerobacter aerogenes	27
Azotobacter vinelandii	174
Bacillus circulans	175
Bacillus stearothermophilus	43, 176 - 178
Bacteroides fragilis	179
Bacteriodes ovatus	180, 181
Bifidobacterium adolescentis	182
Bifidobacterium breve	183
Bifidobacterium longum	184
Clostridium perfringens	185, 186
Diplococcus pneumoniae	187, 188
Escherichia coli	189 - 192
Lactobacillus sp.	193
Lactobacillus fermenti	194
Lactobacillus fermentum	195
Lactobacillus plantarium	195
Micrococcus	196, 197
Pseudoaltermonas sp.	198
Pseudomonas fluorescens	199
Streptococcus bovis	30, 200
Streptococcus mutans	201
Streptomyces olivaceus	172
Streptomyces sp.	202, 203
Thermotoga neapolitana	204
Zymomonas mobilis	205

Table 1.3c : α-Galactosidase producing yeasts

Organism	Reference
Brewer's yeast	15, 16, 206
Candida guillermondii	207, 208
Candida javanica	209
Hansenula polymorpha	210,211
Pichia guilliermondii	212
Saccharomyces carlsbergenesis	7,213, 214
Saccharomuces cerevisiae SU 50B	211,215
Saccharomyces sp.	215, 216

1.7 ISOLATION AND PURIFICATION OF α-GALACTOSIDASES

α-Galactosidases from different sources have been isolated and purified by employing the classical methods of protein purification. In most cases, the enzyme is found in association with many other glycosidases. Since the presence of polyphenols and other gummy polysaccharides is quite common, the purification of α-galactosidases has often proved to be difficult. The common methods employed for the purification of α -galactosidases include ammonium sulfate fractionation [33], acetone precipitation [134], heat treatment [39], ion exchange chromatography [44, 45, 58, 135], gel filtration [156] and chromatofocussing [177]. In few cases, the enzyme extract was concentrated using ultrafiltration either before ammonium sulfate fractionation step [48] or in later stages of purification [33, 43, 146, 217, 218].

The separation of multiple molecular forms of α-galactosidase from microorganisms has also been reported. Among different bacterial sources, *Bacillus stearothermophilus* [43] is reported to produce two different molecular forms (I and II), which have markedly different elution patterns on DEAE-cellulose and hydroxyapatite. The α-galactosidases of *Aspergillus tamarii* [44, 45] exists in three different forms (I, II and III). Forms I and II are intracellular and are induced by raffinose. When raffinose is substituted with guar galactomannan, form III (extracellular) is produced. The forms I and II can be separated by hydroxyapatite and DEAE-cellulose using different elution conditions. Affinity chromatography, owing to its functional purification approach, has also been used for the purification of few α-galactosidases. The commonly used affinity

absorbents are Melibiose-Sepharose and Melibiose-Agarose [83]. Few reports are also available on the use of Concanavalin A-sepharose for purifying plant α-galactosidases [85]. Large scale isolation of α-galactosidase from soybean meal has been reported by Porter *et al* [219]. A novel combination of anion exchange chromatography followed by concanavalin-A affinity chromatography resulted in a highly purified α-galactosidase preparation. However, this procedure was not found practical due to low recovery of the enzyme and high purification costs. Except for the purification of α-galactosidase from *Diplococcus pneumoniae* [187], using red blood cells as affinity sorbent, affinity chromatography has not been utilized for the purification of microbial α-galactosidases.

1.8 PROPERTIES OF α-GALACTOSIDASES

1.8.1 Kinetic properties

[a] ***Effect of substrate concentration***

Effect of substrate concentration on the reaction rate of α-galactosidase follows a typical Michaelis-Menten kinetics [2]. In addition, it is also affected by the increase in chain length of the substrate [44,45]. In general, the natural substrate, melibiose, raffinose and stachyose show lower affinity for the enzyme as compared to *p*NPG and *o*NPG. The effect of substrate concentration on the rate of reaction of *p*NPG and oligosaccharides has been investigated and the K_m and V_{max} values of α-galactosidase from different sources are summarized in Table 1.4.

Civas *et al* [44,45] studied the effect of varying concentration of *o*NPG, raffinose and stachyose on the rate of hydrolysis by α-galactosidase III from *Aspergillus tamarii* and concluded that the affinity decreased with increase in chain length of the substrate. In contrast, α-galactosidase form I and II showed a greater affinity, with a higher value of V_{max} , for all types of substrates. The enzyme from *Trichoderma reesei* [48] showed no profound difference in affinity for α-D-galactosides of varying chain lengths or different aglycons. However, *Aspergillus ficuum* [132] α-galactosidase exhibited substantial difference in the affinity for various α-D-galactosides and oligosaccharides. K_m values for *o*NPG, *m*-nitrophenyl-α-D-galactopyranoside (*m*NPG) and melibiose were reported to be much lower than *p*NPG.

[b] ***Effect of temperature***

α-Galactosidases exhibit different temperature optima and thermal stability depending on the source (Table 1.4). Civas *et al* [45] reported that *Aspergillus tamarii* α-galactosidase form III is stable at 4°C for several months. The α-galactosidase form I was

Table 1.4 Kinetic properties of different α-galactosidases

	Source (M_r)	Optimum		Stability		K_m mM	V_{max} μ moles/mg/min	Substrate	Reference
		pH	Temp, °C	pH	Temp, °C				
1.	*Aspergillus ficuum* (70800)	6.0	60	4.0-8.0	65	1.46	64.4	pNPG	132
2.	*Aspergillus nidulans (Extra and Intracellular) (370,000)*	4.5	50	4.0-10.0	30	0.27 (Extra) 0.28 (Intra)	121	pNPG	47
3.	*Aspergillus niger (45000)*	4.0-4.5	-	3.0-7.5	-	0.5	3	Raffinose	134
4.	*Aspergillus Oryzae (64000)*	4.0	60	3.0-7.5	55	10	-	Raffinose	144
5.	*Bacillus Stearothermophilus*								43
	α-galactosidase I (280000)	6.0	73	-	65	0.59	-	pNPG	
	α-galactosidase II (325000)	7.0	69	-	65	0.23	-	pNPG	
6.	*Bifidobacterium longum* (-)	5.8	40-45	-	50	-	-	pNPG	184
7.	*Candida guillermondii*								208
	α-galactosidase I (270000)	4.5	75	3.5-9.5	70	0.60	3.3	pNPG	
	α-galactosidase II (270000)	4.5	75	2.5-9.5	45	0.61	3.1	pNPG	
8.	*Candida javanica*	4.0	70	-	70	1.11	-	pNPG	209
9.	*Monascus pilosus (150000)*	4.5-5.0	55	3.0-8.0	55	0.8	39	pNPG	156
10.	*Morteirella vinacea (-)*	3.0-5.0	-	6.0-10.0	-	1.83	19.2	Raffinose	39
11.	*Penicillium janthineium* (-)	4.5	60	-	70	0.55	-	pNPG	162
12.	*Pycnoporus cinnabarinus* (210000)	5.0	75	3.0-9.0	75	2.16	10.6	Raffinose	165
13.	*Trichoderma reesei (50000)* (-)	4.0	60	4.5-6.5	60	3.8	15.2	Raffinose	48

stable for one month at 4°C, while form II was not even stable for 10 days [44]. Both these enzymes were unstable to freeze drying, while α-galactosidase form III retained 40 % of its activity. Ohtakara *et al* [165] studied the thermal stability of *Pycnoporous cinnabarinus* α-galactosidase and observed that at pH 5.0 and 75°C the enzyme retained 70% of its activity for 8 h. The α-galactosidase from *Penicillium janthinellum* [162] was thermostable and no loss of activity was observed at 65°C upto 40 min of incubation, but it was inactivated completely at 90°C in 20 min. The enzyme from hyperthermophilic bacterium *Thermotoga neapolitana* [204] had a temperature optimum of 100-105°C and a half life of 130 min at 90°C and 3 min at 100°C. An interesting observation regarding the effect of temperature at different pH values has also been reported by Schuler *et al* [194]. Studies on α-galactosidase from *Lactobacillus* sp. have shown that the enzyme has an optimum temperature of 45°C at pH 5.0 and this optimum value was shifted to 50°C at pH 5.8 indicating the relationship between temperature and pH.

[c] *Effect of pH*

The pH optima of α-galactosidases are generally in the range of 4.0-7.0 (Table 1.4). α-Galactosidases from fungal sources exhibit optimum enzyme activity in the acidic pH range (4.0-6.0) [47, 132], whereas α-galactosidases from bacterial sources have an optimum pH in the range 6.0-7.5 [33, 135]. The effect of pH at different temperatures on α-galactosidase activity from *Lactobacillus fermenti* [194] showed that the pH optimum was 5.0 at 37°C while it was 5.5 at 50°C. In addition to a shift in the optimum pH, there is a pronounced alteration in the shape of pH activity profile on the alkaline side of the pH optima.

[d] *Effect of sugar and their derivatives*

The inhibition of α-galactosidases by various sugars and their derivatives has been reported [31, 34, 39, 43, 135, 165, 202]. D-Galactose is a competitive inhibitor of many α-galactosidases [31, 43, 48]. The inhibition of α-galactosidase from *Mortierella vinacea* [39] by D-galactose was found to be of mixed type, suggesting both competitive and non-competitive binding of D-galactose to the enzyme. The α-galactosidase from *Aspergillus ficcum* [132] was inhibited noncompetitively by glucose and uncompetitively by mannose, whereas D-galactose, D-glucose, melibiose and raffinose acted as competitive inhibitors of α-galactosidase from *Aspergillus nidulans* [47]. The structural analog of D-galactose i.e. L-arabinose is also reported to inhibit the enzyme [39, 135], while 2-deoxy-D-glucose, D-glucose, D-mannose, D-fructose and D-ribose did not show any inhibitory effect. It has been shown that for the attachment of sugar to the enzyme, D-galactose

configuration is required and carbon atoms C_1, C_2, C_4 and C_6 are involved in binding [31,39]. D-Galactal is also known to inhibit α-galactosidase from *Aspergillus niger* [135]. Among various sugars tested for their inhibitory effect on the two forms of α-galactosidase (I & II) from *Bacillus stearothermophilus* [43], lactose was shown to have greater inhibitory effect (35%) on form I, while form II was inhibited by pentoses.

[e] ***Effect of metal ions and group specific reagents***

Most of the metal ions tested for their effect on catalytic activity of α-galactosidases do not appear to have stabilizing / activating effect. However, Mn^{2+} ions have been found to stabilize α-galactosidase from *Escherichia coli* [189, 192], *Penicillium janthinellum* [162] and *Penicillium purpurogenum* [163]. Among various metal ions tested Ag^{2+}, Hg^{2+} and in few cases Cu^{2+} were found to be the most potent inhibitors of a number of α-galactosidases [31, 39, 44, 45, 129, 156, 165, 183]. The inhibition of α-galactosidase with Hg^{2+} ions is usually attributed to its reaction with thiol groups. It has been shown that the inhibition of α-galactosidase from *Aspergillus ficcum* by Hg^{2+}, Ag^{2+}, Cu^{2+} and Zn^{2+} is competitive in nature [132].

Chemical modification of thiol groups by PHMB, iodoacetamide and NEM resulted in loss of activity of α-galactosidase from *Aerobacter aerogenes* [27], *Diplococcus pneumoniae* [187] and *Streptomyces olivaceus* [172], suggesting the involvement of sulfhydryl groups in the catalytic activity. However, the enzymes from *Calvatia cyathiformis* [31] and *Mortierella vinacea* [39] were not inactivated thereby indicating that not all α-galactosidases require -SH group for catalysis. The enzyme from *Mortierella vinacea* [220] was fully inactivated by NBS suggesting that tryptophan is necessary for the catalytic activity. However, PCMB and β-ME failed to inactivate the enzyme.

1.8.2 Molecular properties

[a] Multimolecular forms

An important development in enzyme chemistry has been the demonstration of the existence of multiple forms of enzymes which are designated as isoenzymes. The term isozyme was originally used by Markert and Moller [221] to describe different molecular forms of the protein which showed similar enzyme activity. Recently, the term has been generally used to designate different forms of an enzyme from the same biological source which differ in their physico-chemical and kinetic properties.

The occurrence of multiple forms of α-galactosidase was first reported from coffee beans [41] by chromatography on alumina. Multiple forms of α-galactosidases

from different sources have also been reported [39, 43, 78, 85, 135]. Suzuki *et al* [39] detected three forms of α-galactosidases (I, II & III) from *Mortierella vinacea* differing in their charge. α-Galactosidase from *Aspergillus niger* [135] existing in multiple forms with different isoelectric points have also been reported. The enzyme from *Vicia faba* seeds [99] has been resolved into three molecular forms I, II & III and form I enzyme is a tetramer (M_r 160000 Da) consisting of identical subunits. All the enzyme forms are closely related and display lectin activity with glucose / mannose specificity.

[b] Molecular mass (M_r)

The M_r of α-galactosidases from various microbial sources vary considerably ranging from 32000 Da to 390000 Da [Table 1.5]. *Aspergillus tamarii* produced three different α-galactosidases (M_r , Form I, 265000 Da, Form II, 254000 Da and Form III 56000 Da) when grown on galactomannan [44,45]. *Bacillus stearothermophilus* [43] when grown in a medium containing 1% (w/v) tryptone and 0.5% (w/v) yeast extract produced two different enzymes, form I and II with M_r values 280000 Da and 325000 Da respectively. *Bacteriodes ovatus* [181] produced two different independently regulated enzyme forms (form I and II). Form I (M_r 85000 Da) was produced when grown on guar gum and galactose however, Form II (M_r 80000 Da) was produced when grown on melibiose, raffinose or stachyose. The enzyme from *Candida guilliermondii* [208] when grown on melibiose produced two forms of α-galactosidase having the same M_r (270000 Da) but differing in their pI (6.18 and 6.21).

[c] Amino acid composition

There is a considerable variation in the amino acid composition of the α-galactosidases from different microbial sources [Table 1.6]. But the common feature with most of the α-galactosidases is the presence of large amounts of acidic amino acids like aspartic and glutamic acid, polar amino acids like serine and threonine and non polar amino acid like valine. However, sulfur containing amino acids are relatively low [215, 222, 223, 227]. The α-galactosidase from *Cornebacterium acremonium* [147] has about 33% hydrophobic amino acids and 21% hydroxyl amino acids. The enzyme from *Corynebacterium murisepticum* [222] revealed high percentage of glycine and serine residues. *Escherichia coli* K12 [227] α-galactosidase has a high percentage of leucine in addition to glutamic acid. The enzyme from *Trichoderma reesei* [171] possesses a high content of glycine, alanine and low content of cysteine and methionine. The variation in

Table 1.5: M_r of α-galactosidase from different sources

Source	Form	M_r (Da)	Reference
Aspergillus awamori		130000	129
Aspergillus niger		45000	134
		147000	46
Aspergillus nidulans		370000	47
Aspergillus tamarii	I	265000	44, 45
	II	254000	
	III	56000	
Bacillus stearothermophilus	I	280000	43
	II	325000	
Bacteroides ovatus	I	85000	181
	II	80000	
Bifidobacterium breve		330000	183
Candida guillermondii	I	270000	208
	II	270000	
Cephalosporium acremonium 237		240000	146
Clostridium perfringes		96000	186
Corynebacterium murisepticum		32000	222
Escherichia coli		50000	223
Monascus pilosus		150000	156
Mortierella vinacea		52000	224
Penicillium ochrochloron		57500	58
Penicillium purpurogenum		46334	225
Pichia guillermondii		143000	212
Pseudomonas fluorescence		390000	199
Pycnoporous cinnabarinus		210000	165
Saccharomyces carlsbergensis		270000	7
Saccharum officinarum		46000	94
Thermotoga neapolitina		66000	226
Trichoderma reesei		50000	48

Table 1.6 Amino acid composition of different microbial α-galactosidases

Amino acid	Sources						
	Number of amino acid residues						
	Escherichia coli (*Ref : 223*)	*Saccharomyces carlsbergensis* (*Ref : 7*)	**Cephalosporium acremonium* (*Ref : 147*)	***Corynebacterium murisepticum* (*Ref : 222*)	*Escherichia coli* (*Ref : 227*)	*Trichoderma reesei* (*Ref : 171*)	
						Form A	Form B
Asp	43	121	12.87	2.3	63	62	61
Thr	28	121	12.24	5.10	29	29	29
Ser	14	157	9.25	18.50	39	30	30
Glu	48	75	11.87	9.87	77	27	27
Pro	23	25	5.43	7.89	37	25	25
Gly	28	95	5.69	19.72	55	43	43
Ala	43	95	5.2	9.44	54	46	46
Val	28	35	3.82	1.06	38	7	7
Met	14	15	0.86	3.47	20	5	5
Ile	31	30	2.72	3.7	34	15	15
Leu	40	57	6.49	2.89	71	36	36
Tyr	19	48	3.74	1.50	24	16	16
Phe	11	30	3.62	1.34	30	12	12
His	16	19	9.74	2.65	29	10	10
Lys	19	46	3.3	9.09	18	11	12
Arg	22	16	3.59	1.46	24	15	15
Cys	ND[c]	5	0.5	ND[c]	62	7	7
Trp	ND[c]	-	-	ND[c]	-	-	-

* Figures denote number of residues/mol of the enzyme protein

**Mol % in each sub unit

[c] Not done

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amino acid composition among different α-galactosidases from various sources may be due to genetic variation or proteolytic modification of the native protein [228, 229]. N-terminal amino acids of the enzyme from *Escherichia coli* [223] is serine and methionine. The enzyme from *Phanerochaete chrysosporium* [230] was a glycosylated tetramer of 50 kDa peptide chains and the N-terminal sequence is ADNGLAITPQMG(?W)N-T(?W)NHFG-(?W)DIS(?W)DTI.

[d] Carbohydrate composition

Almost all microbial α-galactosidases have been found to be glycoprotein in nature with varying amounts of carbohydrate content [7, 44, 45, 134, 146]. Although, in case of many α-galactosidases, the carbohydrate content has been estimated, no detailed study on the composition and structure has been carried out. Suzuki *et al* [34] demonstrated that α-galactosidase from *Mortierella vinacea* is a glycoprotein and contains 2.7% of D-glucosamine and about 10.8% of other hexoses. In case of α-galactosidase from *Saccharomyces carlsbergenesis* [7], 90-95% of the carbohydrate is made up of mannose and 1% glucosamine, while the rest is glucose. In case of *Aspergillus niger* α-galactosidase, glycoprotein has been characterized and its structure is $(Man)_n$-$(Glc\ NAc)_2$-Asn [134].

The most significant point of glycoproteins pertain to the functions of the carbohydrate moiety at the molecular level. Many functions have been attributed and one of the possible function of carbohydrate moiety of glycoprotein in α-galactosidases may be in maintaining the three dimensional structure of the enzyme [231]. The carbohydrate composition of few microbial α-galactosidases is listed in Table 1.7.

Table 1.7 : Carbohydrate content of various microbial α-galactosidases

Source	Sugars present	Total %	Reference
Aspergillus tamarii	Glc. NAc. Man. Glc. Gal	23	44
Aspergillus tamarii	Glc. NAc. Man. Gal	13	45
Cephalosporium acremonium	Glc. NAc, Man. Gal. Sialic acid	27	146
Mortierella vinacea	Glc. NAc. Hexose	13.5	39
Saccharomyces carlsbergensis	Man. Glc. Glc. NAc	57	7

Adapted from Srinivas [8]

1.8.3 Specificity

[a] Hydrolase activity

There are two important aspects which govern the rate of hydrolysis of the substrate by α-galactosidases [5]. These enzymes exhibit strict anomeric linkage specificity and hydrolyze α-linked galactosyl residues selectively from the non-reducing end. Primarily, the ring structure of the substrate must be pyranose and the configuration of -H and -OH on carbon atoms 1,2,3 and 4 must be similar to that of α-D-galactose. Like other glycosidases [232], changes at C_6 of the glycosyl moiety of the substrate has no effect on the specificity of α-galactosidases and because of this property, β-L-arabinosides are also hydrolyzed by α-galactosidase from *Bacteriodes ovatus* [181]. However, α-galactosidase from *Streptococcus bovis* [30], *Epidinium ecadatum* [233], and *Calvatia cyathiformis* [31] do not hydrolyze β-L-arabinosides.

α-Galactosidases from several microbial sources act on a number of α-D-galactose linked sugars like melibiose, raffinose, stachyose and manninotriose [31, 33, 34, 40, 45, 134, 135, 145]. Polysaccharides like galactomannans are also hydrolyzed by α -galactosidases from *Streptococcus bovis* [30] and *Phaseolus vulgaris* [30]. *Mortierella vinacea* [39] α-galactosidase is capable of hydrolyzing various glycosides and oligosaccharides containing α-galactoside linkages at their non-reducing ends. The enzyme from *Penicillium ochrochloron* [58] cleaves α-galactose residues from galactomannans more readily than from α-galactose containing oligosaccharides. The α-galactosidase from *Trichoderma reesei* [48] does not show significant difference in its affinity for α-D-galacto-oligosaccharides having different chain lengths or different aglycons. Mycelial α-galactosidase from *Rhizopus hangchow* [234] hydrolyzed *p*NPG and *o*NPG but did not hydrolyze stachyose, melibiose and methyl-α-D-galactose.

[b] Transgalactosylation reactions

Many microbial α-galactosidases have been reported to exhibit transgalactosylation reaction in addition to hydrolysis of α-galactosidic linkages. The main products of galactose transfer reactions are complex carbohydrates. Usually these reactions involve transfer of an element of an alcohol or a sugar to sugar moiety of a glycoside undergoing hydrolysis with the liberation of an aglycon unit.

Gal-OR + H_2O → Gal-OH + R-OH (Hydrolysis)

Gal-OR + R'OH → Gal-OR' + R-OH (Transfer)

Gal-OR + Gal. OH → Gal-O-Gal + ROH (Transfer)

(Gal-: Galactopyranoside, ROH and R'-OH : alcohols, Gal-O-Gal: Galactose disaccharide).

Blanchard and Albon [232] for the first time reported the formation of an unknown product during the hydrolysis of melibiose with yeast α-galactosidase. This unknown compound moved more slowly than melibiose on the chromatogram and was later identified by French [235] as manninotriose, evidently formed by transfer of a D-galactopyranosyl residue to the D-galactose moiety of melibiose. Since then, a number of microbial α-galactosidases have been reported to catalyze transgalactosylation reactions [31, 206, 236]. It has been generally observed that transgalactosylation reactions to aliphatic hydroxyl groups are accompanied by high hydrolytic activity [5]. Prolonged incubation of the reaction mixture of transferase activity results in the disappearance of transformed products by the subsequent hydrolytic action of the enzyme as evidenced by the fact that the liberated galactose retain the same configuration as in the case of the substrate [4].

Studies carried out on the transgalactosylation activity of different α-galactosidases have shown that hexoses are better acceptors of galactose molecule, whereas this property has not been identified with pentoses [5]. Among hexoses, unmodified glucose or galactose are better acceptors. The modification of primary and secondary hydroxyl groups or oxidation or reduction of aldehyde groups of glucose or galactose, destroys the acceptor property of these hexoses [31].

Li and Shetlar [31], using an enzyme preparation obtained from *Calvatia cyathiformis*, reported the formation of new transgalactosylation products with *o*NPG and *p*NPG as substrates. One of the products is shown to be similar to that of galactobiose having an inverted structure. Mitsutomi and Ohatakara [236] reported the isolation and identification of six main oligosaccharides produced from raffinose by the action of α-galactosidase from *Pycnoporous cinnabarinus*. Sugawara *et al* [124] have shown that the α-galactosidase from *Absidia corymbifera* gives only two transfer products from raffinose, but use of melibiose as a substrate resulted in nine transfer products.

In addition to the important hydrolytic as well as transgalactosylation reaction, someα-galactosidases catalyze the synthesis of oligosaccharides when incubated with high concentrations of monosaccharides. The best known example is that of α-galactosidase from yeast which polymerizes galactose to α-1-3, α-1-4 and α-1-5-galactobioses [237, 238].

[c] Crystal structure of α-galactosidase

Purification of α-galactosidase to homogeneity from plants, animals and microorganisms has been achieved. However, there are only few reports on the

crystallization of the enzyme. Suzuki *et al* [39] devised a simple method for the crystallization of α-galactosidase from *Mortierella vinacea*. The crystalline α-galactosidase was free from protease and other glycosidases. It gave a single band when examined by polyacrylamide gel electrophoresis. The crystalline preparation contained 10.8% neutral sugar and 2.7% D-glucosamine, indicating that the enzyme is glycoprotein in nature. Purified α-galactosidase from *Pycnoporous cinnabarinus* [239] was crystallized and the specific activity of this enzyme remains unchanged after crystallization, but its value is higher than that reported for the crystalline α-galactosidase from *Mortierella vinacea* [39]. Crystals of α-galactosidase from *Trichoderma reesei* [240] were obtained from a polyethylene glycol 4000 solution by the hanging drop method.

Human α-galactosidase A, the glycosylated lysosomal enzyme, deficiency of which causes Fabry's disease, has been crystallized as a complex with the inhibitor N-6-aminohexanoyl-α-D-galactopyranosylamine [241]. X-ray diffraction data collected from these crystals indicate that the crystals belong to the orthorhombic space group $C222_1$ with cell dimension of a = 93.8 Å, b=141.1 Å and c= 184.4 Å and diffract to a resolution of 3 Å. However, three dimensional structure of α-galactosidase has not been determined so far.

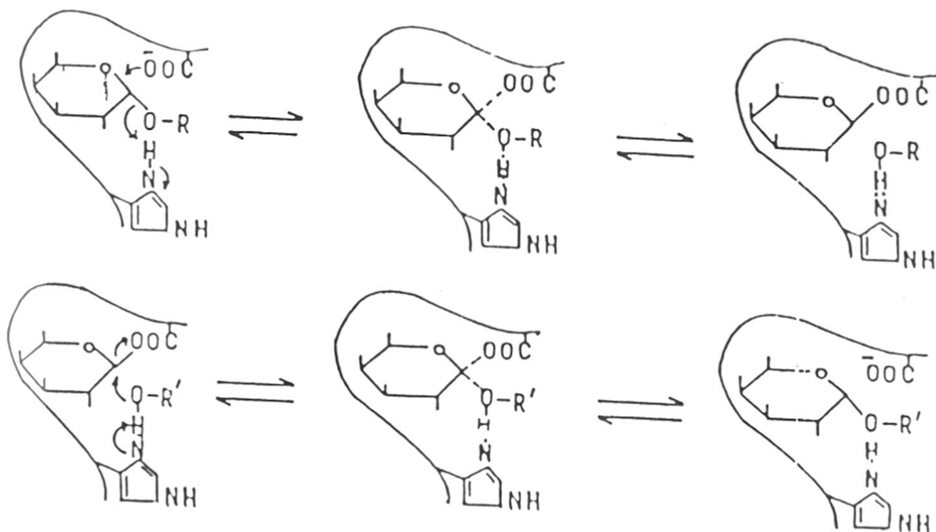
1.9 ACTIVE SITE AND MECHANISM OF ACTION

The inactivation of some α-galactosidases from *Aerobacter aerogenes* [27], *Cucurbito pepo* [242], *Saccharum officinarum* [94], sweet almond [96], mung bean [100] and *Diplococcus pneumoniae* [187] by PHMB and iodoacetamide indicated the involvement of sulfhydryl group at or near the active site of the enzyme. However, α-galactosidases from *Calvatia cyathiformis* [31], coconut [67] and spinach leaves [243] were not inactivated by these reagents. Thus, it is unlikely that all α-galactosidases require an active SH-group for catalysis. The inactivation of α-galactosidases from *Vicia faba* [98], sweet almond [96] and mung bean [100] by photo-oxidation in the presence of Methylene Blue showed the involvement of histidine at the active site. In case of coconut α-galactosidase [67], neither iodoacetamide nor DEPC caused inactivation, suggesting the non-involvement of histidine in the catalytic activity [244, 245]. However, coconut α-galactosidase lost significant amount of its activity on treatment with NBS, TNM and EDC, indicating the probable involvement of tryptophan, tyrosine and carboxylate in the catalytic activity of the enzyme [67]. Recently, Kachurin *et al* [169] identified the presence of methionine at or near the active site of *Trichoderma reesei* α-galactosidase. Moreover, they showed that oxidation of this methionine residue altered the kinetic

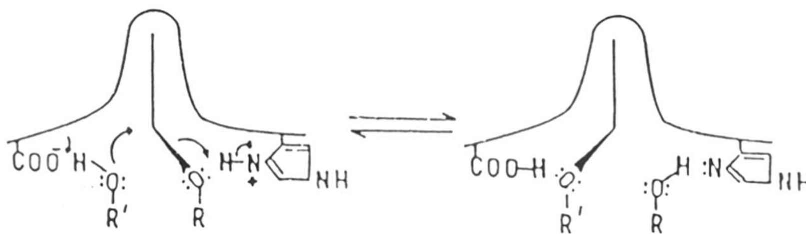
properties of the enzyme and enhanced the activity towards the substrate, *p*NPG. The cDNA for coffee bean α-galactosidase has been cloned and expressed in a baculovirus system by Zhu *et al* [71]. They replaced two tyrosine residues (at positions 108 and 158) with phenylalanine by site-directed mutagenesis. The mutated DNA strands, as well as the wild-type ones were subcloned into pVL vector and transformed into Sf9 insect cells for intracellular expression. The replacement of tyrosine-158 with phenylalanine resulted in a mutant α-galactosidase (Y 158 F) which retained approximately 88% activity of the wild type enzyme. However, the substitution of tyrosine-108 by phenylalanine (Y 108 F) almost abolished the enzymatic activity (1.8% of wild type activity). The V_{max} / K_m value for the mutant of Y108F was 0.027, which was 1000 fold lower than that of wild type α-galactosidase. The above observations suggested that tyrosine-108 is critical for the catalytic activity of this enzyme.

The effect of temperature and pH on the activity of sweet almond [246] and *Vicia faba* [98] α-galactosidases has been studied with melibiose and *p*NPG. Changes in K_m and V_{max} as a function of pH indicated the participation of two ionisable groups with pK_a values 5.75 and 3.0. The above pK_a values suggested the involvement of histidine and carboxylates in the catalytic activity of the enzyme. Studies with *p*NPG also revealed the presence of two groups. Photo-oxidation experiments with *Vicia faba* α-galactosidase I also indicated that histidine is present at the active site.

Based on the photo-oxidation studies, two possible mechanism of action has been proposed for sweet almond α-galactosidase [96]. First, is a "two step" mechanism, wherein the aglycon is cleaved by the concerted action of carboxyl and imidazole groups [Fig 1.1a]. This is then followed by reaction with an acceptor molecule (R'OH), (which could be water or an aliphatic alcohol) resulting in the hydrolysis of the glycosidic bond [96]. It is possible that the electrophilic attack on imidazole group alone is sufficient to cleave the glycosyl oxygen bond with the formation of carbonium ion at C_1 of the galactose moiety [96]. In the "two step"mechanism, two Walden inversions probably occur [5], resulting in retention of the anomeric configuration of the final product [Fig 1.1b]. However, the formation of carbonium ion intermediate need not necessarily lead to racemization. The configuration could be stabilised by a specific binding of intermediate to the enzyme [247]. The "one step" mechanism suggests the formation of a ternary complex, consisting of the enzyme, substrate and the acceptor molecule [248], wherein the histidine and the carboxylate, present in the active site, play a similar role, as indicated in "two step" mechanism. Accordingly, a frontal attack on the anomeric carbon atom of the galactose moiety leads to a product with retention of configuration [96].



a



b

Figure 1.1 : Mechanism of action of α-galactosidase

(a) "two step" mechanism [5]

(b) "one step" mechanism [96]

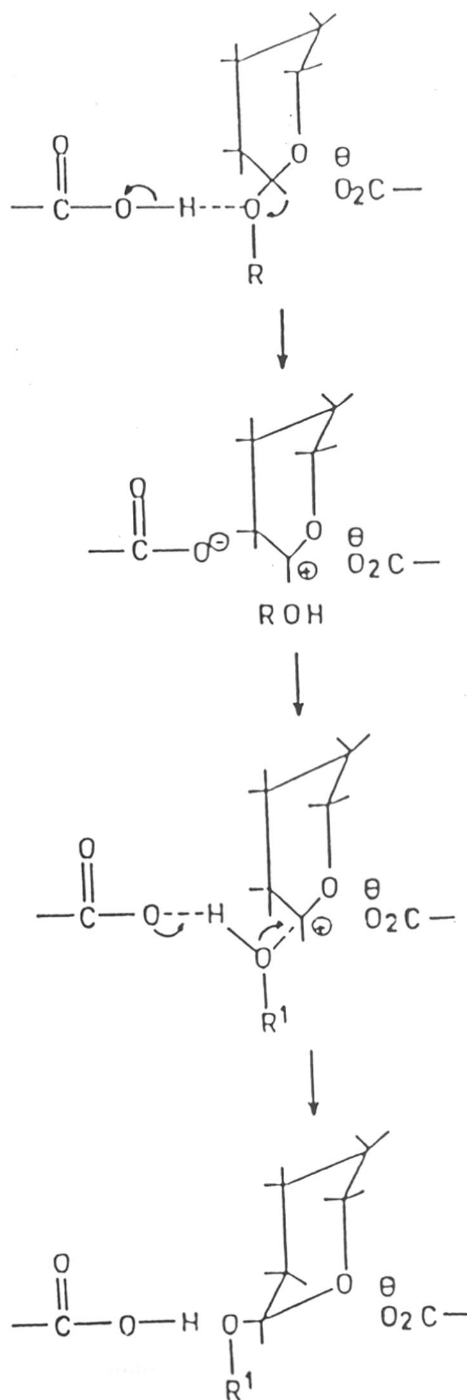
Mathew and Balsubramaniam [68] proposed a modified mechanism of action of coconut α-galactosidase. The effect of pH on K_m and V_{max} values indicated the involvement of two ionizing groups with pK_a values of 3.5 and 6.5 in the catalytic activity. Chemical modification studies indicated the presence two carboxyl groups, a tryptophan and a tyrosine, at or near the active site of the enzyme. Based on these observations a new mechanism of action for α-galactosidase is proposed in which the ionizing group with pK_a of 3.5 is a carboxyl group involved in solubilizing the carbonium ion intermediate and the ionizing group with pK_a, 6.5 is a carboxyl group perturbed due the presence of hydrophobic residues in its vicinity which donates a H^+ ion in catalysis [Fig 1.2].

1.9.1 Lectin nature of α-galactosidase

Proteins or glycoproteins of non-immune origin possessing the ability to agglutinate erythrocytes or precipitate glycoconjugates are termed as hemagglutinins or lectins. Carbohydrate binding proteins which agglutinate erythrocytes and precipitate glycoconjugates are very common in plant tissues. Some specific enzymes with multiple sugar binding sites have the ability to agglutinate erythrocytes [249]. 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 [83, 250, 251].

Agglutination generally requires the presence of multiple binding sites. The binding of lectin to erythrocyte membranes can be reversed using structurally complimentary sugars. Thus, many glycoprotein α-galactosidases being high molecular weight aggregates with multiple binding sites, function as lectins, whereas the monomers, which may have a single binding site, may not be able to cause visible agglutination. In the case of soybean [83] and mung bean [251] α-galactosidases, only the tetrameric form can cause hemagglutination and have catalytic function. In some cases, though hemagglutination occurs initially, on longer incubation the clot gets dissolved due to subsequent enzymatic activity of the protein, 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 by α-galactosidase as proposed by Dey [100] has been shown in Figure 1.3a, wherein the protein binds to the erythrocyte membranes by virtue of its hemagglutination ability but subsequently hydrolyzes the bonds of sugar linkages to which it binds by catalytic function. *Vicia faba* α-galactosidase has been shown to possess hemagglutination Figure



1.2 : Mechanism of action of α-galactosidase

Modified "one step" mechanism [68]

activity with unique glucose / mannose specificity, independent of catalytic activity [Figure 1.3b], even though the catalytic and lectin binding site reside in the same protein molecule [99].

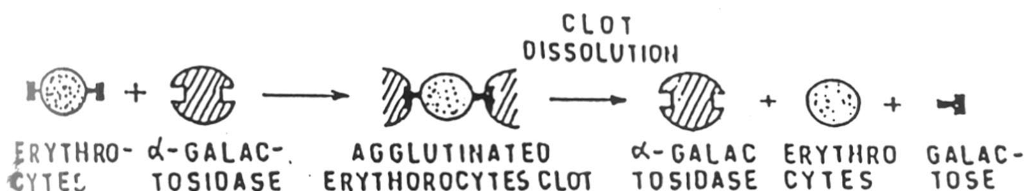


Figure 1.3a : Model to explain hemagglutination and subsequent clot dissolution by the tetrameric form of mung bean α-galactosidase [100]

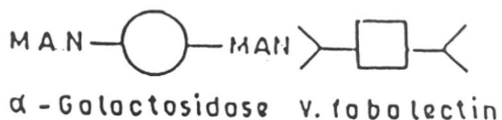


Figure 1.3b : Model to explain *Vicia faba* α-galactosidase-lectin activity [10]

1.10 PHYSIOLOGICAL SIGNIFICANCE

The presence of α-galactosidase has been detected in plants, which contain α-D-galactosidic substrates [5]. It is well established that the primary function of this enzyme in plants is to cleave α-D-galactosyl groups from α-D-galactose containing oligo- and poly- saccharides, which are ubiquitous in nature [252]. The involvement of α-galactosidase in mobilizing the reserve galactose and polysaccharides has been demonstrated in many cases [64, 65, 97, 253]. In many maturing seeds, synthesis of galactosyl sucrose derivatives with a concomitant increase in the α-galactosidase activity have been reported [5, 9]. During germination, it is involved in the hydrolysis of the stored carbohydrates, resulting in the liberation of free sugars, which may serve as a ready energy source for the growing plant [5].

The α-galactosidase may also be involved in the metabolism of galactolipids [93]. A probable role for α-galactosidases in the ultimate breakdown of galactolipids to fatty acids, glycerol and glucose, in conjunction with other enzymes, has been demonstrated in runner bean leaves [93]. Another important function of α-galactosidase involves the protection of plants against α-galactosidic based phytotoxic substances of invading pathogens [254]. In the animal kingdom, the enzyme has been shown to be involved in the hydrolysis of galactolipids. The deficiency of the 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 diseases and is caused by a reduced activity of α-galactosidase, which is normally present in most tissues. Two α-galactosidase isozymes, A and B, are present in normal tissues but form A is absent in Fabry's disease [111]. In addition to the structural differences between forms A and B, both the enzyme forms were found to differ in several properties such as thermal stability [110], antigenicity [117], specificity towards inhibitors [255] and natural substrates [110]. The enzyme also occurs in brain tissues and may be involved in the hydrolysis of monogalactosyl diglycerides and digalactosyl diglycerides [11, 57, 256-258].

1.11 PRODUCTION OF α-GALACTOSIDASE

The production of any industrial enzyme by microbial fermentation offers many advantages such as rapid rate of production, unlimited source of supply and low cost of production [3]. The microbial production of α-galactosidase is mainly restricted to SmF. Although the productivity by SSF is several times higher [34], this technique has not yet been exploited industrially for α-galactosidase production. The titres of α-galactosidase produced by different microorganisms are listed in Table 1.8.

Table 1.8 : Titres of α-galactosidases from different microorganisms

Source	Substrate used	Enzyme activity* (units/ml)	Reference
Aspergillus awamori	*p*NPG	0.122	129
Aspergillus fumigatus	Raffinose	2.0	133
Aspergillus niger	Melibiose	0.125	46
Aspergillus oryzae	*p*NPG	2.58	142
Bacillus stearothermophilus	*p*NPG	0.4	176
Candida guillermondii	*p*NPG	19.0	207
Cephalosporium acremonium	*p*NPG	25.0	146
Mortierella vinacea	Melibiose	1.7	34
Penicillium ochrochloron	*p*NPG	0.14	58
Penicillium purpurogenum	*p*NPG	61.5	225
Penicillium purpurogenum	*p*NPG	4.04	259
Pseudomonas fluorescens	*p*NPG	0.36	199
Saccharomyces carlsbergensis	*p*NPG	1.0	203
Trichoderma reesei	Melibiose	0.064	48

* Units are expressed in μmoles of product liberated / min.
Adapted from Srinivas [8]

1.11.1 Enzyme production by SmF

1.11.1.1 *The technique and its utility*

In SmF, the liquid medium is employed in higher depth in vessels with a diameter to height ratio of 1:2 to 1:3 . In most cases, all the nutrients are dissolved in water except for those involving water insoluble substrates or water immiscible liquids [260]. The free flowing nature of the medium in SmF distinguishes it from SSF, while greater depth of the medium makes it distinct from the liquid surface fermentation (LSF) process [260]. The SmF process is advantageous over LSF in many aspects namely ease of operation, economy, reduced requirement of space, consistent growth pattern, improved containment of contaminants and precise control of parameters [261]. The presence of the resultant product in dilute form and disposal of large quantities of water is a major drawback of SmF [262, 263].

The SmF processes are carried out in shake flasks or aerated agitated fermentors equipped for control of parameters [264, 265]. Development of efficient stirred and aerated fermentor vessels have resulted in more uniform growth of fungal strains under SmF.

1.11.1.2 *α-Galactosidase production by SmF*

The microorganisms preferred for commercial production of α-galactosidase are *Mortierella vinacea* [34], *Bacillus stearothermophilus* [176], *Circenella musae* [266], *Absidia hyalospora* [35] and *Absidia greseiola* [37,267]. The α-galactosidases from *Saccharomyces carlsbergenesis*, *Escherichia coli* and *Aerobacter aerogenes* do not appear to be suitable for commercial exploitation because the enzymes from these sources suffer either from low thermostability (rapid loss of activity at 40°C) or from low activity at neutral pH. In the treatment of soymilk, in particular, the enzyme which is most active at or around neutral pH is preferred [268].

In beet sugar industry, organisms which do not produce invertase along with α-galactosidase are preferred because, the presence of invertase in the system results in the total hydrolysis of raffinose to galactose, glucose and fructose, which is not desirable [12].

1.11.1.3 *Industrial production of α-galactosidase*

According to the available literature, Kitami factory of the Hokkaido Sugar Company in Japan and Great Western Sugar Company in USA are the only two companies manufacturing α-galactosidase for use in beet sugar industry [12].

1.11.1.4 *Factors affecting α-galactosidase production by SmF*

Various factors such as physical, chemical and nutritional parameters are known to affect the productivity of the enzyme to a significant extent [3]. Factors which affect α -galactosidase production in SmF are as follows.

[a] pH

The pH, measure of the hydrogen ion concentration, is particularly critical for microbial growth. Control of pH is important to maintain an optimal environment for growth and product formation [269]. The initial pH of the medium for α-galactosidase production are usually in the range 4.5-8.0 [146, 156, 176, 266, 267]. In most of the cases, pH of the medium is not controlled during fermentation [34]. The use of phosphate buffer, for minimizing changes in the pH of the medium, for α-galactosidase

production by *Streptomyces olivaceus* [172] has been reported. Studies on the effect of initial pH on three different media in the pH range 5.0-7.0 for α-galactosidase production by *Monascus anka* indicated that maximum enzyme levels were obtained when the initial pH of the medium was 6.0 [155].

[b] Temperature

A variety of chemical reactions in the metabolism of a cell is influenced by temperature. Temperature also affects the efficiency of the carbon source and its conversion to cell mass [269]. The effect of incubation temperature on the production of α-galactosidase depends on the particular strain employed for fermentation. The optimum temperature reported for the production of α-galactosidase is 30°C in case of *Mortierella vinacea* [34], *Circenella musae* [266], *Absidia greseiola* [37], *Monascus pilosus* [156], *Corneybacterium murisepticum* [222] and *Trichoderma reesei* [48] and 37° C and above in the case of *Bacillus stearothermophilus* [176, 177], *Lactobacillus fermenti* [194] and *Penicillium dupontii* [161].

[c] Aeration and agitation

The aeration-agitation of the fermentor, especially in the cultivation of fungal strains, is of vital importance in obtaining optimum yield of the product. It plays an important role in maintaining the homogeneity of the medium, transfer of oxygen from gas phase to the liquid and other mass transfer reactions [261]

There are few reports [34, 176, 266], describing the conditions of aeration and agitation and the effect of these parameters on α-galactosidase production. The conditions of agitation and aeration varies from strain to strain and the type of fermentors used. Delente *et al* [176] employed aeration at a rate of 0.8 vvm and agitation of 400 rpm for the production of α-galactosidase from *Bacillus stearothermophilus* in a 14 L fermentor. In another case, an aeration rate of 0.5 vvm and agitation of 240 rpm, was used by Narita *et al* [266] to produce α-galactosidase from *Circenella muscae* in a 20 L fermentor.

[d] Carbon source and induction

Carbon is a major constituent of the microbial cell, which amounts approximately to 50% of the dry wet. An adequate supply of the carbon source is critical for optimal growth and product formation [269]. The α-galactosidase is an inducible enzyme and hence sugars which contain α-galactosyl residues are known to induce the enzyme to a varying extent. A number of reports are available on the effect of carbon source on the

production of α-galactosidase. Among various sugars tested for the production of this enzyme, galactose, melibiose and raffinose were found to be good inducers at 0.4-1% (w/v) [34, 44, 45, 176, 190, 222, 266, 270]. In addition lactose, a β-linked disaccharide, (an inducer of β-galactosidase) is also reported to induce α-galactosidase in many microorganisms [34, 146, 222]. The induction of α-galactosidase by different sugars in *Saccharomyces carlsbergenesis* decreased in the following order : stachyose > raffinose > melibiose > galactose and > lactose [213]. A strain of *Saccharomyces cerevisiae* was found to produce α-galactosidase on a glucose-yeast extract-salt medium, but produced nearly twice the activity when supplemented with melibiose [271]. However, the concentration of each sugar varied depending on the culture requirement.

Galactomannans are the energy reserve polysaccharides in seeds of all endospermic leguminous plants and consist of polymeric chains of β-1,4-linked D-mannosyl residues substituted by single D-galactosyl residues α-linked to mannosyl C_6 sites [272]. A few microorganisms have been reported to utilize these polysaccharides to produce α-galactosidase. Adya and Elbein [134] observed the production of extracellular α-galactosidase when *Aspergillus niger* was grown on guar galactomannan. *Aspergillus tamarii* secreted two forms of α-galactosidase (I and II) when grown in the presence of galactomannan [44, 45]. The 6 fold increase of α-galactosidase activity in *Penicillium ochrochloron*, was observed in presence of guar galactomannan over other α-galactosidic sugars tested [58]. In addition to the above mentioned carbon sources, long chain fatty acids are also known to induce α-galactosidase in *Mortierella vinacea* [273]. Fatty acids with less than 11 carbon atoms inhibited the mold growth whereas, fatty acids (both saturated and unsaturated) with more than 12 carbon atoms markedly enhanced the enzyme production.

Since sugars are expensive, their use is not economical from a commercial point of view. Hence replacement of sugars with cheaper carbon source like wheat bran, rice bran, baggasse etc would be advantageous.

[e] Nitrogen source

The involvement of nitrogen in growth, metabolism and product formation is well known [274]. A variety of nitrogen sources including both organic and inorganic have been evaluated for the production of α-galactosidase. Some of the important observations on the effect of different nitrogen sources in the production of α-galactosidase include, very high yields of the enzyme within the mycelium of *Circenella muscae* with peptone [266] and optimum enzyme production in *Mortierella vinacea* in presence of 0.5% ammonium nitrate or a mixture of 0.3% peptone and meat extract [34,

272]. The effect of various nitrogen sources on the production of α-galactosidase from *Bacillus stearothermophilus* [176] indicated that a 2% water extract of soybean meal and 0.5% yeast extract gave optimum results.

[f] Mineral salts and trace elements

The presence of metal ions (e.g. potassium, magnesium, iron, manganese, zinc, copper and cobalt) in the medium play a significant role in the growth and product formation. Especially, metal ions such as iron, manganese, zinc, copper and cobalt function as effectors of enzymes and coenzymes [269].

Although there are reports on the use of metal ions for the production of α-galactosidase from various microorganisms, very little information is available regarding their effects. The presence of certain metal ions in the fermentation media can markedly influence the productivity of the enzyme in many cases. Stimulatory effect on α-galactosidase production by Zn^{2+}, Mg^{2+} and Fe^{2+} in the fermentation medium has been demonstrated in the case of *Aspergillus tamarii* [44,45]. However, metal ions like Ca^{2+}. Ba^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Hg^{2+} have been shown to inhibit enzyme production in *Monascus* sp. [270].

1.11.1.5 ***Kinetic studies***

The rate of uptake of nutrients from the cell's environment and the release of metabolic end products into the surroundings are associated with cell growth [275]. In general, the growth process has two different manifestations according to the morphology of the microorganisms involved. For unicellular organisms, the increase in biomass is accompanied by increase in the number of cells present. However, in molds, the length and number of mycelia increases as the organism grows [275]. In addition to the influence of external factors such as temperature, pH, aeration and agitation, the growth rate is also influenced by endogenous factors like morphological organization, metabolic patterns, stoichiometry and intracellular control [269].

Very few reports are available on the kinetics of α-galactosidase production [43, 194, 209]. The relationship between cell growth and enzyme production is well studied during the growth of *Lactobacillus fermenti* [194]. Results showed that the enzyme activity is maximum in late growth phase on glucose and in early growth phase on raffinose for both static and agitated culture conditions. Biomass levels are higher for agitated cultures over static cultures due to better mixing conditions for oxygen transfer. The maximum productivity of 17.8 units / L / h was achieved in 24 h with glucose as the carbon source.

1.11.1.6 *Mathematical model for α-galactosidase production*

Studies on microbial growth on mixtures of different substrates have shown that the organisms consume preferentially the substrate supporting most rapid growth. The molecular basis for this kinetic behaviour are the induction, repression, inhibition and activation processes. Several kinetic models have been proposed based on the afore mentioned effects [275].

There is only a single report on reactor modeling and optimization of α-galactosidase production. The effect of culture conditions on α-galactosidase production was studied in detail by Imanaka *et al* [270] using *Monascus* sp. The enzyme is strongly induced by galactose, melibiose, raffinose or stachyose. The optimum conditions for cell growth and enzyme production were determined. In order to assess the relationship between cell growth and sugar consumption, a mathematical model was established in a single stage continuous culture method. According to this model, when glucose was present at a concentration of more than 2.25-10.4 g / L, it repressed galactose consumption and in turn α-galactosidase production. The model for the enzyme production is based on the theory of induction. The specific rate of α-galactosidase synthesis is proportional to the intracellular concentration of mRNA which codes for the enzyme. The constants at the molecular level that could not be determined experimentally were derived from the model [270].

1.11.1.7 *Recovery of enzyme*

The first step in recovery of an intracellular enzyme is to disintegrate the cell to release its contents [276]. In large scale application, the complete disruption of yeasts and bacteria can be obtained by using a French Press, homogenizers or grinding with abrasives [277, 278]. In some cases, ultrasonication is used to disrupt the cells and the extract is centrifuged or clarified by cross flow filtration [279].

In some cases different solvents (acetone, chloroform, ethanol, methanol etc) of various concentrations have also been used to recover the intracellular enzyme. However, in case of extracellular enzymes, the recovery is less expensive because the enzymes are normally recovered by filtration or centrifugation and concentrated either by ultrafiltration or ammonium sulfate precipitation.

1.11.2 Enzyme production by SSF

1.11.2.1 *Technique and its utility*

SSF involves the growth of microorganisms on moist solid substrate in the absence of free flowing water [38]. The traditional "koji" process which involves the growth of filamentous fungi such as *Aspergillus oryzae* on moist mixed substrates such as rice or soy bean to produce a mixture of extracellular amylolytic and proteolytic enzymes, formed the basis for production of enzymes using SSF [280]

The cell growth, product formation, transport phenomena and kinetic aspects in SSF are entirely different from that of SmF [281]. Due to the low moisture content in SSF system, the microorganism is almost in direct contact with the oxygen present in the void spaces between the substrate particles [282]. The absorbed moisture within the solid matrix, in SSF, is reported to have a higher capacity for oxygen transfer under normal aeration conditions. Unlike in SmF system, the moist solid substrate usually acts as a source of carbon, nitrogen, minerals and other nutrients, although, in some cases, it is enriched with additional nutrients for improved productivity [283]. In SSF technique, the growth of microorganisms on moist solids can be accelerated using appropriate physico-chemical and nutritional parameters at their optimal levels during the course of fermentation [283]. In SSF, the bacterial and yeast cultures grow by adhering to the surface of the solid substrate particle, while the filamentous fungi are able to penetrate deep into the solid substrate particles for nutrient uptake there by providing anchorage to the microbial cells [284]. As the fungi, in SSF, grow under conditions closer to their natural habitats, they are more capable of producing certain enzymes and metabolites that usually will not be produced, when grown under SmF [285].

Another principle difference between SmF and SSF is the complete accessibility of the substrate to the microorganisms. In the former case the substrate is present in completely dissolved form whereas, much of the substrate is not easily accessible initially in SSF [286]. Further in SmF process, the net amount of accessible substrate decreases as the fermentation progresses. Many important products such as enzymes, cheese, mushrooms, single cell proteins, organic acids, plant growth regulators, toxins and antibiotics have been successfully obtained using SSF technique [287].

1.11.2.2 *Advantages of SSF over SmF techniques*

The distinct advantage of SSF for fungal enzyme production when compared with SmF are, lower capital investment, high product concentration, reduced expenditure on down stream processing, minimized waste disposal, simpler reactor design with minimum controls and fermentor volume [288]. This is mainly due to high concentration

of the enzyme in the liquid phase in SSF which permits enzyme recovery at considerably lower energy inputs [289, 290]. Other advantages of SSF technique include, simplified procedure for inoculum development, easier scale up of process and reduced solvent requirement for product recovery.

1.11.2.3 *Production of α-galactosidase by SSF*

There has been considerable interest to produce α-galactosidase by SSF processes [34, 46, 128-131, 142, 143, 291, 292]. In most of the cases soybean and wheat bran have been used as the substrates for enzyme production and the microorganisms used in SSF are *Aspergillus awamori* [128, 129], *Aspergillus oryzae* [142] and *Aspergillus niger* [46].

Filho *et al* [291] reported the enzyme production from *Taloromyces emersonii* and *Penicillium capsulatum*, grown in SSF using xylan. Cruz and Park [142] reported the production of α-galactosidase by SSF and its application for the hydrolysis of galacto-oligosaccharides in soybean milk. Soya carbohydrates when added in the fermentation medium were shown to induce enzyme production. Annunzaiato *et al* [143] carried out SSF on wheat bran for α-galactosidase production using *Aspergillus oryzae* QM 6737 for improving enzyme yield and lowering production costs. Enzyme yield increased 3 fold when soy flour or soybeans were used as the substrate, but no enzyme was produced with rice. Somiari and Balogh [46] used a strain of *Aspergillus niger* for α-galactosidase production on wheat bran or rice bran. Srinivas *et al* [139] described the 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* MRRS 234 in SSF. Production of α-galactosidase using *Aspergillus niger* [292] has also been reported for invertase production alongwith α-galactosidase and the enzyme production was growth associated. It was observed that 19.5% of the total enzyme was formed in early stationary phase. Reports on production of microbial α-galactosidases by SSF is scanty and no data is available on the effect of media parameters.

1.11.2.4 *Purification and characterization of α-galactosidase*

To the best of our knowledge, only two reports are available on the purification of α-galactosidase, obtained by SSF [46, 144]. The crude enzyme extract obtained from *Aspergillus oryzae* [144] grown on wheat bran, was purified approximately 35 fold by ethanol precipitation, gel filtration on G-75 and ion exchange chromatography on DEAE-Sephadex. The molecular weight of the enzyme was estimated as 64000 Da. The pH and temperature optima were reported to be 4.0 and 60°C, respectively. The enzyme was

stable over the pH range 3.0-7.5 and temperature upto 55°C (at pH 4.0). The K_m values for *p*NPG and raffinose were 0.40 mM and 10 mM, respectively [144].

In the second report, the enzyme from *Aspergillus niger* [46] was purified using Sephadex G-100 chromatography. Three peaks of activity were obtained after dialysis and chromatography on Sephadex G-100, indicating the presence of multimolecular forms. The specific activity of the pure enzyme was 1567 U μg^{-1} protein and the protein responsible for the highest activity peak consisted of two subunits with relative M_r 78 and 69 kDa, respectively.

1.11.2.5 Hydrolysis of soymilk

Soymilk contains harmful sugars such as raffinose and stachyose which are known to induce flatulence when the milk is consumed by humans, especially children. Therefore it is essential to convert the harmful sugars to simpler forms. Several reports on hydrolysis of soymilk by α-galactosidase from plants and microorganisms have appeared in the literature [138, 142, 144, 145, 197, 293]. However, all these methods suffer from some basic disadvantages with respect to practical applicability of the enzymes in soymilk processing.

The pH optima of α-galactosidase used for the hydrolysis of soymilk lies on the acidic side and not near to the pH of soymilk (6.0) [138, 142, 144, 145, 197, 292, 293]. Therefore, the pH of soymilk has to be adjusted to suit optimum enzyme action to achieve complete hydrolysis or sacrifice the high activity of the enzyme by carrying out the hydrolysis at pH 6.0. Moreover, acidic conditions tends to clot soymilk leading to precipitation of soyaproteins.

The soymilk is pasteurised by heating at 60°C for 30 min. In most cases, hydrolysis of soymilk is carried out using thermostable enzymes at moderate temperature i.e. upto 50°C [292, 293]. Enzymes with higher thermostability and acidic pH optima have also been used. α-Galactosidase having optimum activity at neutral pH is preferred for use in beet sugar stream treatment to avoid unwanted hydrolysis of sugars other than raffinose.

1.12 APPLICATIONS OF α-GALACTOSIDASES

1.12.1 Immobilized α-galactosidases in sugar beet industry

In beet sugar industry, during concentration of beet sugar molasses, the raffinose content increases gradually to 6-10% leading to the inhibition of crystallization of beet sugar and therefore at this stage molasses is discarded [34]. Use of α-galactosidase for the hydrolysis of raffinose in beet syrup helps to eliminate the retardation of sucrose crystallization [12], thereby resulting in an increase in the sucrose content of the beet

syrup [34]. Immobilized α-galactosidases are extensively employed in the beet sugar industry [13, 128, 160, 178, 294]. The development of a technology for the enzymatic hydrolysis of raffinose from beet sugar molasses requires a source of α-galactosidase which is essentially free of invertase [12]. Numerous attempts have been made to immobilize α-galactosidases by various methods. The enzyme from *Mortierella vinacea* entrapped in polyacrylamide gel, agar or konjak mannan was used for the hydrolysis of raffinose in beet molasses in a column reactor [294]. Mycelia of *Mortierella vinacea* [159] containing α-galactosidase were disrupted and entrapped in 7.5% polyacrylamide gel and its general properties were determined. It was observed that there were only slight changes in optimum pH and temperature after immobilization, while the recovery of α-galactosidase activity was approximately 65%. Three different forms (undisrupted, disrupted and entrapped) of *Mortierella vinacea* mycelium were used to hydrolyze the oligosaccharides in soybean milk and among them disrupted mycelia gave the highest hydrolysis ratio. However, it was found that after long term usage at 50°C, the thermal stability of the disrupted mycelia was relatively poor. Reynolds [178] immobilized α-galactosidase on nylon microfibrils suitable for use in large flow through reactor. The immobilized system was stable for 7 months, both under use and storage conditions. The immobilized enzyme behaved similarly to the soluble enzyme, characteristically exhibiting both product and substrate inhibition. The enzyme, from coffee bean [295] was immobilized on cyanogen bromide activated Dextran T-70. The bound enzyme showed higher temperature stability than the soluble enzyme. Japanese and Belgian patents issued to Saimaru *et al* [36, 296] describes glutaraldehyde fixation of α-galactosidase from the genera *Mortierella*, *Absidia* and *Circenella*. Evidence was presented for stabilization of α-galactosidase on the mycelium of *Mortierella vinacea* var raffinoseutilizer [36]. For example, 350 g mycelial pellets were mixed in 3 liters of a slurry containing 1% glutaraldehyde by weight of mycelia at 20°C and pH 7.0 for 1.5 h. The fixed pellets were washed with water and allowed to react with beet molasses under conditions simulating its practical use for 21 days.

Chitin and Chitosan are good alternative carriers for immobilization of enzymes [297]. Use of chitosan is more advantageous than chitin because of its inherent cationic properties. The enzyme from *Pycnoporous cinnabarinus* [297] was immobilized on chitosan beads cross linked with glutaraldehyde. Efficiency of the immobilized enzyme improved when chitosan beads of increased deacetylation were used. The immobilized beads were packed in a column reactor and used for the continuous hydrolysis of raffinose in beet molasses [297]. Similarly, α-galactosidase immobilized on chitin [298] could be used repeatedly for 20 times for the hydrolysis of raffinose.

1.12.2 Processing of legume based foods

Soybeans are used largely as low cost and high quality protein supplement [293]. However, it contains raffinose, stachyose and verbascose which cause flatulence when ingested by humans [299]. The absence of α-galactosidase in human intestinal track prevents the hydrolysis of these sugars present in soybean and other legumes [299, 300]. After ingestion of these foods, the undigested sugars pass into the large intestine where they are fermented anaerobically by α-galactosidase producing bacteria, resulting in the production of gas and gastro-intestinal distress [142].

Many attempts have been made to eliminate the oligosaccharides from soybean by soaking, germination [190, 301] and fermentation [193]. Enzymatic treatment of soymilk by microbial α-galactosidase offers a promising solution for the elimination of these oligosaccharides, especially in soymilk [128, 129, 144, 145, 159]. Recently, Somiari and Balogh [46] reported the hydrolysis of stachyose in cowpea flour using a crude preparation of α-galactosidase from *Aspergillus niger* and found that the enzyme treatment is more effective than soaking and cooking.

1.12.3 Guar gum processing

The commercially produced guar galactomannan has 38% galactose and 62% mannose [272]. The useful commercial properties of galactomannan are due to its high viscosity in dilute aqueous solutions and co-gelation with other polysaccharides such as carrageenan, agar and xanthan gum.

To convert guar galactomannan into a locust bean galactomannan equivalent, some of the side chain α-1-6-linked-D-galactosyl residues need to be removed without significant cleavage of the galactomannan backbone [272]. This can be achieved by partial enzymatic hydrolysis of guar gum by α-galactosidase [81]. Studies with purified α -galactosidase have shown that the galactose content of the polysaccharide can decrease without affecting the backbone [272]. The enzymatically modified guar galactomannan was found to exhibit physical and functional properties similar to that of locust bean gum. A process for the modification of galactomannan polymers with plant α-galactosidase has been reported [272].

1.12.4 Other potential applications

1.Type B erythrocytes which contain 3-O-α-D-galactopyranoside [77, 84], can be transformed into type O erythrocyte by exposure to α-galactosidase [14], for transfusional purposes [202].

2.Treatment of Fabry's disease, a metabolic disorder resulting from deficiency of lysosomal α- galactosidase A [108, 109].

3.Elucidation of structure of some complex carbohydrates [209].

4.Transgalactosylation of different α-galactose linked sugars for obtaining different products [124].

5.Enhancing cellulolytic conversion of wood polysaccharides to hexose [168].

6.Facilitating the enzymatic biopulping and bleaching of softwood in paper industries [177].

7.Increasing the efficiency of fermentation feed stocks for the consequent lowering of biochemical oxygen demand (BOD) of the process effluent [215].

1.13 SCOPE AND OBJECTIVES OF THE PRESENT WORK

α-Galactosidase finds commercial use in beet sugar industry, in the hydrolysis of raffinose, for improving crystallization of the sugar. It also has a number of other potential uses. Recently, α-galactosidase has assumed significant industrial importance due to the increased processing of soy and legume based food products.

The enzyme is presently produced by SmF technique. Few reports are also available on the production of the fungal enzyme by SSF. The scope of the present work was targeted to the following aspects.

(a) Screening of fungal isolates, especially thermophilic fungi for α-galactosidase production and identification of *Humicola* sp. as α-galactosidase producer.

(b) Formulation of a medium for the optimum production of α-galactosidase in SmF and SSF, determination of the physico-chemical properties of the crude enzyme and its applicability in the removal of raffinose and stachyose from soymilk.

(c) Purification of α-galactosidase from *Humicola* sp. and its extensive characterization.

(d) Active site studies of purified α-galactosidase to evaluate its structure function relationship.

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CHAPTER - II

Production of thermostable α-galactosidase from the thermophilic fungus *Humicola* sp. (NCIM 1252) by submerged fermentation (SmF)

2.1 SUMMARY

The thermophilic fungus *Humicola* sp. isolated from soil, when grown in a medium containing wheat bran and yeast extract secreted α-galactosidase in culture filtrate. The culture filtrate also contained small amount of β-galactosidase activity. α-Galactosidase production was highest in a medium containing 5% wheat bran extract and 0.5% (w/v) beef extract as a carbon and nitrogen source, respectively. Addition of Zn^{2+} and Mg^{2+} in the fermentation medium stimulated the enzyme production. Secretion of the enzyme was strongly inhibited by the presence of Cu^{2+}, Ni^{2+} and Hg^{2+} in the fermentation medium. The production of enzyme, under stationary conditions, was 10 fold higher than that under shaking conditions. The temperature range for production of enzyme was 37-55°C, with maximum enzyme activity (5.54 U ml^{-1}) at 45°C. Optimum pH and temperature of the crude enzyme were 5.0 and 60°C, respectively. *Humicola* α-galactosidase, when incubated at 60°C for 60 min retained its full activity. Enzyme activity was strongly inhibited by 5 mM concentration of Hg^{2+}. The K_m and V_{max} values for *p*NPG were 0.6 mM and 33.6 μmole min^{-1} mg^{-1} and that for raffinose were 10.52 mM and 1.82 μmole min^{-1} mg^{-1}, respectively.

2.2 INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is ubiquitous in distribution [1-4]. The enzyme catalyzes the hydrolysis of α-linked galactose moieties present in galacto-oligo and polysaccharides, especially those which occur in legume seeds [5,6]. The enzyme hydrolyzes galactosaccharides and liberates free sugar which may serve as a ready energy source [1]. α-Galactosidase has been used in the structural analysis of complex carbohydrates [7], to eliminate or reduce the flatus inducing factors from ingestion of edible beans [8] and reduce the raffinose content, which can approach 10% of beet sugar during processing [9]. Other industrial applications of α-galactosidase are, removal of raffinose and stachyose from soy products including soy milk and soy whey [10] as well as commercial hydrolysis of beet sugar molasses [11]. Several patents have been awarded for the production of α-galactosidase from mesophiles namely *Pseudomonas fluorescens* H-601 [12], *Candida guilliermondii* [13] and *Paecilomyces varioti* HS-1001 [14]. Process for the production of thermostable α-galactosidase from *Bacillus stearothermophilus* [15] by SmF has also been demonstrated.

Molds and yeasts have been examined for α-galactosidase activity, and enzyme characteristics have been documented for a number of fungal species [10,16-18].

Although there are several reports on α-galactosidase from mesophilic fungi [18-21], extracellular α-galactosidase has not been reported from thermophilic fungi. Among other thermophiles, only *Bacillus stearothermophilus* [22] is reported to produce an extracellularα-galactosidase.

A thermophilic fungus *Humicola* sp. (NCIM 1252) was found to produce large amount of extracellular α-galactosidase. In this chapter, studies on the optimization of fermentation parameters for growth and enzyme production by SmF has been described.

2.3 MATERIALS AND METHODS

MATERIALS

Chemicals

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG), *o*-nitrophenyl-α-D-galactopyranoside (*o*NPG), *p*-nitrophenyl-β-D-galactopyranoside, raffinose, stachyose and melibiose were purchased from Sigma Chemical Company, USA. Malt extract and yeast extract were from Difco Laboratories, Michigan, USA. Soluble starch was from BDH and all other chemicals used were of analytical grade.

Microorganism

The thermophilic fungus *Humicola* sp. (NCIM 1252) was isolated from soil samples around decaying plant material. It was maintained on yeast extract soluble starch (YpSs) and potato dextrose agar (PDA) slants and subcultured quarterly. YpSs medium contained (per litre of distilled water) : yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4 . 7H_2O$ 0.5 g; soluble starch, 15 g and agar 20 g. PDA medium contained (per litre of distilled water) : extract from 200 g potatoes; glucose, 20 g; yeast extract, 1 g and agar 20 g. The *Humicola* sp. is deposited with the National Collection of Industrial Microorganisms (NCIM), Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India, with accession number 1252.

MEDHODS

Basal medium

The basal medium contained (per litre of distilled water) : K_2HPO_4, 3 g; $MgSO_4.7H_2O$, 0.5 g; yeast extract, 5 g and wheat bran extract, 20 g. Wheat bran extract was prepared by steaming 100 g wheat bran in 1 litre distilled water for 30 min. The supernatant was collected by decantation, its volume adjusted to 2 liters and then sterilized by autoclaving at 121°C for 40 min. The pH of the basal medium was 6.0 and

was not further adjusted. The medium was inoculated with a piece of (1 x 1 cm) 7 day old sporulated culture from either PDA or YpSs slant. Cultivation was carried out in 250 ml or 500 ml Erlenmeyer flasks containing 50 ml or 100 ml of medium, respectively. The cultures were incubated at 45°C on a rotary shaker at 200 rpm or kept under static conditions. After the fermentation period the mycelium was removed by filtration through muslin cloth and then the clear filtrate was clarified by passing through Whatman No. 1 filter paper. The clear liquid was used for determining α-galactosidase activity.

Optimization of fermentation parameters

Optimization studies were carried out in 250 ml conical flasks using 50 ml of the basal medium as the control medium. The basal medium was inoculated and then incubated at 45°C as described above. The effect of various organic nitrogen sources, namely yeast extract, malt extract, cornsteep liquor, peptone, liver extract, beef extract, tryptone and casamino acid was studied by supplementing the basal medium with the respective nitrogen source at 0.5% (w/v) concentration. Similarly, the effect of various carbon sources was studied by supplementing the basal medium with various sugars (glucose, sucrose, xylose, starch, lactose, galactose, fructose, maltose, cellobiose, raffinose and melibiose, each at 2% (w/v) and wheat bran extract (2%, 5% and 7%). The effect of initial pH on growth and enzyme production was studied by adjusting the initial pH of the medium (beef extract 0.5% w/v, wheat bran extract 5%) from 4.0 to 9.0. The optimum temperature for the growth of the organism and production of enzyme was studied by carrying out the fermentation at various temperatures between 37-60°C. Similarly, the effect of different metal ions was studied by supplementing 1 mM concentration of each metal ion in the fermentation medium.

Time course

Time course for α-galactosidase production under shaking condition was carried out in the production medium by incubating the cultures at 45°C for 72 h. Aliquots were removed after every 12 h and assayed for α-galactosidase activity. In a similar way, time course for α-galactosidase production under static conditions was carried by incubating the cultures at 45°C for 12 days and estimating the enzyme activity every 2 days.

Enzyme production

A piece of (1 x 1 cm) 7 day old sporulated culture was transferred to 500 ml flasks containing 100 ml of fresh medium (5% wheat bran extract, 0.5% w/v beef extract, 0.3% w/v KH_2PO_4 and 0.05% w/v $MgSO_4.7H_2O$). The flasks were incubated at 45°C for 10

days under static conditions. Subsequently, the supernatant was collected by filtration through muslin cloth and Whatman filter paper No. 1. The enzyme in the filtrate was then concentrated by ammonium sulfate precipitation (95% saturation). The precipitate was collected by centrifugation, dissolved in minimum volume of 100 mM potassium phosphate buffer, pH 7.0, dialyzed extensively against 20 mM potassium phosphate buffer, pH 7.0 and used as the source of the enzyme.

Enzyme assays

α-Galactosidase

α-Galactosidase was assayed by incubating 100 μl of suitably diluted enzyme with 50 μl of 666 μM *p*NPG and 850 μl of 100 mM citrate phosphate buffer pH 5.0, at 50°C for 10 min. 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. When raffinose and stachyose were used as substrates, the reducing sugars liberated were measured at 520 nm as described by Nelson [23]. But when melibiose was used as a substrate, the reducing sugars liberated following its hydrolysis were measured at 540 nm as described by Miller [24]. One unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 μmole of product (*p*-nitrophenol or reducing sugar) / min under the standard assay conditions. All experiments were carried out in quadruplicate and the values reported are a mean of four such experiments in which 3-5% variability was observed.

Other enzyme activities

β-Galactosidase

β-Galactosidase was assayed by incubating 100 μl of suitably diluted enzyme with 50 μl of 666 μM *p*-nitrophenyl-β-D-galactopyranoside and 850 μl of 100 mM of citrate phosphate buffer pH 5.0 at 50°C for 10 min. 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. One unit (U) of the enzyme activity is defined as the amount of enzyme required to liberate 1 μmole of *p*-nitrophenol / min under the standard assay conditions.

Invertase

The total reaction mixture of 3 ml contained 1 ml of 0.5 M sucrose and 1 ml of acetate buffer (0.2 M, pH 4.5) preincubated at 50°C and 1 ml of appropriately diluted enzyme. The reaction was initiated by the addition of the enzyme, followed by incubation at 50°C for 15 min [25]. After the incubation period the reaction was

terminated by the addition of 1 ml DNSA reagent and heated in a boiling water bath for 5 min. After cooling, 10 ml of distilled water was added and colour was read at 540 nm [24]. One unit (U) of invertase activity is defined as the amount of enzyme required to liberate 1 μmole of reducing sugar / min under standard assay conditions.

Xylanase

The total reaction mixture of 1 ml contained 0.5 ml of suitably diluted enzyme in 50 mM sodium phosphate buffer, pH 6.0 and 0.5 ml of 2% (w/v) xylan solution. The reaction mixture was incubated at 50°C for 30 min and then the reaction was terminated by the addition of 1 ml 3,5-dinitrosalicylic acid (DNSA). The reducing sugars liberated were determined by measuring the absorbance at 540 nm as described above [24]. One unit (U) of xylanase activity is defined as the amount of enzyme required to liberate 1 μ mole of xylose equivalent / min under standard assay conditions.

Protease

The protease activity was determined according to Kunitz [26]. The reaction mixture contained an aliquot of suitably diluted enzyme solution and 2 ml 0.5% (w/v) casein in 100 mM citrate phosphate buffer, pH 5.0. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% (v/v) TCA. The precipitate formed was filtered through Whatman No. 1 filter paper and after standing for 30 min at room temperature, the absorbance of the filtrate was measured at 280 nm. One unit (U) of the protease activity is defined as the amount of enzyme required to bring about an increase of 1.0 absorbance unit / ml of reaction mixture / min under the standard assay conditions.

Amylase

Amylase activity was determined according to Bernfeld [27]. The reaction mixture contained 0.5 ml of 1% w/v soluble starch in 0.05 M acetate buffer pH 5.0 and 0.5 ml of suitably diluted enzyme. The mixture was incubated at 50°C for 15 min and the reducing sugars liberated were determined as glucose equivalents by the DNSA method [24]. One unit (U) of the enzyme activity is defined as the amount of enzyme required to liberate 1 μmole of reducing sugars / min under the standard assay conditions.

Determination of protein

Protein was estimated according to the method of Lowry *et al* [28] using BSA as standard. The blue colour developed after the addition of Folin phenol reagent was read at 660 nm.

Kinetic parameters

The kinetic parameters (K_m and V_{max}) were determined under standard assay conditions by using substrate concentrations in the range of 33.3-333.0 μM for *p*NPG and 0.5-10.0 mM for raffinose. The values were determined by Lineweaver Burk plots [29].

2.4 RESULTS

Fermentation parameters

A number of potential thermophilic isolates from soil samples were screened for the production of extracellular α-galactosidase in 2% wheat bran extract and 0.5% (w/v) yeast extract liquid medium. One of the isolate, identified as *Humicola* sp. (NCIM 1252) produced maximum levels of α-galactosidase and therefore selected for the present work.

β-Galactosidase, invertase, xylanase, protease and amylase activities in the crude broth were assayed as described in Methods. Only trace amounts of β-galactosidase activity was detected in the culture filtrate. More importantly, invertase was not detected in the crude broth.

Effect of carbon source

Effect of different carbon sources on the production of α-galactosidase in stationary culture is summarized in Table 1. All the carbon sources tested, supported the growth and enzyme production. However, maximum enzyme activity (3.47 U/ml) was observed when raffinose was used as the sole carbon source. Sucrose and maltose also favored cell growth and enzyme production. However, when lactose was used as the carbon source very low enzyme levels were observed. The enzyme production (2.59 U/ml) was also considerably high in a medium containing 5% wheat bran extract. Increase in wheat bran concentration (>5%) supported the growth but not enzyme production. Since 5% wheat bran extract gave fairly high activity and is an economical carbon source, it was selected for further fermentation studies.

Effect of nitrogen source

All the organic nitrogen sources (0.5% w/v) namely, yeast extract, malt extract, cornsteep liquor, peptone, liver extract, beef extract, tryptone and casamino acid

supported growth and enzyme production. Enzyme production was higher in a medium containing liver extract and yeast extract as the nitrogen source. However, maximum enzyme level (5.54 U/ml) was observed in presence of beef extract [Table 2].

Table 1 : Effect of various carbon sources on production of α-galactosidase

Carbon source (2% w/v)	Final pH	Enzyme activity ($U\ ml^{-1}$)
Glucose	6.8	0.78
Sucrose	7.1	1.46
Xylose	6.7	0.64
Starch	6.8	0.85
Lactose	6.7	0.51
Galactose	6.8	0.93
Fructose	6.7	0.88
Maltose	6.8	1.03
Cellobiose	6.6	0.68
Raffinose	7.8	3.47
Melibiose	6.8	0.75
Wheat bran extract		
2.0%	7.0	0.94
5.0%	7.4	2.59
7.0%	7.4	2.50

The *Humicola* sp. was grown without shaking at 45°C as described under Methods. Wheat bran in the medium was replaced by other carbon sources as listed above.

Table 2 : Effect of nitrogen sources on production of α-galactosidase

Nitrogen source (0.5% w/v)	Final pH	Enzyme activity ($U\ ml^{-1}$)
Yeast extract	7.8	3.93
Malt extract	7.6	2.80
Corn steep liquor	7.7	3.44
Peptone	7.1	2.33
Liver extract	7.8	4.57
Beef extract	7.8	5.54
Tryptone	7.2	2.61
Casamino acid	7.0	2.07

The *Humicola* sp. was grown in stationary culture at 45°C as described under Methods. Yeast extract in the medium was replaced by other nitrogen source as listed above.

Effect of initial pH

The effect of initial pH of the medium on *Humicola* sp. α-galactosidase production is shown in Figure 1. It was observed that *Humicola* sp. could grow and produce the enzyme over a wide range of pH 5.0-9.0. However, maximum enzyme levels were obtained when the initial pH of the medium was adjusted to 6.0. The pH of the medium increased upto 7.8 during growth.

Effect of temperature

Effect of temperature on the production of α-galactosidase under stationary and shaking conditions is shown in Figure 2. At 37°C, α-galactosidase activity under shaking and stationary condition was 0.38 and 2.6 U/ml, respectively. Enzyme secretion was maximum at 45°C with or without shaking. A decrease in the enzyme activity was detected above 50°C in shake flask cultures and at 60°C the fungus did not grow.

Effect of metal ions

The effect of different metal ions (1 mM) on production of α-galactosidase was examined. Presence of Zn^{2+} in the fermentation medium had a marginal stimulatory effect on the production of the enzyme whereas Cu^{2+}, Co^{2+}, Ni^{2+} and Hg^{2+} were inhibitory [Table 3].

Table 3 : Effect of metal ions on production of α-galactosidase

Metal ion (1 mM)	Relative activity (%)
Mg^{2+} (control)*	100
Cu^{2+}	6
Ca^{2+}	20
Zn^{2+}	118
Ni^{2+}	4
Mg^{2+}	100
Hg^{2+}	3
Co^{2+}	10
Mn^{2+}	26

**Humicola* sp. was grown in the basal medium in presence of Mg^{2+}. Mg^{2+} in the medium was replaced by the above listed metal ions at 1 mM concentration and the fermentation was carried out at 45°C in stationary cultures as described under Methods.

Time course

The time course of α-galactosidase production in a medium containing 5% (w/v) beef extract in stationary and shaken cultures is shown in Figure 3a and 3b, respectively. Under both the conditions, enzyme production was growth associated. In stationary cultures, the maximum enzyme activity, 5.3 U ml^{-1} (2.34 U mg^{-1} protein) was obtained on the 10th day of fermentation while in shaken culture, the maximum enzyme activity was 0.53 U ml^{-1} (0.74 U mg^{-1} protein) after 48 h. Increase in biomass was rapid in shaken culture compared to stationary culture. This may be due to increased oxygen availability to the fungus in shake flask conditions.

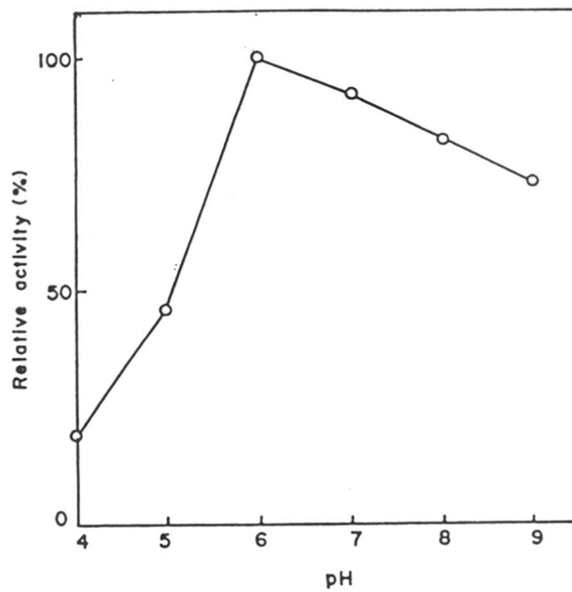

Figure 1 : α-Galactosidase production by *Humicola* sp. at different initial pHs. pH was adjusted with 0.1 N NaOH or 0.1 M HCl.

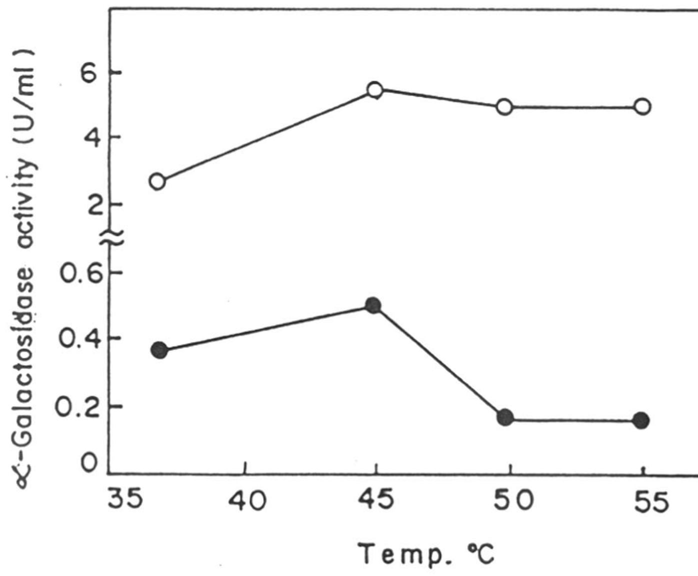

Figure 2 : Effect of temperature on the production of α-galactosidase in stationary (o) and shaken (•) cultures.

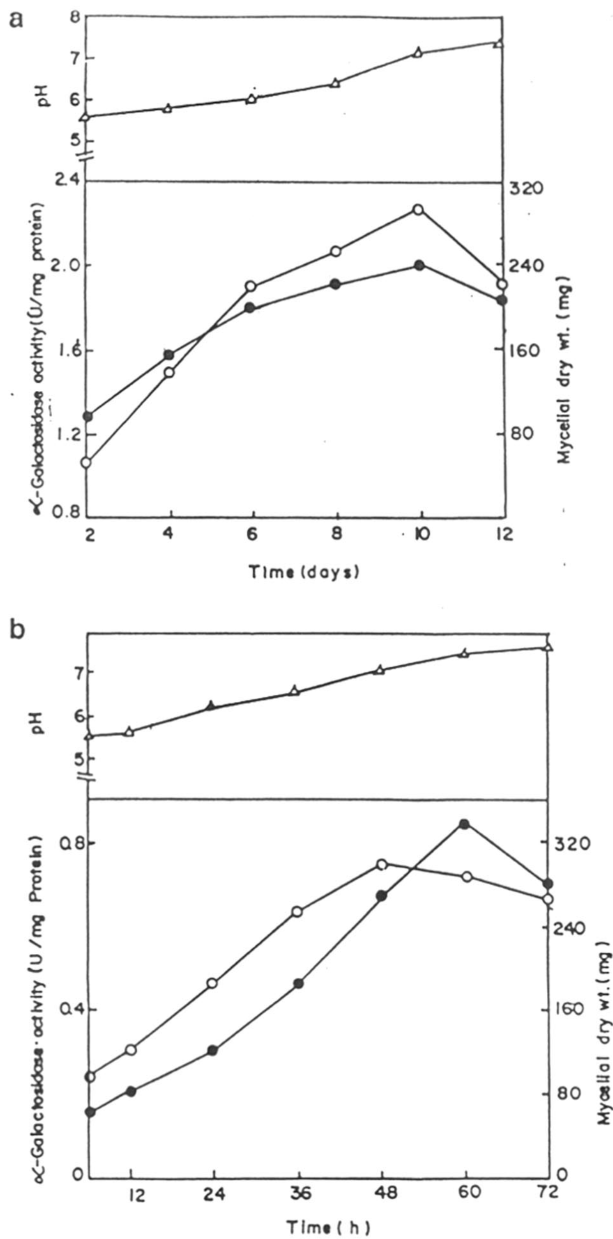

Figure 3 : Time course of α-galactosidase production by *Humicola* sp. using 5% wheat bran extract medium. (a) Stationary cultures : specific activity (o), pH (Δ) and biomass (•); **(b) Shaken cultures** : specific activity (o), pH (Δ) and biomass (•

Properties of the crude enzyme

Effect of pH on enzyme activity and stability

As shown in Figure 4, the optimum pH of the crude enzyme was 5.0 and no activity was detected at pH 3.0. However, 75% and 55% of the maximum activity was found at pH 3.5 and 7.0, respectively. Though the enzyme was stable only in the pH range 4.0-5.0, it retained more than 85% of its maximum activity between pH 3-7. Only 12% inactivation was observed at pH 3.0 and 7.0 when enzyme was incubated at 4°C for 16 h at the respective pH [Figure 4].

Effect of temperature on enzyme activity and stability

Effect of temperature on the activity and stability of the enzyme is shown in Figure 5. The optimum temperature for enzyme activity was 60°C. At 50°C, only 50% enzyme activity was observed and at 70°C, no activity was detected. The crude enzyme was stable at 60°C for 1 h and only 15% of its original activity was lost when incubated at 65°C for 1 h. The enzyme was rapidly inactivated above 65°C.

Effect of metal ions on enzyme activity

The effect of metal ions on the activity of α-galactosidase is given in Table 4. Mg^{2+} had no apparent effect on activity. The enzyme was strongly inhibited by Hg^{2+}. However, in presence of Mn^{2+} and Cu^{2+} the enzyme showed 21% and 50% of its maximum activity, respectively. No significant inhibition was observed in presence of other metal ions.

Kinetic parameters

The enzyme hydrolyzed raffinose in addition to *p*NPG. The K_m and V_{max} values of *Humicola* α-galactosidase for *p*NPG and raffinose were 0.6 and 10.52 mM and 33.6 and 1.82 μmole min^{-1} mg^{-1}, respectively.

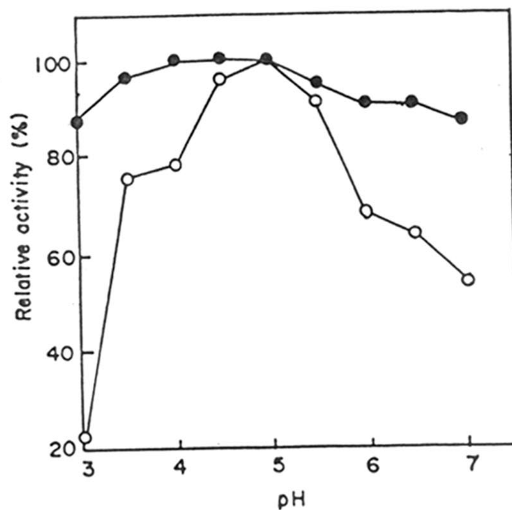

Figure 4 : Effect of pH on α-galactosidase activity (o) and stability (•).

For optimum pH, 100 μl of crude α-galactosidase was incubated at 50°C for 10 min in the pH range 3.0-7.0 by using 100 mM citrate phosphate (2.6-7.0) and potassium phosphate (5.7-8.0) buffers.

For pH stability, 100 μl of the enzyme solution was kept at the desired pH at 4°C for 16 h and then the residual activity was measured under standard assay conditions.

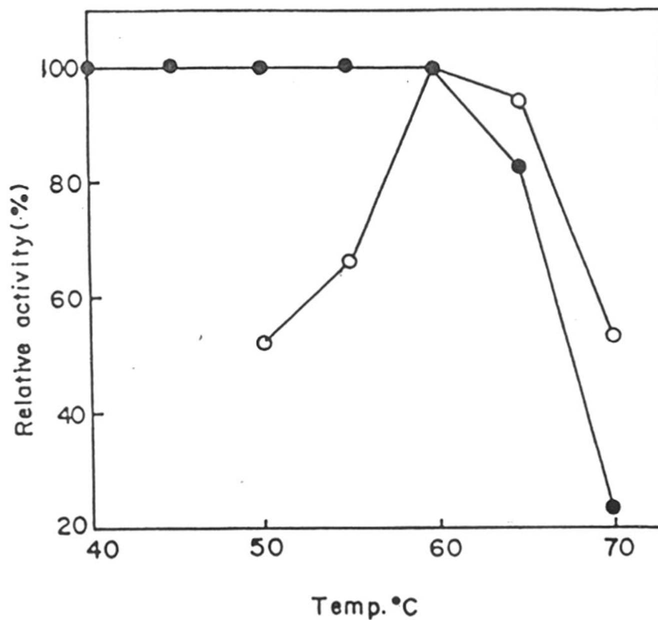

Figure 5: Effect of temperature on α-galactosidase activity (O) and stability (•).

For optimum temperature, 100 μl of the crude enzyme in 100 mM citrate phosphate buffer pH 5.0, was incubated at 50°C for 10 min at various temperature (40-70 °C) in water bath.

For thermal stability, 1 ml of the enzyme solution was incubated at the temperature indicated for 1 h, then cooled immediately in ice bath and residual activity was determined under standard assay conditions.

Table 4 : Effect of metal ions on α-galactosidase activity

Metal ion (5 mM)	Relative activity (%)
Control	100
Hg^{2+}	9
Mn^{2+}	21
Ni^{2+}	82
Cu^{2+}	50
Ca^{2+}	84
Zn^{2+}	90
Co^{2+}	72
Mg^{2+}	100

The crude enzyme (100 μl) was incubated in 100 mM citrate phosphate buffer pH 5.0 and 50°C for 10 min in presence of metal ions and the residual activity determined under standard assay conditions. The results are expressed as a percentage of control.

2.5 DISCUSSION

α-Galactosidase is an inducible enzyme [19,21,30-32] and hence sugars which contain α-galactosyl residues are known to induce the enzyme to a varying extent. The enzyme from *Humicola* was induced in presence of glucose, sucrose, xylose, starch, lactose, galactose, fructose, maltose, cellobiose, raffinose, melibiose and wheat bran. Among the α-galactosidically linked oligosaccharides, such as raffinose and melibiose and monosaccharide galactose, only raffinose was the most effective inducer of α-galactosidase in *Humicola* sp. Among the other inducers, lactose with the β-1,4 configuration of its glycosidic bond also induced the enzyme formation in *Humicola* sp. The disaccharide sucrose induced enzyme production but to a lesser extent as compared to raffinose. Maximum activity of α-galactosidase from *Aspergillus fumigatus* [33] was after 6 h of growth, detected in a medium containing sucrose and raffinose as the inducers. Similarly, α-galactosidase production from *Monascus anka* [34] was enhanced in presence of raffinose. In addition, lactose the β-linked disaccharide, an inducer of β-galactosidase, is also reported to induce α-galactosidase production in many microorganisms [21,35,36]. There are reports of induced synthesis of the enzyme in a media containing galactose, melibiose and raffinose at concentrations 0.4-1% (w/v) [16,30-32,36-38]. The induction of α-galactosidase by different sugars in the cells of

Saccharomyces carlsbergenesis decreased in the following order : stachyose > raffinose > melibiose > galactose > lactose [39].

Humicola sp. produced α-galactosidase constitutively. Wheat bran extract (5%) gave fairly high activity (2.59 U/ml) when used in combination with 0.5% (w/v) yeast extract as the nitrogen source. But, when yeast extract was replaced by 0.5% (w/v) beef extract, the enzyme activity increased more than two fold (5.54 U/ml). Inorganic nitrogen supplements like ammonium nitrate did not support growth and enzyme production. A very high yield of α-galactosidase within the mycelium of *Circenella muscae* in presence of peptone has been reported [38]. The use of ammonium nitrate at 0.5% (w/v) concentration or a mixture of 0.3% (w/v) peptone and meat extract resulted in optimum enzyme production in *Mortierella vinacea* [40,41].

The thermophilic fungus, *Humicola* sp. grew over a wide range of pH (5-9), with an optimum pH of 6.0. Studies on the effect of initial pH on three different media in the pH range 5.0 to 7.0 for α-galactosidase production by *Monascus anka* [34] indicated that the maximum enzyme was produced when the initial pH of the medium was 6.0. Similarly, the fermentation for α-galactosidase production from other microorganisms carried out in the pH range 4.5-8.0 have also been reported [30,42,43]. In all the above reports, the pH of the fermentation medium was not controlled in most of the cases. However, in case of α-galactosidase production from *Streptomyces olivaceus*, changes in pH were minimized by using phosphate buffer [43].

The optimum temperature for growth and enzyme production of *Humicola* was 45°C. Optimum temperature for the production of α-galactosidase was 30°C in case of *Mortierella vinacea* [40], *Circenella muscae* [38], *Monascus pilosus* [42] and *Trichoderma reesei* [21] and 37°C and above in the case of *Bacillus stearothermophilus* [22,30], *Lactobacillus fermenti* [44] and *Penicillium dupontii* [45]. The production of α-galactosidase from *Humicola* sp. was enhanced by the addition of Zn^{2+} and Mg^{2+} in the fermentation medium. Such kind of stimulatory effect on α-galactosidase production by Zn^{2+} and Mg^{2+} has been demonstrated in case of *Aspergillus tamarii* [31,32]. However, metal ions like Ca^{2+}, Co^{2+}, Ni^{2+} and Hg^{2+} have been shown to inhibit enzyme production in *Monascus* sp. [41].

Isolation of α-galactosidases from mesophilic bacteria, yeast and fungi have been well documented. Among thermophilic fungi, there is only a single report of an intracellular α-galactosidase from *Penicillium dupontii* [45]. However, no reports exist on extracellular α-galactosidases from thermophilic fungi. Our results indicate that the newly isolated thermophilic fungus, identified as *Humicola* sp. (NCIM 1252), produces higher levels of extracellular α-galactosidase (5.54 U ml^{-1} when 33.3 μM *p*NPG is used

as the substrate or 0.389 U ml^{-1} when 25 mM raffinose is used as the substrate) compared to mesophilic fungi namely *Trichoderma reesei* (specific activity 0.9 U mg^{-1}) [21], *Aspergillus nidulans* (specific activity 0.1 U mg^{-1}) [46]. Moreover, extract from a cheap agriculture residue like wheat bran can be used instead of raffinose, melibiose and stachyose as a carbon source for enzyme production. The crude enzyme from *Humicola* sp. had a pH optima of 5.0 but it retained 50% of its maximum activity at pH 7. Similar observations were reported for the enzyme from *Aspergillus nidulans* [46]. However, the enzyme from *Humicola* is highly thermostable compared to the enzyme from *Trichoderma reesei* RUT C-30 [21] and *Aspergillus nidulans* [46] which were inactivated above 40°C. The α-galactosidase from *Humicola* sp. had a temperature optimum of 60°C and was completely stable at 60°C for 1 h. It also retained 90% and 50% of its maximum activity at 65°C and 70°C for 1 h, respectively. Temperature optima of 60°C has also been reported for enzymes from *Aspergillus ficcum* NRRL 3135 [19], *Aspergillus oryzae* [47] and *Trichoderma reesei* RUT C-30 [21].

In summary, *Humicola* sp. produces high levels of invertase free extracellular α-galactosidase, stable at 60°C for 1 h and therefore, it has the potential for commercial exploitation.

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CHAPTER - III

Increased production of α-galactosidase from *Humicola* sp. by solid state fermentation (SSF) and its application in soymilk hydrolysis

1.1 SUMMARY

Humicola sp. when grown on various agricultural residues in SSF, produced α-galactosidase. Maximum enzyme activity (44.6 U/g) was obtained at 45°C when soy flour was used as the carbon source and the moisture content in the substrate was 86%. At 95% humidity, maximum amount of α-galactosidase was secreted irrespective of the substrate used for the enzyme production. The crude enzyme could hydrolyze raffinose and stachyose in soymilk. Scanning electron microscopic (SEM) studies showed high density growth of fungal mycelia on wheat bran particles.

3.2 INTRODUCTION

Industrially important enzymes have traditionally been obtained by SmF because of easy handling and greater control of environmental factors such as temperature and pH. SSF constitutes an interesting alternative since metabolites so obtained are more concentrated and purification procedures are less expensive. Some of the advantages of SSF over conventional SmF for work involving fungi are, simplicity of equipment and low moisture content which prevents bacterial contamination [1-3]. In addition, less solvent is needed to extract the enzymes, which greatly reduces energy requirements and pollution problems [4,5]. The high concentrations of the enzyme in the liquid phase, in SSF, permits enzyme recovery at considerably lower energy inputs [6,7]. Other advantages of SSF technique are, a simplified procedure for inoculum development, easier scale up of process and reduced solvent requirement for product recovery.

There has been considerable interest to produce α-galactosidase by SSF processes [8-17]. The first report on α-galactosidase production from *Mortierella vinacea* by koji method was reported in 1969 by Suzuki *et al* [8]. It was observed that α-galactosidase production was maximum when soybean koji was used. The organisms which are reported to produce α-galactosidase in SSF are *Aspergillus awamori* [10-13], *Aspergillus niger* [9] and *Aspergillus oryzae* [14]. However, no data is available on the effect of media parameters on enzyme production. Different solid substrates such as wheat bran [8-11,14,15], soybean [8,14] and rice bran [8] have been used for the production of α-galactosidase in SSF. Although, soybean is reported to be the best solid substrate for α-galactosidase production, wheat bran is preferred as it is highly cost intensive. The

added advantage of using wheat bran is that, it provides larger surface area for the growth of microorganisms [18].

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) has a number of food processing and medical applications. The enzyme is very important in the processing of beet sugar, where it is used to remove raffinose which inhibits normal crystallization of sucrose [19]. One of the potential applications of α-galactosidase is in the hydrolysis of galacto-oligosaccharides such as raffinose and stachyose in soymilk. The potential of soymilk as a substitute for cow's or human milk has been emphasized over the years especially for infants and children who have low levels of lactase in their intestine. Moreover, soymilk can be used as an alternative economical protein beverage to cow's milk as it has low fibre content and the nutritional value of soymilk is comparable to that of cow's milk [20]. The high protein nature of the soymilk can help in overcoming the wide spread protein deficiencies [21].

One of the problems associated with the consumption of soymilk is its tendency to induce flatulence, which is accompanied by an uncomfortable feeling of fullness and intestinal activity [21-23]. It has been suggested that flatulence is caused by microbial fermentation of oligosaccharides such as raffinose and stachyose, which are not digested by humans as they do not have α-galactosidase in their digestive tract. α-Galactosidases from microbial sources have been used for soymilk processing [11,21,24,25].

In this chapter, the enhanced production of α-galactosidase by SSF, and a preliminary study on the effect of pH and temperature on the hydrolysis of soymilk using a partially purified (ammonium sulfate precipitated) α-galactosidase from *Humicola* sp. has been described.

3.3 MATERIALS AND METHODS

MATERIALS

Microorganism

Growth and maintenance of *Humicola* sp. (NCIM 1252) was carried out as described in Chapter II.

Substrates for SSF

Substrates with various particle sizes, used for SSF are as follows : wheat bran (1300 μm), coconut cake (1186 μm), groundnut cake (1073 μm), soy residue (1028 μm) and soyflour (395 μm). They were obtained locally.

METHODS

SSF technique

For solid state culture, 10 g substrate in 250 ml Erlenmeyer flasks was moistened with 20 ml tap water (moisture content 86%) and sterilized by autoclaving at 121°C for 40 min. The flasks were inoculated with 1 x 1 cm pieces of seven day old sporulated culture from a PDA slant or a 1% inoculum (w/v) prepared by suspending spores from PDA slants in sterile distilled water. The contents of each flask were mixed thoroughly with a sterile inoculating needle before and after inoculation for uniform distribution of fungal spores in the medium. The flasks were incubated at 45°C in a humidity control incubator (Indian Equipment Company) and were removed periodically and the contents mixed by gentle tapping. Humidity was monitored by humidity measurement meter (Casella, London). The content of the flasks were harvested and assayed for α-galactosidase activity every 24 h for a period of 10 days. Each experiment was done in triplicate and results in each Table are mean value of three such experiments.

In case of shake flask experiments, cultivation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium and incubated, at 45°C, on a rotary shaker at 200 rpm.

The optimization of fermentation time and substrate : water ratio was carried out by using commercial wheat bran as the solid substrate.

Effect of various surfactants on enzyme production was studied by adding Tween-80, Triton X-100 and SDS at concentrations ranging from 0.1-1.0% (w/v).

For tray fermentation, enamel coated metallic trays (28 x 24 x 4 cm) containing 50 and 100 g of wheat bran and trays (45 x 30 x 4 cm) containing 250 and 500 g of wheat bran were moistened with tap water (ratio 1 : 2 w/v). Trays were covered with aluminum foil and autoclaved at 121°C for 40 min and inoculated with 10 % of seven day old inoculum (w/w) prepared in 250 ml Erlenmeyer flasks containing 10 g wheat bran and 20 ml tap water. The contents of the trays were mixed with a sterile stab needle before and after inoculation and were incubated in a humidity controlled incubator at 45°C.

Enzyme extraction

After six days of incubation, flasks were removed and enzyme was extracted by shaking with 50 ml of citrate phosphate buffer (20 mM pH 5.0) for 2 h at 45°C as described by Ebune *et al* [26]. Solids were separated by filtration through cheesecloth and the filtrate was subjected to centrifugation at 10,000 rpm for 20 min. The clear supernatant was used for α-galactosidase activity measurements.

In case of tray fermentation, the enzyme from fermented bran was extracted with 20 mM citrate phosphate buffer, pH 5.0 (ratio of fermented bran to buffer was 1 : 5 w/v).

Enzyme assay

This was carried out as described in Chapter II. The *p*-nitrophenol liberated following the hydrolysis of *p*NPG at pH 5.0 and 50°C was measured spectrophotometrically, at 405 nm. One unit (U) is defined as the amount of enzyme that liberates 1 μmole of *p*-nitrophenol / min under the standard assay conditions.

pH measurement

Liquid pH measurements were carried out with a standard pH meter (Global, Hyderabad, India). pH measurements of fermented solids were made by suspending 10 g biomass in 50 ml distilled water in a conical flask. The flasks were kept under mild agitation for 30 min at 30°C, solids were separated by centrifugation (3000 rpm, 10 min) and the pH of the filtrates were measured.

Moisture content

The influence of initial moisture level on enzyme production was evaluated by varying the ratio (w/v) of wheat bran to tap water (1 : 0.5, 1 : 1, 1 : 2, 1 : 3, 1 : 4 and 1 : 5). The percentage moisture in solid substrate was determined gravimetrically by placing the samples in an oven at 105°C for 24 h till constant weight.

SEM

The growth and morphology of *Humicola* sp. during SSF was studied by SEM. Samples for SEM were fixed with ultra-violet light and were mounted on brass stubs. Specimens were then coated with a thin layer of gold (100Å) 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 high resolution unit.

Preparation of soymilk

Soybean seeds were purchased from the local market and soymilk was prepared according to Mulimani and Ramlingam [27]. Twenty five grams of dry seeds were boiled in 400 ml of distilled water for a period of 60 min and cooled to room temperature. The boiled seeds were ground in a mortar with pestle for 15 min with addition of 125 ml of

distilled water and the mixture was then filtered through four layers of cheese cloth. The filtrate was centrifuged at 7,000 rpm for 20 min and the supernatant was used as soymilk.

Effect of pH on the hydrolysis of galacto-oligosaccharides in soymilk

The pH of the freshly prepared soymilk was adjusted to different pH values in the range of 4.0-7.0. To soymilk sample (2 ml), crude α-galactosidase (2 units) was added and incubated at 50°C for 2 h. The reaction was stopped by keeping the tubes in boiling water bath for 5 minutes. The liberated galactose was estimated according to Nelson [28].

Effect of temperature on the hydrolysis of galacto-oligosaccharides in soymilk

To 2 ml soymilk, α-galactosidase (2 units) was added and incubated at different temperatures (40-70°C) for 2 h. The reaction was stopped by keeping the tubes in a boiling water bath for 5 min. The liberated galactose was estimated according to Nelson [28].

Enzymatic hydrolysis of galacto-oligosaccharides in soymilk

The method of Mulimani and Ramlingam [27] was adopted to study the extent of hydrolysis of raffinose and stachyose present in soymilk using partially purified (ammonium sulfate precipitated) α-galactosidase. Ten ml of soymilk and 10 units of α-galactosidase were incubated for 3 h at different temperatures. Periodically, 2 ml aliquots were removed and the reaction was terminated by adding 0.2 ml of 0.3 M barium hydroxide and 0.2 ml of 0.18 M zinc sulfate. The precipitated proteins were removed by centrifugation and the galactose liberated was estimated as reducing sugar according to Nelson [28].

Isolation of galacto-oligosaccharides from soymilk

Twenty five grams of dry soy bean seeds were boiled in 400 ml of distilled water for a period of 60 min and cooled to room temperature. Twenty five ml of the boiled reaction mixture was poured into 60 ml of absolute ethanol to partially precipitate the soy proteins. Subsequently, 0.25 ml of barium hydroxide and 0.25 ml of zinc sulfate were added to this mixture to precipitate the proteins completely. This mixture was then filtered using Whatman No. 1 filter paper and the filtrate was extracted with 40 ml of chloroform. The aqueous phase was concentrated to 2.5 ml under vacuum and subjected to TLC.

TLC of hydrolyzed soymilk

TLC was performed on glass plates (5 x 18 cm) coated with 0.2 mm layer of silica gel G. Plates were incubated at 110°C for 1 h before use. The mobile phase consisted of n-propanol : ethyl acetate : water (6 : 1 : 3, v/v) [29]. The TLC plates were developed in an ascending direction. After the run, the plates were dried and the carbohydrates were identified by spraying with 1% (w/v) α-naphthol in absolute alcohol containing 10% (v/v) orthophosphoric acid, according to the method of Albon and Gross [30]. The standard sugars used were sucrose, raffinose and stachyose.

3.4 RESULTS

Choice of the substrate

α-Galactosidase was produced by *Humicola* sp. (NCIM 1252) on a wide range of solid substrates. The highest enzyme activity (44.6 U/g) was obtained with soy flour [Table 1]. Tap water used for moistening solid substrates promoted the enzyme titre. Supplementation of soy flour with wheat bran, coconut cake and groundnut cake (7 : 3, 5 : 5 w/w) had no beneficial effect [Table 1]. On the contrary, in case of wheat bran and soyflour (7 : 3 w/w), an increase in the enzyme yield was noticed. A comparison of the enzymatic levels by solid state, semi-solid state and submerged fermentation conditions is summarized in Table 2.

Table 1 : Effect of different substrates on α-galactosidase production by *Humicola* sp.

Carbon source (10 g each)		Final pH	Enzyme activity (U/g)
Soyflour		8.5	44.6
Wheat bran (W.B.)		8.2	22.8
W.B. + Soyflour	(7 : 3, w/w)	8.2	34.5
	(5 : 5, w/w)	7.3	20.1
Coconut cake (C.C.)		8.4	41.5
C. C. + Soyflour	(7 : 3, w/w)	7.4	31.8
	(5 : 5, w/w)	7.4	38.8
Groundnut cake (G.C.)		8.2	34.5
G. C. + Soyflour	(7 : 3, w/w)	7.3	15.2
	(5 : 5, w/w)	7.3	20.1

The fungus was grown at 45°C as described under Methods. Wheat bran in the medium was replaced by other substrates as listed above.

Table 2 : Effect of culture conditions for production of α- galactosidase

Substrate	Stationary (Activity U/g)		Shaking (Activity U/g)	
	Solid-state	Semi-solid state	Semi-solid state	Submerged
Coconut cake	40.9	19.4	17.2	12.6
Wheat bran	25.2	16.1	12.5	7.2
Groundnut cake	30.4	14.9	14.5	10.0
Soyflour	44.6	31.8	30.7	13.9
Soyresidue	14.7	7.5	6.1	4.9

The fungus was grown as described under Methods. Wheat bran in the medium was replaced by other substrates as listed. Water : substrate ratio for solid-state, semi-solid and SmF was 2, 5 and 10, respectively.

Effect of humidity on α-galactosidase production

The effect of humidity on production of α-galactosidase is shown in Table 3. It was observed that, at 95% humidity maximum enzyme was secreted irrespective of the substrate used.

Table 3 : Effect of humidity on production of α-galactosidase

Substrate (10 g each)	Humidity (Activity U/g)			
	0%	50%	75%	95%
Wheat bran	10.5	13.9	17.9	22.8
Coconut cake	14.8	18.9	27.8	41.5
Groundnut cake	12.4	18.5	25.9	34.5
Soyflour	14.9	18.4	29.6	44.6

The fungus was grown at 45°C as described under Methods. Wheat bran in the medium was replaced by other substrates as listed.

Effect of initial moisture content of the substrate

The effect of initial moisture content of the substrate on enzyme yield is shown in Figure 1. Initial moisture content of wheat bran medium on the production of α-galactosidase indicated an increase in enzyme titres with an increase in moisture content upto 86% after 6 days of fermentation. The enzyme production at 65% and 95% initial moisture content was considerably low.

Time course of α-galactosidase production in SSF

Time course of α-galactosidase production, using various substrates is shown in Figure 2. It was observed that maximum enzyme activity was found on the 6th day of fermentation in all the substrates used. Enzyme production increased with increase in time upto 6 days and then decreased on 8th and 10th day. Among all the substrates used, soyflour gave maximum enzyme yield of 44.6 U/g [Figure 2]. This could be due to higher content of proteins, carbohydrates and oil in soyflour. Coconut cake and groundnut cake yielded lower levels of the enzyme activity (37 and 30 U/g, respectively)

Tray fermentation

When SSF was carried out in trays of various sizes containing 50, 100, 250 and 500 g wheat bran moistened with appropriate amounts of tap water, the time course of enzyme production was found to be similar to that in 250 ml Erlenmeyer flasks containing 10 g of wheat bran. It can be seen from Figure 3, that there was no substantial decrease in the enzyme production in a tray containing 500 g of wheat bran.

SEM studies

SEM was carried out to study the nature of growth of *Humicola* sp. on wheat bran. The cell structure of uninoculated wheat bran is shown in Figure 4a. It was observed that, the growth of the fungus started after 24 h. The mycelium grew, spreading over the surface of the solid substrate and the substrate was completely covered with fungal mycelium on the 3rd day [Figure 4b]. On the 6th day, the solid substrate was totally invisible with the fungal mycelium forming a thick mat and penetrating the solid substrate. The high density growth of mycelia or spore formation on the surface of solid substrate was more evident on the 6th day [Figure 4c].

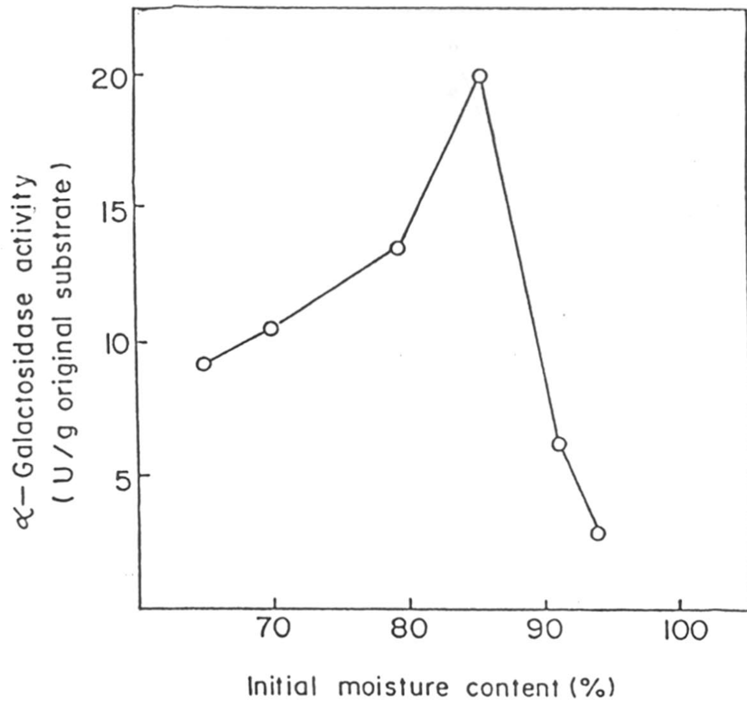

Figure 1 : Effect of initial moisture content on α-galactosidase production at 45°C as described in Methods.

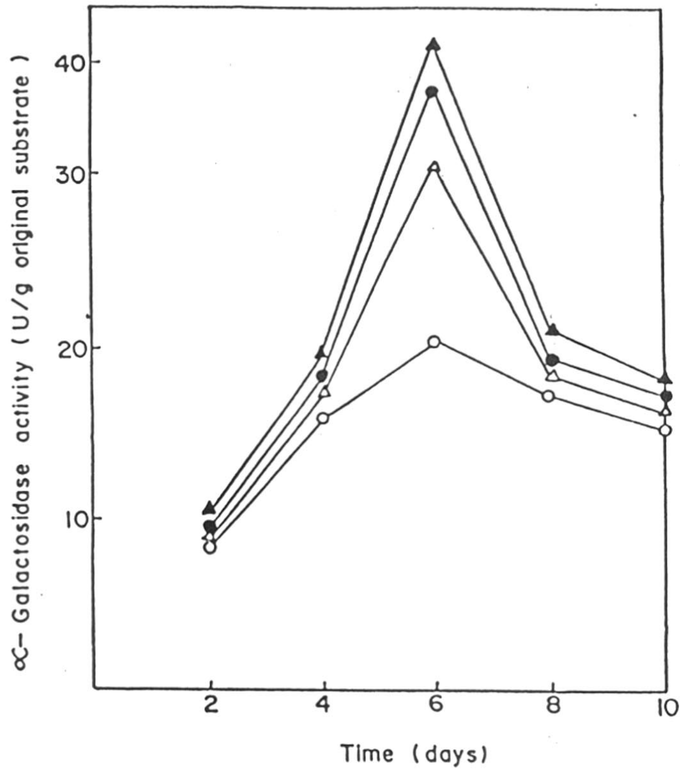

Figure 2 : Production of α-galactosidase on different substrates by *Humicola* sp. at 45°C.

Wheat bran (o); Groundnut cake (Δ); Coconut cake (•); and Soyflour (▲).

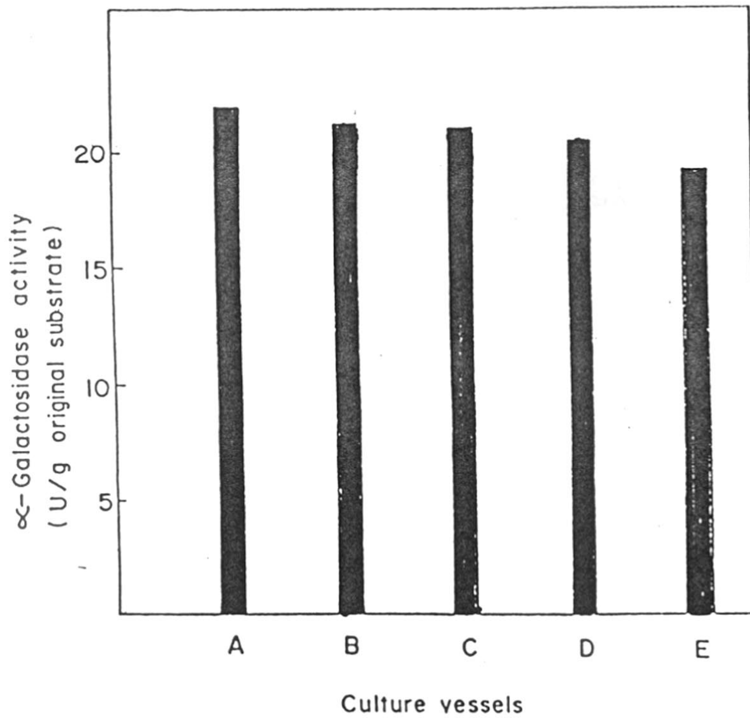

Figure 3 : Cultivation of *Humicola* sp. in trays :

(A) Flask (250 ml) (10 g substrate)

(B) Tray (28 x 24 x 4 cm) (50 g substrate)

(C) Tray (28 x 24 x 4 cm) (100 g substrate)

(D) Tray (45 x 30 x 4 cm) (250 g substrate)

(E) Tray (45 x 30 x 4 cm) (500 g substrate)

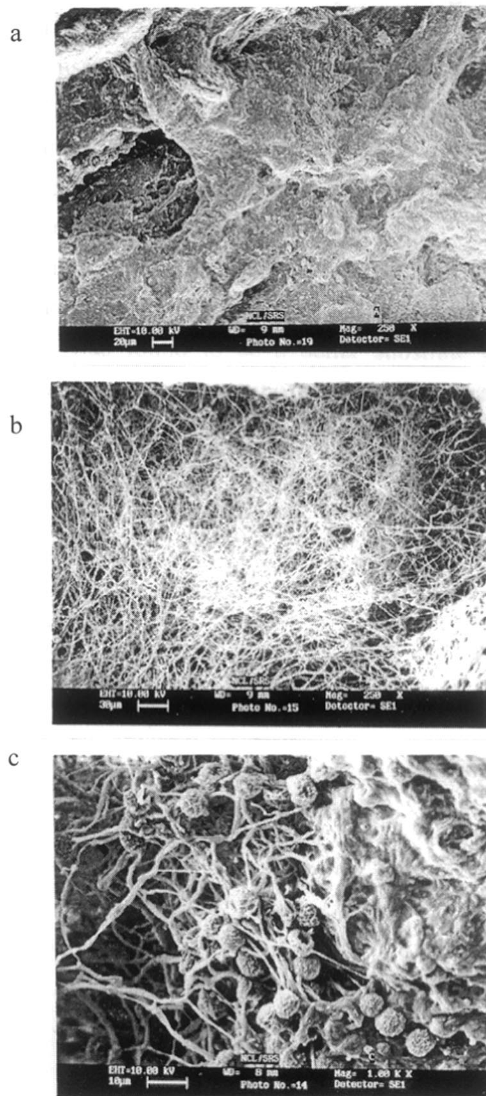

Figure 4 : Scanning electron micrographs of wheat bran degraded for different periods by *Humicola* sp. during SSF.

(a) Control (uninoculated)

(b) After 3 days of fermentation

(c) After 6 days of fermentation.

Application of α-galactosidase in soymilk processing

The potential use of *Humicola* sp. α-galactosidase in soymilk processing was evaluated. Maximum hydrolysis of galacto-oligosaccharides in soymilk was observed at pH 5.0 and 50°C [Figure 5]. The relative hydrolysis at 40°C and 50°C was 70% and 65%, respectively. Therefore, soymilk hydrolysis was carried out at 50°C even though optimum temperature of this enzyme is 60°C.

Qualitative analysis of the products liberated by TLC indicated that the *Humicola* sp. α-galactosidase could completely hydrolyze stachyose and raffinose present in soymilk. Stachyose was found to be a better substrate (with complete hydrolysis occurring within 1 h) as compared to raffinose (~2 h for complete hydrolysis) [Figure 6].

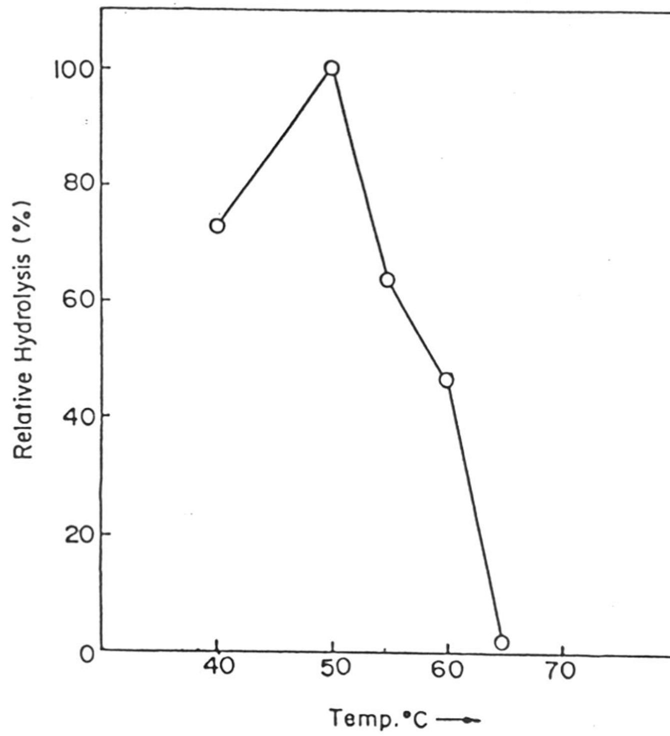

Figure 5 : Effect of temperature on soymilk hydrolysis

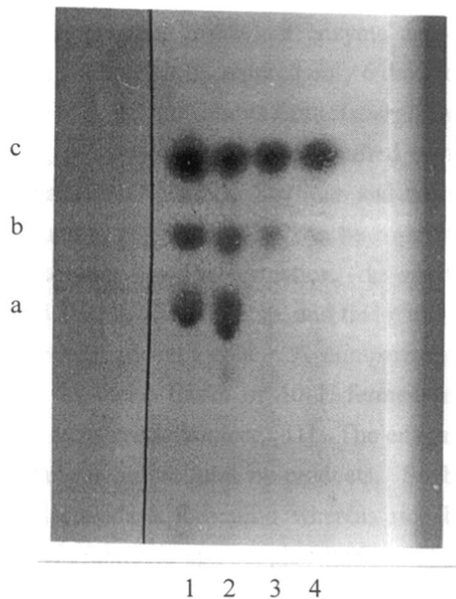

Figure 6 : TLC analysis of the hydrolytic products of soybean milk (for details refer to Methods).

(1) Standard sugars : (a) Stachyose (b) Raffinose (c) Sucrose

(2) Control (without enzyme treatment)

(3) 1 h incubation with enzyme

(4) 2 h incubation with enzyme.

3.5 DISCUSSION

The amount of α-galactosidase produced by *Humicola* sp. on different solid substrates, was found to be much higher than the amount of enzyme produced by the same fungus under stationary submerged and semi-solid fermentation conditions in a medium containing 5% wheat bran extract and 0.5% beef extract [Chapter II]. Moreover, the time required to produce maximum enzyme activity under stationary submerged culture was 10 days whereas, SSF required only 6 days to give maximum enzyme activity (44.6 U/g). In case of α-galactosidases from *Aspergillus niger*, *Aspergillus awamori* and *Aspergillus oryzae*, the fermentation time required to achieve maximum enzyme titres ranged from 4-7 days [9-11,14,15]. Soyflour and coconut cake were effective for α-galactosidase production [Table 1]. This can be correlated to higher raffinose content in soyflour leading to more enzyme formation. In addition, coconut cake was a better substrate for sporulation of *Humicola* sp. and this may be due to better oxygen diffusion into the voids between coconut cake. A comparison of solid substrate culture with submerged culture in shake flasks or 10 L fermentor has been made in case of α-galactosidase from *Mortierella vinacea* [31]. The enzyme formation by koji method was compared using various agricultural by-products. Soybean and cotton seed cake were effective for α-galactosidase formation whereas rice bran and wheat bran were less effective. In case of *Humicola* sp., supplementation of soyflour to other solid substrates did not enhance enzyme production [Table 1], although there are reports where the supplementation of carbon and nitrogen source, enhances α-galactosidase production [14,15]. Cruz and Park [14] reported that, the synthesis of α-galactosidase from *Aspergillus oryzae* was induced by the addition of soybean carbohydrates to wheat bran and it was also noted that the productivity of enzyme by SSF is greater than SmF. Higher yields of α-galactosidase from *Aspergillus oryzae* [15] were obtained by adding glucose (73% increase), sucrose (39% increase), or melibiose (39% increase) to the bran, but raffinose and stachyose had little or no effect. Enzyme yield increased three times when soyflour or soybean was used as substrate but no enzyme production was observed using rice. Enzyme production on soybean decreased with increased initial moisture levels and with decreased particle size. The secretion of α-galactosidase from *Taloromyces emersonii* [16] and *Penicillium capsulatum* [16] grown in SSF, in a medium containing xylan, has also been reported.

For the extraction of α-galactosidase from plant sources, a variety of conditions have been used to improve the enzyme yield [32]. In the present studies, the use of various surfactants like Tween-80, Triton X-100 and SDS did not enhance the enzyme

production from *Humicola* sp. However, the addition of Triton X-100 in the extraction buffer has been shown to improve the yield of α-galactosidase from *Vicia faba* [33]. Although Triton X-100 had no significant effect on the extraction of guar α-galactosidase [34], it had beneficial effect in terms of recovery during various stages of purification.

Humicola sp. does not produce invertase, which is advantageous because invertase present in the enzyme preparation can hydrolyze the raffinose and sucrose. The enzyme preparation has no protease activity, which again is an advantage because protease contamination causes many undesirable effects on soymilk such as, coagulation, precipitation of protein and formation of bitterness [11,21]. Studies on α-galactosidase from *Aspergillus awamori* [11] and *Aspergillus saitoi* [21] indicated that the enzyme preparation contained both invertase and protease activities. Invertase present in the enzyme preparation hydrolyzed sucrose leading to increased sweetness of the product.

The initial moisture content is known to affect hydrolytic enzyme production in SSF significantly [4,35]. Lower moisture level leads to sub-optimal growth, a lower degree of substrate swelling and high water tension whereas, higher moisture level decreases porosity, changes particle structure, promotes development of stickiness, lowers oxygen transfer and enhances formation of aerial mycelium [36,37]. Hasseltine [1] reported that, the basic difference between SSF and SmF is the variation in their water levels. The same amount of substrate under stationary and shaking conditions was fermented under solid-state, semi-solid and submerged fermentation. As shown in Table 2, higher amount of enzyme activity was obtained in solid-state than in semi-solid state fermentation under stationary conditions. But under shaking conditions, semi-solid fermentation as well as SmF gave comparatively less activity than SSF. To maintain a constant level of substrate to moisture during cultivation, the flasks were incubated in humidity control incubator at 45°C. It was observed that, at 95% humidity maximum amount of enzyme was secreted irrespective of the substrate used for the enzyme production [Table 3]. According to Lonsane *et al* [36] initial moisture content in the fermentation medium changes during the course of fermentation due to evaporation and metabolic activities. This loss can be compensated by keeping the fermentor atmosphere humidified at about 90-97% relative humidity [36].

The results obtained in tray fermentation is encouraging for large scale production of the enzyme. Similar trials for large scale production of α-amylase from thermophilic *Bacillus coagulans* in enamel trays have been reported by Babu and Satyanarayana [37]. Crude *Humicola* α-galactosidase exhibited an optimum pH and temperature of 5.0 and 50°C, respectively for the hydrolysis of galacto-oligosaccharides. Similar observations were made for the α-galactosidases from *Aspergillus awamori* [11] and

Aspergillus saitoi [21]. Smiley *et al* [10], using a crude water extract of a culture of *Aspergillus awamori* NRRL 4869 grown on wheat bran, were able to convert the undesirable galacto-oligosaccharides in soywhey to acceptable monomeric sugars in a hollow-fiber recirculating batch reactor. Silman *et al* [12] have also reported detailed studies on the parameters influencing the conversion of raffinose in a hollow fiber reactor using a mixed crude enzyme system consisting of α-galactosidase and invertase from *Aspergillus awamori*. In the present studies, α-galactosidase from *Humicola* sp. hydrolyzed raffinose and stachyose in soymilk and the TLC analysis revealed that stachyose was hydrolyzed within 1 h whereas raffinose takes 2 h for hydrolysis [Figure 6]. The decrease in the concentration of stachyose is more important than decrease in the concentration of raffinose since stachyose induces more flatulence than raffinose [23,27].

Humicola sp. grown on cheap and economical solid substrates produce higher levels of α-galactosidase in SSF in 6 days. The liquid used to moisten solid substrates is just a tap water and no expensive ingredients are needed to produce enzyme in large quantity. Investigation by means of TLC indicated that the addition of small amounts of this enzyme preparation resulted in complete hydrolysis of raffinose and stachyose which are the main cause of flatulence of soymilk. Therefore the use of *Humicola* α-galactosidase in hydrolyzing galacto-oligosaccharides from legume based products will prove economical from an industrial point of view.

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CHAPTER- IV

Purification and characterization of α-galactosidase from *Humicola* sp.

4.1 SUMMARY

The extracellular invertase free α-galactosidase from the thermophilic fungus Humicola sp. was purified to homogeneity by ammonium sulfate precipitation, ethanol precipitation, DEAE cellulose and Sephacryl S-300 chromatography with a 202 fold increase in specific activity and 13.8% recovery. The molecular mass of the enzyme by gel filtration on Sephacryl S-300 was 354.8 kDa and 87.1 kDa by SDS polyacrylamide gel electrophoresis. The enzyme had an optimum pH and temperature of 5.0 and 60°C, respectively. The enzyme was stable in the pH range 4.5 to 6.0. It retained its full activity for 2 h at 55°C and for 50 min at 60°C. *Humicola* α-galactosidase is a glycoprotein with 6.2% carbohydrate content and acidic is in nature with a pI of 4.90. The enzyme activity was strongly inhibited by Hg^{2+}, Mn^{2+} and Cu^{2+}. D-Galactose, D-glucose and D-mannose inhibited α-galactosidase noncompetitively and the inhibition constant (K_i) for galactose was 15.5 mM. The purified enzyme hydrolyzed *p*NPG, *o*NPG, melibiose, raffinose and stachyose exhibiting a K_m value of 0.31, 0.54, 0.70, 3.3 and 7.6 mM, respectively. The energy of activation (E_a) of *Humicola* α-galactosidase was found to be 35.87 Kcal / mole, for *p*NPG. Amino acid composition revealed that the enzyme is rich in glycine and alanine and low in cysteine and methionine.

4.2 INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase, E.C.3.2.1.22) is widely distributed in nature [1] and is known to hydrolyze α-galactosyl linkages in oligo- and polysaccharides. Purification of α-galactosidase from plants [2-4], animals [5-7] and microorganisms [8-10] has been reported. In general, α-galactosidases are high molecular weight enzymes, composed of two or four subunits, the association of which plays an important role in exhibiting the catalytic activity. The α-galactosidase from *Aspergillus nidulans* (M_r 370 kDa) [11] is a tetramer composed of four monomers (M_r 87 kDa) and the α-galactosidase from *Pseudomonas fluorescens* H-601 (M_r 390 kDa) [12] is also a tetramer consisting of four equal subunits of M_r 86 kDa. Many divalent cations inhibit α-galactosidase activity. However, only Mn^{2+} has been found to stabilize α-galactosidase from *Escherichia coli* [13,14], *Penicillium janthinellum* [15] and *Penicillium purpurogenum* [16]. α-Galactosidase from *Aspergillus nidulans* [11] has a low affinity for raffinose (K_m 13.60 mM) and displayed the highest affinity (K_m 0.28 mM) and rate of reaction (V_{max} 132 U mg^{-1} protein) for *p*NPG. *Pseudomonas fluorescens* H-601 α-galactosidase [12] also displayed high affinity (K_m 0.38 mM) for *p*NPG and showed very

low affinity (K_m 39 mM) for stachyose. Transgalactosidase activity for α-galactosidase preparation from *Diplococcus pneumoniae* [17] has been demonstrated.

The common methods employed for the purification of α-galactosidase include ammonium sulfate fractionation, ethanol precipitation, heat treatment, ion exchange chromatography, gel filtration and chromatofocussing. The homogeneous enzyme has been obtained from plant sources [3,18,19] and their activities against oligosaccharides and galactomannans have been studied. Microbial α-galactosidases have been purified from bacteria [12,20], yeast [21,22] and fungi [16,23], but extensive characterization of the enzyme has been reported only from few sources.

Microorganisms have the advantage of high production yields, and among them fungal α-galactosidases are more suitable for technological applications mainly due to their extracellular localization, acid pH optima and broad stability profiles [11,15,24,25]. The thermophilic fungus *Humicola* sp. secretes α- and β-galactosidase in the culture filtrate and among the two enzymes, amount of α-galactosidase produced is considerably more (Chapter II). In this chapter, the purification and characterization of α-galactosidase produced by *Humicola* sp. has been described.

4.3 MATERIALS AND METHODS

MATERIALS

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG) was used as the substrate throughout the present work. *p*NPG, *o*NPG, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-N-acetyl-α-D-glucosaminide, *p*-nitrophenyl-N-acetyl-α-D-galactosaminide, *o*-nitrophenyl-α-D-maltopentaside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-D-mannoside, *p*-nitrophenyl-α-D-galactoside, *m*-nitrophenyl-α-D-galactoside, raffinose, stachyose, melibiose, galactose, glucose, mannose, low and high molecular weight markers, Coomassie Brilliant Blue and Bromophenol Blue were purchased from Sigma Chemical Company, U.S.A. All other chemicals were of analytical grade having highest purity and procured locally.

METHODS

Organism and culture conditions

Growth and maintenance of *Humicola* sp. (NCIM 1252), was carried out as described in Chapter II.

α-Galactosidase assay

α-Galactosidase activity was measured as described in Chapter II. The *p*-Nitrophenol liberated following the hydrolysis of *p*NPG at pH 5.0 and 50°C was measured, spectrophotometrically, at 405 nm. When raffinose, stachyose and melibiose were used as substrates, the reducing sugars liberated were determined by the method of Nelson [26] and Miller [27], respectively. One unit (U) is defined as the amount of enzyme that liberates 1 μmole of *p*-nitrophenol or reducing sugar / min under standard assay conditions.

Protein determination

Protein concentration, in the culture broth and during purification steps was determined as described by Lowry *et al* [28] with BSA as standard.

Protein determination in samples obtained after gel filtration on Sephacryl S-300 was carried out by the method of Bradford [29] with BSA as standard. The absorbance of Coomassie Brilliant Blue G-250 bound protein was read at 595 nm.

Purification of α-galactosidase

Unless otherwise mentioned all the purification steps were carried out at 4°C.

Step I. Ammonium sulfate precipitation : The culture filtrate (1 liter) containing 2630 units of α-galactosidase (3084 mg protein) was brought to 95% saturation by slow addition of solid ammonium sulfate under mild stirring and the mixture was allowed to stand overnight at 4°C. The precipitated protein was collected by centrifugation (7000 x g, 20 min), suspended in 50 ml of 100 mM potassium phosphate buffer, pH 7.0 and dialyzed for 48 h against 2 litres of 20 mM potassium phosphate buffer, pH 7.0 with changes after 8-10 h and stored untill further use.

Step II. Ethanol precipitation : To the dialyzate obtained from step I three volumes of chilled distilled ethanol was added slowly under mild stirring. The mixture was allowed to stand overnight at 4°C and the resulting precipitate was collected by centrifugation (7000 x g, 20 min) and dissolved in 50 ml of 100 mM potassium phosphate buffer, pH 7.0. The solution was then dialyzed for 48 h against 2 litres of 20 mM phosphate buffer, pH 7.0 with changes after 8-10 h, concentrated to 30 ml by ultrafiltration using Amicon YM-10 membrane and used for the next step.

Step III. DEAE cellulose chromatography I : The enzyme obtained from step II was further purified by batchwise treatment with DEAE cellulose. The anion exchanger (50 g, wet weight) was equilibrated with 20 mM phosphate buffer, pH 7.0. The dialyzate (step II) was applied to DEAE cellulose at 4°C. After 1 h of occasional stirring, it was filtered through Whatman No. 1 filter paper by applying mild vacuum and filtrate was checked for enzyme activity. Subsequently the DEAE cellulose cake was washed with 20 mM phosphate buffer, pH 7.0, to remove any unabsorbed material and the bound enzyme was eluted by a step wise addition of 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl. The 0.2 M eluate containing highest activity was pooled, concentrated by ultrafiltration using Amicon YM-10 membrane and dialyzed extensively against the same buffer.

Step IV. DEAE cellulose chromatography II : A second chromatography on DEAE cellulose was performed as described above and the 0.2 M NaCl eluted fractions rich in α-galactosidase activity were pooled, dialyzed against 20 mM potassium phosphate buffer, pH 7.0, concentrated by ultrafiltration, and used for gel filtration.

Step V. Gel filtration on Sephacryl S-300 : Two ml of the concentrated enzyme solution (9 mg / ml) was chromatographed on a Sephacryl S-300 column (2 x 120 cm), pre-equilibrated with 20 mM potassium phosphate buffer pH 7.0 containing 0.15 M NaCl. Fractions of 1.5 ml were collected at a flow rate of 10 ml / h and analyzed for protein content and α-galactosidase activity. Active fractions were pooled, concentrated by ultrafiltration, dialyzed against deionised water, lyophilized and stored at 0°C till further use.

Electrophoresis

Polyacrylamide gel electrophoresis under nondenaturing conditions was performed on 7.5% (w/v) acrylamide using Tris-glycine buffer pH 8.3 [30] and the bands were visualized by silver staining [31]. Analytical isoelectric focusing was carried out in BIO-RAD Rotofer cell (2°C, 3 h, 10 w) over the pH range 3.0 to 10.0.

Determination of M_r

Gel filtration: The native M_r of the enzyme was determined by gel filtration essentially as described by Andrews [32]. A Sephacryl S-300 column (2 x 120 cm) was equilibrated with 20 mM potassium phosphate buffer, pH 7.0 containing 0.15 M NaCl and calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and albumin bovine serum (66 kDa).

SDS/PAGE: The subunit M_r of the enzyme was determined using (10% w/v) acrylamide gel (pH 8.3) according to Laemmli [30]. Sigma high molecular weight standard mixture (SDS-6H) was used as reference proteins. The gels were stained in 0.25% Coomassie Brilliant Blue R-250 in methanol : acetic acid : water (45 : 9 : 46) overnight and destained in ethanol : acetic acid : water (30 : 10 : 60).

Glycoprotein nature and carbohydrate content

The glycoprotein nature of the enzyme was assessed by subjecting the purified protein (50 μg) to nondenaturing rod gel electrophoresis (7.5% w/v polyacrylamide gel, under Tris-glycine buffer system, pH 8.3) [30] followed by staining with thymol sulfuric acid [33].

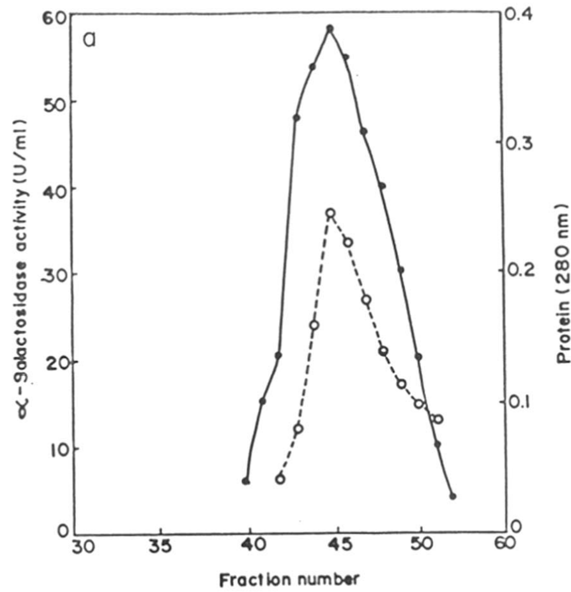
The carbohydrate content was determined by the phenol sulfuric acid method of Dubois *et al* [34] using mannose as the standard. The purified protein (100 μg in 400 μl deionised water) was mixed with an equal volume of 5% (w/v) phenol for 10 min at room temperature. Subsequently two ml of concentrated sulfuric acid was added to the reaction mixture and allowed to cool for 20 min. The orange colour developed was then measured spectrophotometrically at 490 nm.

Amino acid analysis

Lyophilized salt free protein (200 μg) was hydrolyzed with 200 μl of 6N HCl containing 0.05 % phenol and 0.025% β-ME *in vacuo* at 110°C for 22 h. Analysis was carried out on an automated amino acid analyzer (Hewlett Packard series 1050, with HP fluorescence detector). Total tryptophan and cysteine in the protein was determined spectrophotometrically by the method described by Spande and Witkop [35] and Habeeb [36], respectively.

4.4 RESULTS

Purification of α-galactosidase : The elution profiles of the enzyme from Sephacryl S-300 column (2 x 120 cm) and isoelectric focusing are shown in Figure 1a and 1b, respectively. The α-galactosidase obtained after Sephacryl S-300 was found to be homogeneous by native PAGE [Figure 2a] and SDS-PAGE [Figure 2b]. The purification of extracellular α-galactosidase from *Humicola* sp. is summarized in Table 1. The specific activity of the enzyme increased from an average of 0.85 U/mg of protein in the crude broth to 172.4 U/mg after Sephacryl S-300. Thus, the resultant purification is 202.8 fold with 13.8% overall recovery and the yield of the enzyme after the final step is 2.1 mg.



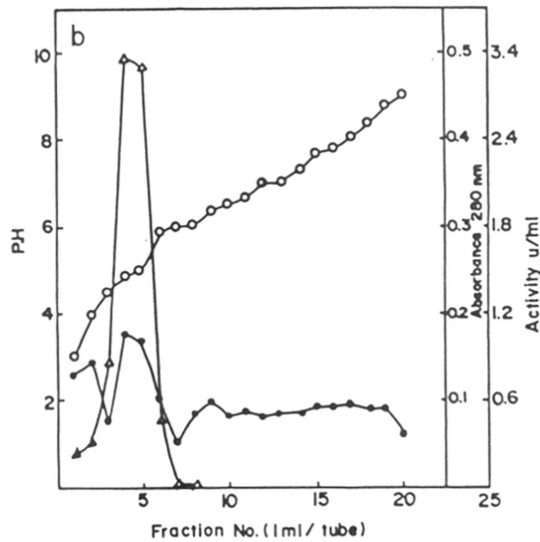


Figure 1 : Elution profiles of *Humicola* sp. α-galactosidase.
(a) From Sephacryl S-300 : (o---o), A 280 nm; (•---•), α-galactosidase activity.
(b) From isoelectric focusing : (•---•), A 280 nm; (o---o), pH; (Δ---Δ), α-galactosidase activity

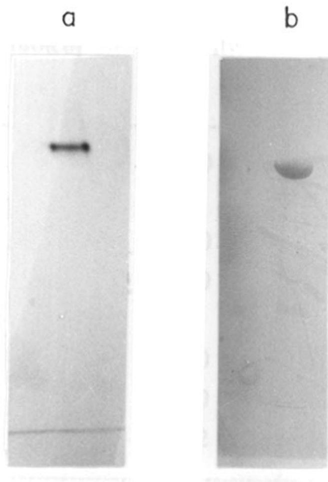


Figure 2 : Electrophoresis of purified α-galactosidase

(a) Native PAGE : 7.5% (w/v), polyacrylamide gel, Tris-glycine buffer, pH 8.3, current 20 mA, protein loaded 10 μg.

(b) SDS-PAGE : 10.0% (w/v), polyacrylamide gel, Tris-glycine buffer pH 8.3, SDS 0.1 %, current 20 mA, protein loaded 5 μg.

Table 1 : **Summary of purification of α-galactosidase from *Humicola* sp.**

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Culture filtrate	3084	2630	0.85	-	100.0
Ammonium sulfate precipitation	920	2130	2.3	2.7	80.9
Ethanol precipitation	194	1680	8.7	10.2	63.9
Chromatography on DEAE (1st)	70	1040	14.9	17.4	39.5
DEAE (2nd)	18	620	34.4	40.4	23.6
Sephacryl S-300	2.1	362	172.4	202.8	13.8

Physicochemical properties : Determination of M_r of the purified α-galactosidase by gel filtration using Sephacryl S-300 gave a value of 354800 Da [Figure 3a]. However, the subunit M_r of the enzyme as determined by SDS-PAGE was 87100 Da [Figure 3b] indicating the homotetrameric nature of the enzyme. The enzyme stained as a red band on yellow background suggesting that it is a glycoprotein. The carbohydrate content of the native enzyme was 6.2%. The enzyme was found to be an acidic protein with a pI of 4.90 [Figure 1b].

Optimum pH and pH stability : The pH activity profile is shown in Figure 4. The highest enzyme activity was recorded at pH 5.0 and the relative activity at pH 4.0 and 6.0 were 62% and 75%, respectively. *Humicola* α-galactosidase, at pH 3.0, lost 85% of its activity but it retained 38% of its activity at pH 8.0. The enzyme was completely stable in the pH range 5.0 to 6.0. However, the enzyme showed comparatively high pH stability and retained significant amount of its activity (> 65%) between pH 3.5 and 8.0 [Figure 4].

Optimum temperature and temperature stability : The optimum temperature for enzyme activity was 60°C [Figure 5a]. α-Galactosidase retained its full activity upto 60°C for 50 min. At 65°C it retained approximately 50% of its original activity for 30 min but was completely inactivated in 30 min at 70°C [Figure 5b]. The Arrhenius plot of α-

galactosidase was found to be linear. The affinity of the enzyme towards the substrate (*p*NPG) decreased with decrease in temperature and the energy of activation (E_a) of the enzyme was 35.87 Kcal / mol [Figure 6].

Effect of metal ions and reagents : The effect of metal ions and reagents on the enzyme activity is shown in Table 2. The enzyme was strongly inhibited by Hg^{2+}, PCMB and PHMB. Mn^{2+} and Cu^{2+} inhibited the enzyme activity to a lesser extent and there was no activation by any of the metal ions or reagents tested. Moreover, iodoacetate and β-ME did not inhibit the enzyme activity.

Table 2 : Effect of metal ions and reagents on purified α-galactosidase activity

Metal ions and reagents	Relative activity (%)
None	100.0
Fe^{2+}	91.6
Ca^{2+}	85.5
Zn^{2+}	84.3
Mg^{2+}	81.8
Co^{2+}	79.2
Ni^{2+}	78.7
Cu^{2+}	38.1
Mn^{2+}	20.5
Hg^{2+}	8.7
Sodium azide (1 mM)	101.0
Urea (10 mM)	105.0
Iodoacetate (1 mM)	105.0
EDTA (10 mM)	102.0
Sodium fluoride (1 mM)	96.0
β-ME (1 mM)	95.0
PHMB (1 mM)	7.0
PCMB (1 mM)	5.0

The enzyme (100 μl) was incubated in presence of different metal ions (5 mM final concentration) and reagents (as indicated) in 100 mM citrate phosphate buffer pH 5.0, for 16 h and the residual activity was determined under standard assay conditions.

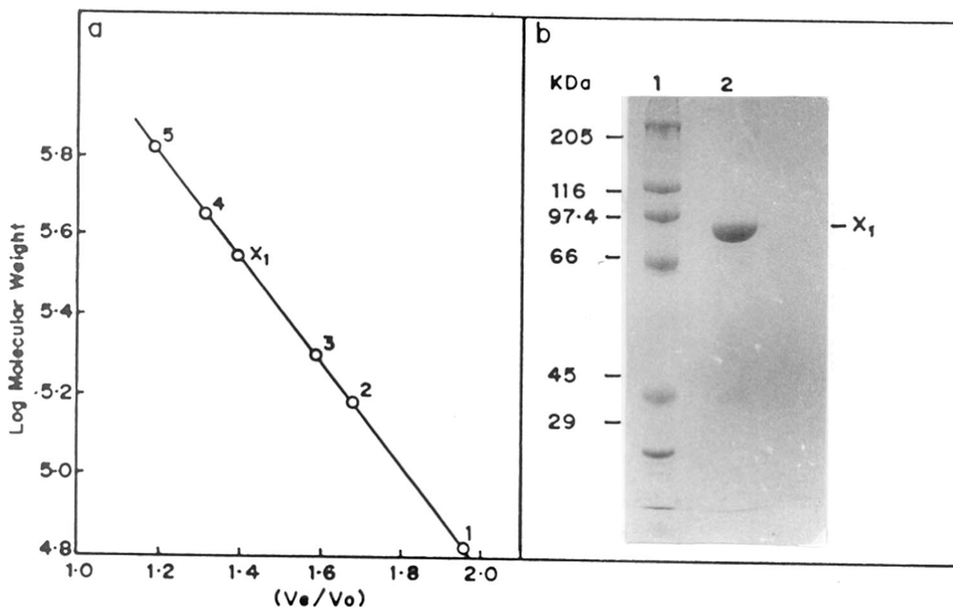


Figure 3 : M_r determination of *Humicola* sp. α-galactosidase

(a) **Gel filtration.** Sephacryl S-300 column (2 x 200 cm) was equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl and gel was calibrated with **(1)** Albumin bovine serum [66 kDa] **(2)** Alcohol dehydrogenase [150 kDa] **(3)** β-Amylase [200 kDa] **(4)** Apoferritin [443 kDa] **(5)** Thyroglobulin [669 kDa]. Vo= Void volume, V_e= Elution volume and X_1= α-galactosidase.

(b) **SDS-PAGE** : Purified protein (5 μg) was electrophoresed on 10% (w/v) polyacrylamide slab gel and stained with Coomassie Blue R-250. Lane 1 : Sigma high molecular weight standard mixture (SDS-6H) viz. Myosin (205 kDa), β-galactosidase (116 kDa), posphorylase (97.4 kDa), albumin bovine (66 kDa), albumin egg (45 kDa) and carbonic anhydrase (29 kDa). Lane 2 : X_1, 5 μg denatured α-galactosidase.

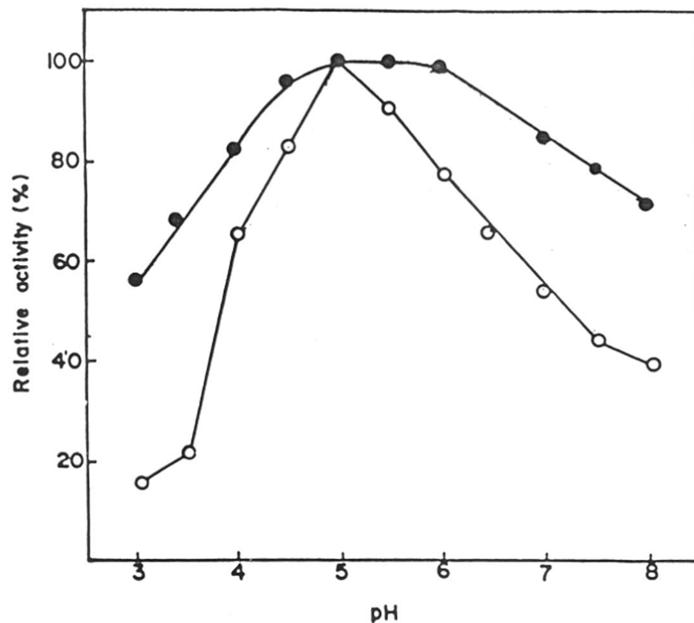


Figure 4 : Effect of pH on the activity and stability of α-galactosidase.

For optimum pH, 100 μl of purified enzyme was incubated at 50°C for 10 min in the pH range of 3.0 to 8.0 by using citrate phosphate (2.6-7.0), potassium phosphate (5.7-8.0) and Tris-HCl (7.2-9.0) buffers.

The pH stability was determined by incubating 100 μl of the enzyme in a pH range as above at 4°C for 16 h and the residual activity was determined under standard assay conditions. Symbols used are, (o---o), optimum pH; (•---•), pH stability.

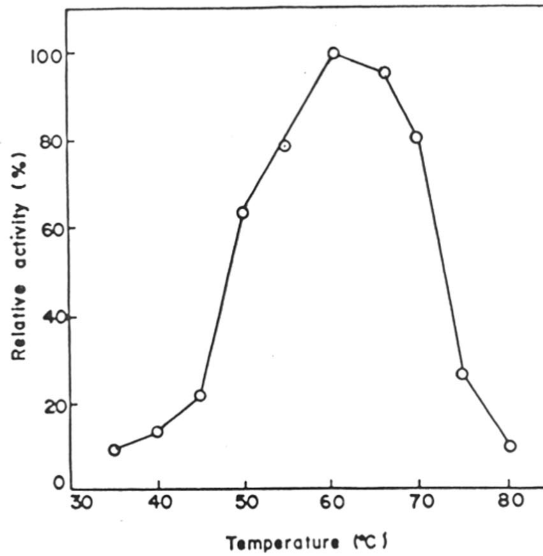


Figure 5a : Optimum temperature for the *Humicola* sp. α-galactosidase

Purified α-galactosidase was assayed in a water bath in 100 mM citrate phosphate buffer, pH 5.0 at various temperatures (35-80°C) and residual activity was determined under standard assay conditions.

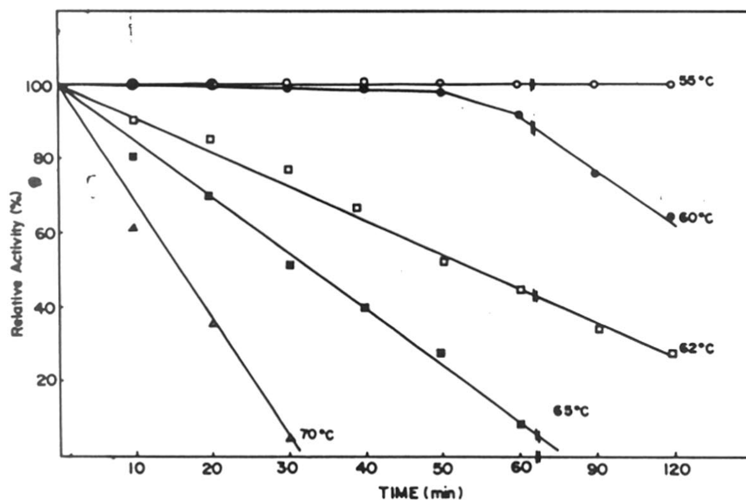


Figure 5b : Effect of temperature on the stability of α-galactosidase.

The α-galactosidase (100 μg) was incubated in citrate phosphate buffer, pH 5.0 at different temperatures (55-70°C) and aliquots withdrawn at definite time intervals (0- 120 min). The aliquots were immediately quenched in an ice bath. Subsequently, the substrate *p*NPG was added and the reaction carried out under standard assay conditions.

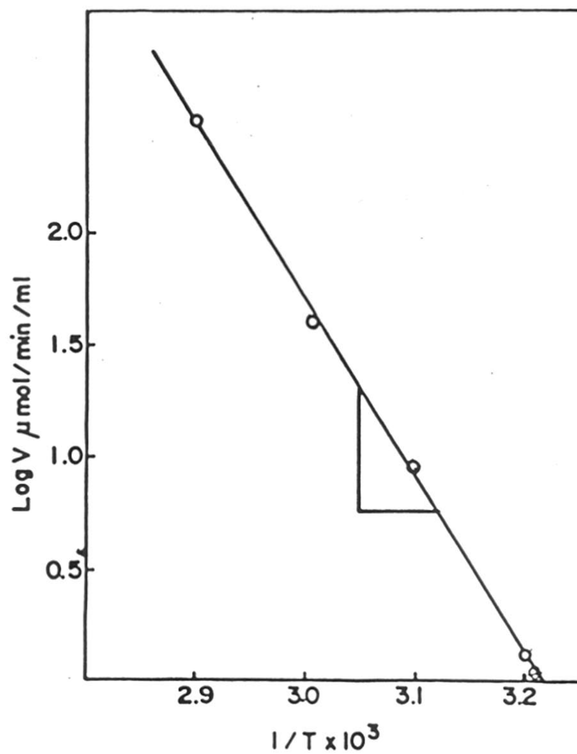


Figure 6 : Arrhenius plot : The K_m and V_{max} values were determined at different temperatures (40-70°C) using *p*NPG at saturating concentrations (33.3 - 333.0 μM) and by fitting the data to linear regression using Lineweaver-Burk plots [38]. Log V_{max} was plotted against 1/T and the energy of activation was calculated by using the equation; E_a= -0.219 x slope where, E_a is the energy of activation in calories.

Effect of sugars and sugar alcohols : The effect of sugars are shown in Table 3. Galactose, glucose and mannose were noncompetitive inhibitors of α-galactosidase as determined by the Dixon plot. In the presence of 10 mM concentration of galactose, glucose and mannose, the enzyme showed 52%, 93% and 88% of the original activity respectively. The K_i for galactose was 15.5 mM [Figure 7], and that for glucose and mannose were 37 mM and 47 mM, respectively [Table 3]. All the other sugars and sugar alcohols showed inhibition levels similar to that of glucose. No inhibition was observed with mannitol and sorbitol.

Table 3 : Effect of sugars and sugar alcohols on purified α-galactosidase activity

Sugars and sugar alcohols (10 mM)	Relative activity (%)	K_i (mM)
None	100.0	-
Galactose	52.0	15.5
Glucose	93.0	37.0
Mannose	88.0	47.0
Arabinose	93.0	-
Xylose	91.0	-
Sucrose	93.0	-
Sorbitol	97.0	-
Mannitol	98.0	-
Glycerol	94.0	-

The enzyme (100 μl) was incubated in presence of sugars or sugar alcohols (10 mM) and the residual activity was determined under standard assay conditions. Inhibition constants (K_i) were determined according to Dixon [37] using *p*NPG as substrate.

Substrate specificity : The rate of hydrolysis of various oligosaccharides by the enzyme were determined from the initial velocities (Table 4). *p*NPG was most susceptible. *o*NPG, melibiose, raffinose, stachyose and *p*-nitrophenyl-α-D-glucopyranoside were hydrolyzed to a lesser extent.

Kinetic parameters : *Humicola* sp. α-galactosidase hydrolyzed various glycosides and oligosaccharides containing α-galactosidic linkages at their non reducing ends i.e. *o*NPG, *p*NPG, melibiose, raffinose and stachyose. The enzyme also hydrolyzed *p*NP-α-D-

glucopyranoside (K_m 10.0 mM) and *p*NP-α-D-mannoside (K_m 20.0 mM) but to a lesser extent [Table 4]. It did not hydrolyze β-galactosides and N-acetyl-α-D-galactopyranoside. α-Galactosidase was found to have more affinity towards *p*NPG and *o*NPG as compared to raffinose and stachyose which are the natural substrates of the enzyme. The K_m and V_{max} values for *p*NPG were 0.31 mM and 24.8 μmole min^{-1} ml^{-1}, respectively [Figure 8] and that for *o*NPG were 0.54 mM and 14.1 μmole min^{-1} ml^{-1}, respectively. K_m and V_{max} values for other substrates are shown in Table 4.

Table 4 : Substrate specificity and kinetic parameters of α-galactosidase from *Humicola* sp.[#]

Substrate*	Relative activity (%)	K_m (mM)	V_{max} (μmol min^{-1} ml^{-1})	k_{cat} (min^{-1})
*p*NPG	100.0	0.31	24.8	1654.4
*o*NPG	27.3	0.54	14.1	940.0
Melibiose	53.0	0.70	0.23	15.3
Raffinose	10.6	3.3	0.07	4.5
Stachyose	31.8	7.6	0.10	6.7
*p*NP-α-D-glucopyranoside	12.9	10.0	0.10	6.7
*p*NP-α-D-mannoside	4.5	20.0	0.03	2.0

* [*o*NP-α-D-maltopentaside, *o*NP-N-acetyl-α-D-galactopyranoside, *p*NP-N-acetyl-α-D-glucosaminide, *p*NP-α-D-galactoside, *m*NP-α-D-galactoside, *p*NP-β-D-galactopyranoside were not hydrolyzed].

[#] Substrates were prepared in 100 mM citrate phosphate buffer pH 5.0. A suitable quantity of enzyme was added to produce a linear increase of p-nitrophenol, o-nitrophenol, glucose and galactose during the first 10 minutes of reaction. The kinetic parameters were determined under standard assay conditions by using the concentration in the range of 33.3-333.0 μM for synthetic substrates and 0.5-10.0 mM for natural substrates. The values were determined by fitting the data to linear regression using Lineweaver-Burk.

Amino acid composition **:** Amino acid composition of α-galactosidase revealed that enzyme was rich in glycine (12.3%), alanine (10.2%) while it was low in sulfur

containing (cysteine 0.7% and methionine 0.7%), basic (histidine 0.8%) as well as in aromatic (tryptophan 0.9%) amino acids [Table 5].

Table 5 : A comparison of amino acid composition of α-galactosidase from reported microbial sources

Amino acid	*S. cerevisiae* (Res/mol)	*E. coli* (Res/mol)	*C. acremonium* (Res/mol)	*T. reesei* (Res/mol)	*Humicola* sp. present investigation (Res/mol)
Ref. No →	[58]	[49]	[51]	[59]	
Asx	121	43	271	62	397
Thr	121	28	291	29	200
Ser	157	14	255	30	277
Glx	75	48	223	27	363
Pro	25	23	134	25	116
Gly	95	28	239	43	423
Ala	95	43	175	46	350
Val	35	28	93	21	242
Met	15	14	16	5	24
Ile	30	31	58	15	132
Leu	57	40	138	36	294
Phe	30	11	59	11	153
His	19	16	171	10	26
Tyr	48	19	55	16	109
Lys	46	19	61	12	135
Arg	16	22	55	15	142
Trp	40	ND	-	19	32* a
Cys	5	ND	12	7	2* b
M_r (Da)	300000	100000	240000	-	354800

* Determined spectrophotometrically

a Spande and Witkop [35]

b Habeeb [36]

ND - not determined

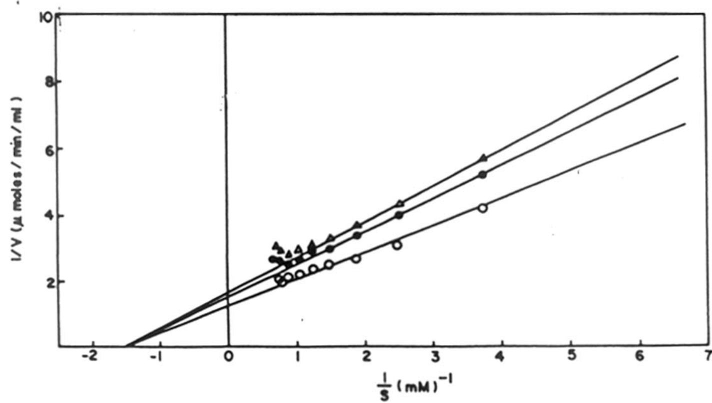


Figure 7 : Inhibition of α-galactosidase by galactose at pH 5.0.

The purified enzyme (100 μl) was incubated at 50°C for 10 min in presence of 10 and 20 mM galactose. Inhibition constant (K_i) was determined according to Dixon [37] using *p*NPG as substrate. Galactose concentration: (o---o), control (0 mM) ; (•---•), 10 mM ; (Δ---Δ), 20 mM.

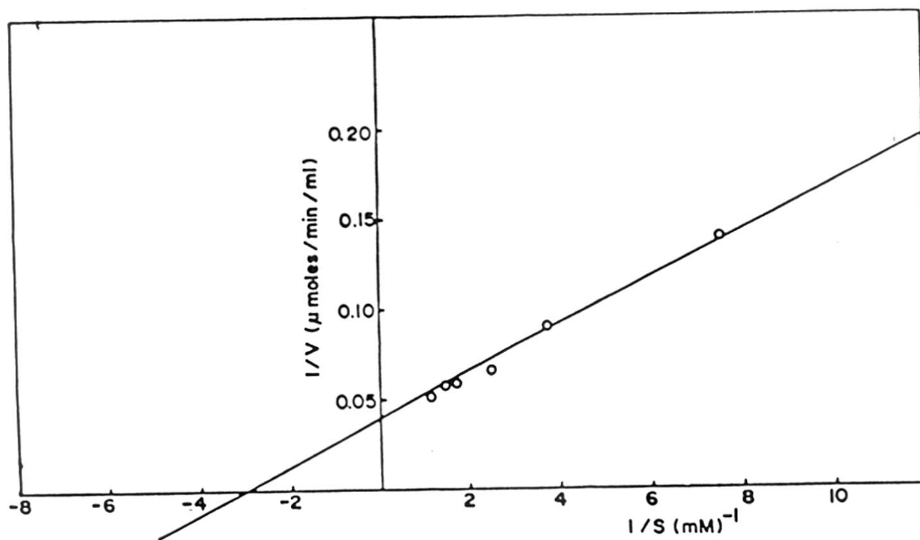


Figure 8 : Lineweaver-Burk plot for the hydrolysis of *p*NPG by Humicola α-galactosidase.

The enzyme was assayed in a series of substrate concentration (33.3-333.0 µM) under standard assay conditions (pH 5.0, 50°C).

4.5 DISCUSSION

α-Galactosidases have been studied with respect to their purification and characterization from quite a few mesophilic microorganisms. However except for *Penicillium duponti* [39] no reports exist on an α-galactosidases from thermophilic fungi. A homogeneous α-galactosidase preparation from *Humicola* sp. was obtained after 5 steps of purification and the yield of the enzyme was 13.8% [Table 1]. In case of mesophilic fungus *Pycnoporous cinnabarinus* α-galactosidase [40] the yield of the purified enzyme increased when heat treatment was used as one of the steps in the course of purification.

The M_r of *Humicola* α-galactosidase is 354800 Da by gel filtration [Figure 3a] and 87100 Da by SDS-PAGE [Figure 3b] indicating that the enzyme is a homotetramer. The existence of multimolecular forms of α-galactosidases has been reported from various sources [18,41-44]. However, no multiple forms appeared during purification of α-galactosidase from *Humicola* sp. The M_r of microbial α-galactosidases vary considerably and are in the range of 32000 Da to 390000 Da. The highest M_r of 390000 Da has been reported for the enzyme from *Pseudomonas fluorescence* [12]. *Bacillus stearothermophilus* exhibited two forms of the enzyme i.e. form I and form II with M_r values of 280000 Da and 325000 Da, respectively [43]. The α-galactosidase purified from *Aspergillus niger* [45] when examined by electrofocusing suggested the presence of several components having pI ranging from pH 4.1-4.7. The isoelectric point of *Humicola* enzyme is 4.90 and is similar to other reported microbial α-galactosidases which are in the range of 4.4 to 6.3 [11,21,46]. The amino acid composition of *Humicola* sp. α-galactosidase showed that the enzyme is rich in glycine and alanine but low in sulfur containing amino acids (cysteine and methionine), basic (histidine) as well as in aromatic (tryptophan) amino acids [Table 5]. The amino acid composition of microbial α-galactosidases shows considerable variation. The common feature with most of the α-galactosidases is the presence of large amounts of acidic amino acids like aspartic and glutamic acid, polar amino acids like serine / threonine and non polar amino acid like valine. Sulfur containing amino acids are relatively low [47-50]. The α-galactosidase from *Cornebacterium acremonium* has about 33% hydrophobic amino acids and 21% hydroxyl amino acids [51]. The enzyme from *Cornebacterium murisepticum* [50] revealed high percentage of glycine and serine residues. *Escherichia coli* K12 [52] α-galactosidase has a high percentage of leucine in addition to glutamic acid. The enzyme from *Trichoderma reesei* [53] possesses high content of glycine, alanine and low content of cysteine and methionine.

The pH and temperature optima for the *Humicola* sp. α-galactosidase was found to be 5.0 and 60°C, respectively. This is similar to α-galactosidases reported from yeasts, molds and bacteria [21,54], although α-galactosidases from *Candida javanica* [22] and *Pycnoporous cinnabarinus* [24] exhibited an optimum temperature of 70 and 75°C, respectively. The enzyme from *Humicola* showed considerable activity (>75%) between pH 4.5-6.0 and retained 40% of its maximal activity at pH 8.0 [Figure 4]. The enzyme showed high stability (>80%) over the pH range 4.0 to 7.0 for 16 h which is similar to other microbial α-galactosidases. α-Galactosidase from *Humicola* sp. exhibited high thermostability and retained 90% of its activity at 60°C for 60 min and at 65°C the enzyme retained 50% of its activity for 30 min [Figure 5b]. The high temperature stability could be useful for commercial application in soymilk hydrolysis. Of the α-galactosidases studied from other sources, few enzymes were found to be stable against high temperatures [4,55,56]. Among them, the α-galactosidases from *Pycnoporous cinnabarinus* [24] retained its full activity when incubated at 75°C for 15 min whereas the corresponding enzyme from *Vicia sativa* [57] retained > 84% of its activity for 40 min at the same temperature.

Influence of divalent cations on the activity revealed that Hg^{2+} and Mn^{2+} strongly inhibited the enzyme while Cu^{2+} had only a marginal effect. None of the of the other metal ions and EDTA had any significant effect [Table 2], suggesting that the *Humicola* α -galactosidase is neither a metal requiring enzyme nor a metalloenzyme. However, among the divalent cations only Mn^{2+} has been observed to stabilize the α-galactosidases from *Escherichia coli* [13,14], *Penicillium janthenellum* [15] and *Penicillium purpurogenum* [16]. The inactivation of *Humicola* α-galactosidase by thiol group modifiers like PCMB or PHMB [Table 2] indicates the involvement of thiol group in enzyme activity. D-Galactose, D-glucose and D-mannose at 10 mM concentration are inhibitory to the enzyme and they act as noncompetitive inhibitors [Table 3, Figure 7]. Although competitive inhibition by D-galactose and D-glucose is expected, noncompetitive inhibition by D-galactose and D-glucose have been reported for α-galactosidase from *Mortierella vinacea* [56] and *Aspergillus tamarii* [41]. However, in case of *Candida javanica* α-galactosidase, these sugars (10 mM) did not have any effect [22].

Humicola α-galactosidase hydrolyzes *p*NPG, *o*NPG, melibiose, raffinose and stachyose but *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-mannoside are poor substrates. The kinetic constants (K_m and V_{max}) for hydrolysis of the α-galactosides indicate pronounced difference in affinity for α-D-galactosides having different aglycon substituents [Table 4]. The K_m of *Humicola* α-galactosidase for *p*NPG, *o*NPG, melibiose, raffinose and stachyose are much lower than the values reported for

Pseudomonas fluorescens α-galactosidase [12]. The *Humicola* enzyme was unable to hydrolyze *p*-nitrophenyl-β-D-galactopyranoside or p- and m-nitrophenyl-α-D-galactoside indicating that the anomeric linkages of the substrate play an important role in the catalytic efficiency of the enzyme. The hydrolysis of raffinose and stachyose shows that the affinity of α-galactosidase for the tetrasaccharide is approximately 50% of the trisaccharide whereas, the value of V_{max} is higher for stachyose (0.10 μmole min^{-1} ml^{-1}) compared to raffinose (0.07 μmole min^{-1} ml^{-1}). The K_m and V_{max} of purified enzyme for nitrophenyl substrates are comparable to that previously reported for *Aspergillus nidulans* [11], *Candida guilliermondii* [21] and *Candida javanica* [22]. The K_m values of α-galactosidase for *p*NPG from various microorganisms ranges from 0.16-0.60 mM while for *o*NPG it ranges from 0.30 to 11.0 mM. Only in case of α-galactosidase from *Mortierella vinacea* [56], the K_m for *o*NPG (0.36 mM) was found to be lower than that for *p*NPG (0.43 mM). The *Humicola* α-galactosidase is a glycoprotein, as has been reported for purified α-galactosidases from *Mortierella vinacea* [56], *Candida javanica* [22] and *Aspergillus tamarii* [41,42]. Most of the microbial α-galactosidases reported are glycoproteins with varying amount of carbohydrate. The carbohydrate content of *Mortierella vinacea* α-galactosidase [56] is 13.5% and it is higher than that of *Humicola* sp. α-galactosidase (6.2%).

The important feature of *Humicola* sp. α-galactosidase is the absence of invertase activity, its higher temperature stability at 60°C and pH optima of 5.0. The enzyme therefore has the potential for application in the degradation of raffinose and stachyose. It may also find an application in the hydrolysis of oligosaccharides having α-galactoside linkages such as raffinose, stachyose and verbascose which have been found in soyflour and seeds of legume plants.

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CHAPTER - V

Active site **characterization** of **α-galactosidase from** ***Humicola*** sp.

5.1 SUMMARY

Chemical modification studies on the purified α-galactosidase from *Humicola* sp. with group specific modifiers, spectral analysis and kinetic data suggested the involvement of a single tryptophan and lysine and two carboxyl residues in the catalytic activity of the enzyme. The involvement of two carboxyl residues in the catalytic activity of the enzyme indicates that *Humicola* enzyme also follows the classical glycosidase catalytic mechanism. However, active site nature of *Humicola* α-galactosidase is atypical compared to other reported α-galactosidases in that, it shows the involvement of lysine in the catalytic activity of the enzyme.

5.2 INTRODUCTION

Identification of specific amino acid residues involved in the catalytic activity of enzymes and the specific role played by them is important in understanding their mechanism of action and structure function relationship. The effect of chemical modifiers on enzyme activity, the kinetic measurement of inactivation and determination of amino acid residues involved, can lead to important information on active site and structure function relationship of the enzyme. Though α-galactosidases have been studied with respect to their functional properties and potential industrial applications, the structure function relationship of this enzyme has not been studied in detail. α-Galactosidases from *Mortierella vinacea* [1], *Pycnoporous cinnabarinus* [2], *Trichoderma reesei* [3] and Human [4] have been crystallized but their crystal structure has not been resolved so far. In the absence of crystallographic data, chemical modification of the amino acid side chains provide information on the groups involved in the catalytic activity of the enzyme. Inhibition of α-galactosidases from *Aerobacter aerogenes* [5], *Cucurbito pepo* [6], *Saccharum officinarum* [7], mung bean [8], *Diplococcus pneumoniae* [9] and sweet almond [10] by sulfhydryl reagents and certain divalent metal ions have been reported. Kinetics of chemical modification of mung bean α-galactosidase [8] showed the probable involvement of twelve carboxylate and nine histidine residues, per molecule of enzyme, in the catalytic activity. *Pneumococcal* α-galactosidase [9] was sensitive to sulfhydryl reagents and metal ions. PCMB (0.1 mM) caused 80% inhibition and the enzyme was also completely inhibited by 0.2 mM $CuSO_4$. Studies on the inhibition of α-galactosidase from sweet almond [10] by metal ions and photooxidation indicated the involvement of carboxylate and histidine in the catalytic activity. The chemical

modification of α-galactosidase from coconut [11] implicated a single tryptophan and tyrosine and two carboxylate residues in its catalytic activity. Recently, Kachurin *et al* [12] reported the presence of methionine near the active site of α-galactosidase from *Trichoderma reesei*. Further, they showed that oxidation of methionine altered the kinetic properties of the enzyme and enhanced its activity.

In this chapter, the results on the investigation of the amino acid residues involved in the catalytic activity of *Humicola* α-galactosidase by chemical modification are presented.

5.3 MATERIALS AND METHODS

MATERIALS

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG), N-bromosuccinimide (NBS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), citraconic anhydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 3-nitro-L-tyrosine ethylester hydrochloride (NTEE), diethylpyrocarbonate (DEPC), 2,3-butanedione (diacetal), *p*-nitrophenyl glyoxal, tetranitromethane (TNM), N-acetylimidazole (NAI), N-ethylmaleimide (NEM), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), *p*-hydroxymercury benzoate (PHMB), iodoacetate, phenylmethylsulfonyl fluoride (PMSF), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 2[N-morpholino] ethanesulfonic acid (MES) were from Sigma Chemical Company, U.S.A. All other chemicals and reagents used were of highest purity available from commercial sources.

METHODS

Purification of α-galactosidase

The extracellular α-galactosidase from *Humicola* sp. (NCIM 1252) was purified to homogeneity as described in Chapter IV. Prior to the modification reactions, the purified enzyme was dialyzed against the respective reaction buffers for 24 h at 4°C. Insoluble material, if any, was removed by centrifugation (5000 x g, 5 min) and the protein concentration and specific activity determined.

α-Galactosidase assay

This was carried out as described in Chapter II. The *p*-nitrophenol liberated following the hydrolysis of *p*NPG, at pH 5.0 and 50°C, was measured

spectrophotometrically, at 405 nm. One unit (U) of the enzyme is defined as the amount of enzyme that liberated 1 μmole of *p*-nitrophenol / min under standard assay conditions. The kinetic constants (K_m and k_{cat}) of native and partially inactivated enzyme samples were determined under standard assay conditions using substrate concentrations in the range 33.3-333.0 μM of *p*NPG and fitting the data to linear regression using Lineweaver Burk plot [13].

Protein estimation

Protein concentration was determined according to Lowry *et al* [14] using BSA as standard.

CHEMICAL MODIFICATION STUDIES

Modification of carboxyl groups

Reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) : The enzyme (1 mg) in 1 ml of 50 mM MES/HEPES buffer (75:25 v/v) pH 6.0, was incubated with a total of 35 mM EDC and 21 mM NTEE at 25°C for 30 min. Samples were removed at suitable intervals and the residual activity was determined under standard assay conditions. To determine the incorporation of number of nitrotyrosyl groups, the EDC/NTEE treated enzyme samples were precipitated by the addition of an equal volume of 10% (w/v) TCA and the mixture was left at 4°C for 30 min. The precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air dried and dissolved in 2 ml 100 mM NaOH. The number of nitrotyrosyl groups incorporated were determined spectrophotometrically, at 430 nm, by assuming a molar absorption coefficient of 4600 M^{-1} cm^{-1} [15]. Enzyme samples incubated in the absence of EDC/NTEE served as control.

Modification of tryptophan residues

Reaction with N-bromosuccinimide (NBS) : This was carried out by titrating 1 ml of α-galactosidase (0.5-1.0 mg), in 100 mM sodium acetate buffer pH 4.5, with freshly prepared NBS (2 mM). The reagent was added in 6 installments (10 μl each) and after each addition , an aliquot (10 μl) was removed, quenched by the addition of 20 μl of L-tryptophan (25 mM) and checked for residual activity under standard assay conditions. The NBS mediated inactivation was followed by monitoring the decrease in absorbance at 280 nm and the number of tryptophan residues modified was calculated by assuming a

molar absorption coefficient of 5500 M^{-1} cm^{-1} [16]. Enzyme samples incubated in the absence of NBS served as control.

Modification of lysine residues

Reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) : The enzyme (500 μg), in 1 ml of 4% (v/v) sodium bicarbonate pH 8.4, was incubated with different concentrations (1-12 mM) of TNBS at 25°C for 20 min in dark. Aliquots were withdrawn at suitable intervals and the reaction was terminated by adjusting the pH to 4.6. The residual activities were determined under standard assay conditions. Subsequently, the samples were dialyzed against 20 mM citrate phosphate buffer, pH 5.0 and the number of amino groups modified was determined spectrophotometrically, at 335 nm, by assuming a molar absorption coefficient of 9950 M^{-1} cm^{-1} for the trinitrophenylated lysine [17]. Enzyme samples incubated in the absence of TNBS served as control.

Reaction with citraconic anhydride : The amino groups of α-galactosidase were reversibly blocked with citraconic anhydride according to Dixon and Perham [18]. The purified enzyme (100 μg) in 1 ml of 100 mM sodium bicarbonate buffer pH 8.4, was treated with a total of 100 μl of 10 mM citraconic anhydride (diluted with dioxane) at 25° C for 20 min. The reagent was added in 5 installments and the pH of the reaction mixture was maintained at 8.4 by the addition of 1 M NaOH. Aliquots were removed after each addition and the residual activities were determined under standard assay conditions. The control consisted of enzyme samples incubated under identical conditions without citraconic anhydride. Decitraconylation was achieved by incubating the modified enzyme samples at pH 4.0 and 4°C for 15 h, followed by determining the enzyme activity.

Modification of histidine residues

Reaction with diethylpyrocarbonate (DEPC) : Purified α-galactosidase (100 μg), in 1 ml of 50 mM sodium phosphate buffer pH 7.5, was incubated with 2 mM DEPC (freshly diluted with absolute ethanol) at 25°C for 30 min and the residual activity was determined under standard assay conditions. The modification of the enzyme was monitored, spectrophotometrically, by measuring the increase in the absorbance at 240 nm as described by Ovadi *et al* [19]. Enzyme samples incubated in the absence of DEPC served as control. DEPC concentration was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM imidazole buffer pH 7.5, followed by monitoring the

increase in the absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 M^{-1} cm^{-1} [20].

Modification of arginine residues

Reaction with 2,3-butanedione (diacetal) : Purified α-galactosidase (100 μg), in 1 ml of 50 mM sodium borate buffer pH 7.8, was incubated with 2 mM butanedione at 25°C for 30 min [21]. After the incubation period, the residual activity was determined under standard assay conditions. Enzyme samples incubated in the absence of 2,3-butanedione served as control.

Reaction with p-nitrophenyl glyoxal : Purified α-galactosidase (100 μg), in 1 ml of 50 mM sodium pyrophosphate buffer pH 9.0, was incubated with 2 mM *p*-nitrophenyl glyoxal at 25°C for 30 min. Subsequently, the residual activity was determined under standard assay conditions. The number of arginine residues modified was determined according to Yamasaki *et al* [22] using L-arginine as standard. Enzyme samples incubated in the absence of *p*-nitrophenyl glyoxal served as control.

Modification of tyrosine residues

Reaction with N-acetylimidazole (NAI) : The purified enzyme (100 μg) in 1 ml of 50 mM sodium borate buffer pH 7.5, was incubated with 1 mM NAI at 25°C for 30 min followed by estimation of the residual activity under standard assay conditions. The enzyme incubated in the absence of NAI was taken as control. The number of tyrosine residues modified was calculated by using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm [23].

Reaction with tetranitromethane (TNM) : TNM was diluted with 95% ethanol and the concentration of the diluted stock was 2 mM. The enzyme (100 μg), in 1 ml of 50 mM Tris-HCl buffer pH 8.5, was incubated with 2 mM TNM at 25°C for 1 h. The reaction mixture was then dialyzed extensively against 20 mM citrate phosphate buffer, pH 5.0 and the residual activity was determined under standard assay conditions. The enzyme incubated in the absence of TNM served as control. The number of tyrosine residues modified was estimated spectrophotometrically, by using a molar absorption coefficient of 4200 M^{-1} cm^{-1} for nitrotyrosine at 428 nm [24].

Modification of cysteine residues

Reaction with N-ethylmaleimide (NEM) : Purified α-galactosidase (100 μg), in 1 ml of 50 mM sodium phosphate buffer pH 7.5, was incubated with 5 mM NEM for 30 min at 30°C [25] and the residual activity was measured under standard assay conditions. Enzyme samples incubated without NEM served as control.

Reaction with p-hydroxymercurybenzoate (PHMB) : The enzyme solution (100 μg), in 1 ml of 50 mM sodium acetate buffer pH 5.5, was incubated with 2 mM PHMB at 25°C for 30 min and the residual activity was determined under standard assay conditions. PHMB reaction was followed by monitoring the increase in absorbance at 250 nm accompanying mercaptide formation [26]. Enzyme samples incubated without PHMB served as control.

Reaction with 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) : The α-galactosidase (100 μg), in 1 ml of 50 mM sodium phosphate buffer pH 8.0, was incubated with 2 mM DTNB at 30°C for 30 min and the residual activity was measured under standard assay conditions. The number of cysteine residues modified was determined by using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} at 412 nm [27]. Enzyme samples incubated in the absence of DTNB was taken as control.

Reaction with iodoacetate : The enzyme (100 μg), in 1 ml of 50 mM Tris-HCl buffer pH 8.6, was incubated with 2 mM iodoacetate at 30°C for 30 min [28] and the residual activity was determined under assay conditions. Enzyme samples incubated without iodoacetate served as control.

Modification of serine residues

Reaction with phenylmethylsulfonyl fluoride (PMSF) : Purified α-galactosidase (100 μg), in 1 ml of 50 mM sodium phosphate buffer pH 7.2, was incubated with 2 mM PMSF at 30°C for 60 min [29]. After the incubation period, the residual activity was determined under standard assay conditions. Enzyme samples incubated without PMSF served as control.

Substrate protection studies

In all chemical modification reactions, the effect of substrate protection was studied by preincubating the enzyme with an excess amount of *p*NPG followed by treatment with various modifying reagents under similar experimental conditions.

5.4 RESULTS

The importance of specific functional groups for the activity of α-galactosidase was determined by the use of various chemical modifiers. The results of the initial inactivation studies are shown in Table 1. No inactivation was observed when the enzyme was incubated with reagents specific for sulfhydryl groups except PHMB, indicating the non-involvement of cysteine residues in the activity of the enzyme. However, strong inactivation by PHMB can be correlated to the presence of mercury ion since Hg^{2+} was found to inhibit the enzyme activity [Chapter IV]. Absence of inactivation with DEPC ruled out the possibility of the involvement of histidine in the catalytic activity of the enzyme. Similarly, treatment of the enzyme with 2,3-butanedione, TNM and PMSF did not result in any loss of enzyme activity suggesting that arginine, tyrosine and serine do not have a role in the catalytic activity of the enzyme. The loss of enzyme activity in the presence of EDC, NBS and TNBS suggested the involvement of carboxyl groups, tryptophan and lysine in the catalytic activity of *Humicola* sp. α-galactosidase.

Modification of carboxyl group

Purified α-galactosidase when incubated with EDC/NTEE lost 90% of its initial activity. However, no loss of activity was observed in control samples. EDC/NTEE mediated inactivation of the enzyme was time as well as concentration dependent. A plot of percent residual activity versus the number of carboxyl groups modified showed the involvement of two carboxyl residues per monomer of the enzyme [Figure 1]. EDC/NTEE mediated inactivation could be prevented to a great extent by preincubating the enzyme with excess amounts of the substrate, *p*NPG [Table 2a]. Furthermore, the kinetic parameters of the partially inactivated (EDC/NTEE) enzyme samples showed decrease in the k_{cat} and an increase in K_m values [Table 2b].

Modification of tryptophan

Purified α-galactosidase when incubated with 2 mM NBS at pH 4.5 and 30°C for 60 min, lost all of its activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. The NBS mediated inactivation of the enzyme was accompanied by a decrease in the absorbance of the modified protein at 280 nm. Based on a molar absorption coefficient, at 280 nm, to be 5500 M^{-1} cm^{-1} [16] and the subunit M_r of the enzyme to be 87100 Da, the total number of tryptophan residues modified per monomer of the enzyme was found to be 4.2.

However, the plot of percent residual activity versus the number of tryptophan residues modified showed that the loss of activity resulted from the modification of single tryptophan residue per monomer of the enzyme [Figure 2]. The NBS mediated inactivation of α-galactosidase could be prevented to a considerable extent by incubating the enzyme with excess amount of substrate, *p*NPG, prior to the modification reaction [Table 3a]. The kinetic analysis of partially inactivated enzyme (NBS) samples showed a decrease in the k_{cat} and no change in the K_m values [Table 3b].

Modification of lysine

Purified α-galactosidase when incubated with 10 mM TNBS at pH 8.4 for 30 min lost 80% of its initial activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. A plot of percent residual activity versus the number of lysine residues modified showed that the loss of activity resulted from the modification of single lysine residue per monomer of the enzyme [Figure 3]. The TNBS mediated inactivation of α-galactosidase could be prevented by incubating the enzyme with excess amounts of *p*NPG prior to the modification reaction [Table 4a]. The kinetic analysis of partially inactivated enzyme (TNBS) samples showed a decrease in the k_{cat} and an increase in the K_m values [Table 4b].

Citraconylation of α-galactosidase resulted in 85% loss in its initial activity. Citraconylated enzyme on decitraconylation regained approximately 50% of its initial activity.

Table 1 : Effect of different chemical modifying agents on the activity of α-galactosidase from *Humicola* sp.

Modifying agent	Concentration (mM)	Residual activity (%)	Reaction buffer
None	-	100	Citrate phosphate, 100 mM, pH 5.0
NBS	2	0	Sodium acetate, 100 mM, pH 4.5
Citraconic anhydride	10	15	Sodium bicarbonate, 100 mM, pH 8.4
TNBS	10	20	Sodium bicarbonate, 4% (v/v), pH 8.4
EDC	35	10	MES/HEPES (75:25), 50 mM, pH 6.0
DEPC	2	97	Sodium phosphate, 50 mM, pH 7.5
2,3-Butanedione	2	93	Sodium borate, 50 mM, pH 7.8
p-Nitrophenyl glyoxal	2	92	Sodium pyrophosphate,50 mM, pH 9.0
NAI	1	95	Sodium borate, 50 mM, pH 7.5
TNM	2	95	Tris-HCl, 50 mM, pH 8.5
NEM	5	96	Sodium phosphate, 50 mM, pH 7.5
PHMB	2	6	Sodium acetate, 50 mM,pH 5.5
DTNB	2	93	Sodium phosphate, 50 mM, pH 8.0
Iodoacetate	2	90	Tris-HCl, 50 mM, pH 8.6
PMSF	2	96	Sodium phosphate, 50 mM, pH 7.2

Enzyme (100 μg, 1 ml) was incubated with various modifying reagents at appropriate temperatures and after terminating the reaction, the residual activity determined under standard assay conditions.

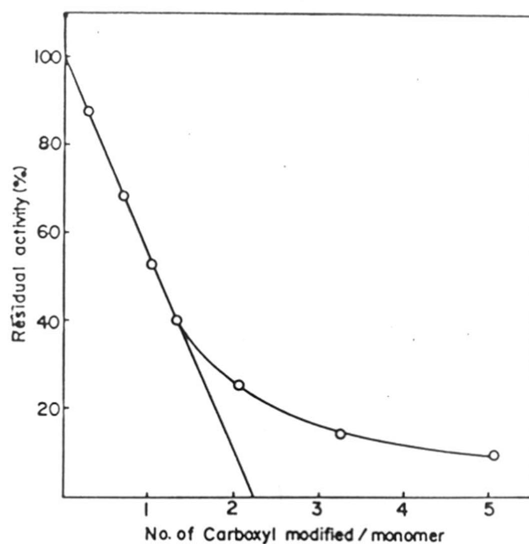

Figure 1 : Plot of percent residual activity versus the number of carboxyl groups modified by EDC/NTEE. The number of carboxyl groups modified were determined as described under Methods.

Table 2a: Influence of carboxyl group modification on the activity of α-galactosidase: Substrate protection studies.

Reaction mixture	Residual activity (%)
Control	100
Enzyme + EDC/NTEE	10
Enzyme + *p*NPG (2 mg) + EDC/NTEE	59

Table 2b : Kinetic parameters of partially inactivated *Humicola* α-galactosidase*.

Reagent used	Percentage activity	K_m (mM)	k_{cat} (min^{-1})
None	100	0.307	1654
EDC/NTEE	70	0.434	888
-	31	0.476	701

*The kinetic constants were determined as described under Methods.

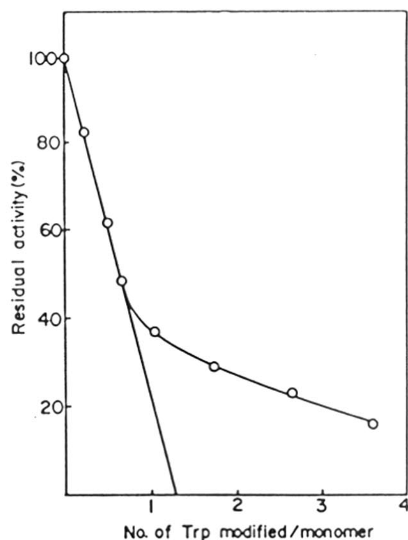

Figure 2 : Plot of percent residual activity versus the number of tryptophan residues modified by NBS. The number of tryptophan residues modified were determined as described under Methods.

Table 3a : Influence of tryptophan modification on the activity of α-galactosidase: Substrate protection studies

Reaction mixture	Residual activity (%)
Control	100
Enzyme + NBS (60 μM)	9
Enzyme + *p*NPG (2 mg) + NBS	54

Table 3b : Kinetic parameters of partially inactivated *Humicola* α-galactosidase*.

Reagent used	Percentage activity	K_m (mM)	k_{cat} (min^{-1})
None	100	0.307	1654
NBS	73	0.333	951
-	27	0.347	741

*The kinetic constants were determined as described under Methods.

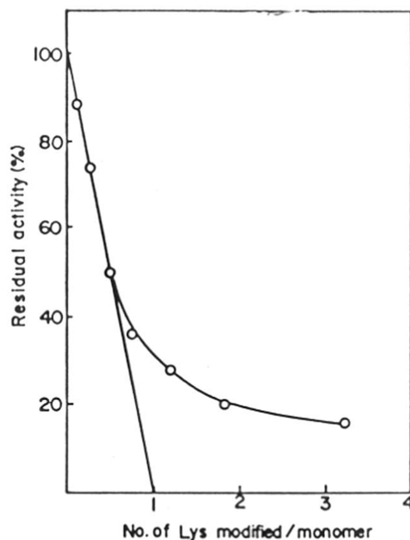

Figure 3 : **Plot of percent residual activity versus the number of lysine residues modified by TNBS.** The number of lysine residues modified were determined as described under Methods.

Table 4a : Influence of lysine modification on the activity of α-galactosidase: Substrate protection studies

Reaction mixture	Residual activity (%)
Control	100
Enzyme + TNBS (10 mM)	20
Enzyme + *p*NPG (2 mg) + TNBS	70

Table 4b : Kinetic parameters of partially inactivated *Humicola* α-galactosidase*.

Reagent used	Percentage activity	K_m (mM)	k_{cat} (min^{-1})
None	100	0.307	1654
TNBS	77	0.400	1110
-	26	0.416	369

*The kinetic constants were determined as described under Methods.

5.5 DISCUSSION

In case of α-galactosidases, carboxylate [8,11], tryptophan [11], tyrosine [11], histidine [8] and methionine [12] have been implicated in the catalytic activity of the enzyme. However, in case of *Humicola* sp. α-galactosidase, initial studies ruled out the probable involvement of histidine, arginine, tyrosine, serine and cysteine residues in the catalytic activity of the enzyme.

The modification of carboxyl groups by EDC/NTEE resulted in a significant loss of activity suggesting that, carboxylate may have a role in the catalytic activity of *Humicola* α-galactosidase. Spectral analysis of inactivation showed that the loss of activity of the enzyme resulted from the modification of two carboxyl residues per monomer of the enzyme [Figure 1]. Substrate protection studies on EDC/NTEE modified enzyme revealed that the inactivation could be prevented to a great extent by preincubating the enzyme with excess amounts of *p*NPG [Table 2a]. Additionally, kinetic analysis of the partially inactivated enzyme samples showed an increase in the K_m with a decrease in k_{cat} values [Table 2b]indicating that the loss of enzyme activity is due to specific modification of carboxyl groups. The above results point towards the involvement of carboxylate in the catalytic activity of *Humicola* α-galactosidase.

Participation of carboxyl groups in the catalytic function has been reported for a variety of glycosyl hydrolases including lysozyme [30,31], cellulase [32-39], cellobiohydrolase [40], glucoamylase [41-44], α-amylase [45], α-galactosidase [8,10,46,47], β-galactosidase [48], cyclodextrin glycosyl transferase [49-51] and xylanase [52-56]. Moreover, carboxyl groups seem to be an integral part of the catalytic site of those enzymes where acid-base catalysis is involved. The possibility of all glycosidases sharing a common mechanism, with the classic paradigm of hen egg white lysozyme, has been intensively investigated for a variety of glycosidases and many subsequent mechanistic studies have been reviewed [57,58]. In coconut α-galactosidase [11], the involvement of two carboxyl groups in the catalytic activity of the enzyme has been demonstrated. Involvement of two carboxyl residues in the catalytic activity of *Humicola* α-galactosidase suggests that it also follows the classical glycosidase catalytic mechanism.

Treatment of *Humicola* α-galactosidase with NBS resulted in significant loss of activity and inactivation of the enzyme was dependent on the concentration of the reagent. The NBS mediated inactivation was accompanied by a decrease in the absorbance of the modified protein at 280 nm which is characteristic of tryptophan

modification. Determination of number of tryptophan residues following NBS modification indicated that, modification of a single tryptophan residue per monomer of the enzyme is responsible for the loss of activity [Figure 2]. Substrate protection studies revealed that the NBS mediated inactivation of the enzyme could be prevented to a large extent by incubation of the enzyme in presence of excess amounts of *p*NPG, prior to the modification reaction [Table 3a]. This suggests that the inactivation of the enzyme is due to specific modification of tryptophan residues. Moreover, kinetic analysis of partially inactivated enzyme samples showed no change in the K_m values but a decrease in the k_{cat} suggesting the involvement of tryptophan residues in the catalytic function of the enzyme [Table 3b]. Our results are comparable to that of the α-galactosidase from coconut [11].

When lysine residues of the enzyme were modified by TNBS, it lost approximately 80% of its activity. Spectral analysis of TNBS inactivation revealed that the loss of activity is due to the modification of a single lysine residue per monomer of the enzyme [Figure 3]. The involvement of lysine in the catalytic activity of *Humicola* α-galactosidase was also ascertained by acylation. Citraconylation of the purified enzyme lead to significant loss (approximately 85%) of its initial activity and decitraconylation restored significant amount (approximately 50%) of its original activity. The above results indicate the presence of lysine at or near the active site of *Humicola* α-galactosidase. Substrate protection studies on TNBS modified enzyme showed that the inactivation could be prevented to a considerable extent by preincubating the enzyme with excess amounts of *p*NPG [Table 4a]. Additionally, increase in the K_m of the partially inactivated enzyme with a concomitant decrease in k_{cat} suggested that the inactivation is due to lysine modification [Table 4b]. The above results suggest the involvement of lysine in the catalytic activity of *Humicola* sp. α-galactosidase.

Reports on the inhibition of α-galactosidases from plants and microorganisms by different chemical compounds which are specific to certain amino acids are available [5-12,30]. The presence of essential catalytic groups in sweet almond α-galactosidase has been investigated using metal ions and photo-oxidation [10]. From the inhibition data, it was postulated that carboxylate and histidine were involved in metal binding. Two possible mechanisms of action have been suggested. Mathew and Balsubramaniam [46] proposed a modified mechanism of action, based on chemical modification and kinetic studies, for the catalytic function of coconut α-galactosidase. The effect of pH on K_m and V_{max} values of coconut α-galactosidase indicated the involvement of two ionizable groups with pK_a values 3.5 and 6.5 in the catalytic activity. Chemical modification studies indicated the presence of two carboxyl groups, a single tryptophan and a tyrosine,

at or near the active site of the enzyme. Based on these observations, a new mechanism of action of α-galactosidases has been proposed wherein the ionizing group with a pK_a of 3.5 is probably the carboxyl group involved in solubilizing carbonium ion intermediate and the ionizing group with a pK_a of 6.5 is a carboxyl group perturbed due to the presence of hydrophobic residues in the vicinity which donates a H^+ ion for catalysis. The effect of pH on K_m and V_{max} of mung bean α-galactosidase [8] indicated the importance of carboxylate (pK_a 4.0) and histidine (pK_a 6.5) for activity. This was confirmed by amino acid modification experiments in the absence and presence of the substrate. The stoichemistry of the enzyme inactivation showed the probable presence of 12 carboxyl and 9 histidine residues per molecule of enzyme in the active site.

The inactivation of α-galactosidases from *Aerobacter aerogenes* [5], *Cucurbito pepo* [6], *Saccharum officinarum* [7], mung bean [8], *Diplococcus pneumoniae* [9], sweet almond [10] and *Cassia serecea* [57] by PHMB, iodoacetic acid and iodoacetamide suggested the possible involvement of sulfhydryl group at or near the active site of the enzyme. However, in case of α-galactosidases from coconut [11], *Calvatia cyathiformis* [58] and spinach leaves [59], these reagents failed to inactivate the enzyme. Thus, it is unlikely that all α-galactosidases require an active SH-group for their catalytic activity. The inhibition of α-galactosidase from mung bean [8], sweet almond [10] and *Vicia faba* [60] by photo-oxidation in the presence of methylene blue indicated the involvement of histidine in the catalytic activity. In case of coconut α-galactosidase [11], neither iodoacetamide nor DEPC caused inactivation, indicating the non-involvement of histidine in the catalytic activity [61,62]. The α-galactosidase from *Mortierella vinacea* IBT-3 [63] was completely inhibited by NBS suggesting that, tryptophan residues are essential for the enzyme activity. However, PCMB and iodoacetamide did not inhibit enzyme activity indicating that sulfhydryl group is not involved in the catalytic activity. *Aerobacter aerogenes* α-galactosidase was inactivated by oxygen, iodoacetamide, PCMB and NEM and was reactivated by thioglycolate, cysteine, sodium sulfide, sodium cyanide or β-ME [5]. The enzyme from *Saccharum officinarum* [7] was inhibited by low concentrations of PCMB while iodoacetamide / iodoacetic acid showed weaker inhibition. This discounts the possibility of Hg^{2+} forming complexes with histidine residues at the active site of the enzyme at pH 4.5. The α-galactosidase from *Trichoderma reesei* [12] when treated with H_2O_2 showed 12-fold increase in activity towards *p*NPG. NMR studies and amino acid analysis showed that the oxidation of one of the five methionines to methionine sulphoxide is sufficient to activate the enzyme. Binding of galactose prevented H_2O_2 mediated activation of the enzyme. Oxidative activation did not result in the conversion of other H_2O_2 sensitive amino acid residues

such as, histidine, tyrosine, tryptophan and cysteine. The catalytically important cysteine thiol group could be quantitatively titrated after oxidative activation of the enzyme. Further oxidation of methionines (upto four out of five residues) could be achieved by increasing the oxidation time and /or by prior denaturation of the protein, suggesting that a methionine residue located at the active site is selectively oxidized.

In conclusion, the active site nature of *Humicola* sp. α-galactosidase is similar to that of the coconut enzyme except that, the *Humicola* enzyme shows the involvement of lysine instead of tyrosine in the catalytic activity. The active site studies also revealed that, *Humicola* α-galactosidase is the only enzyme reported so far, exhibiting the involvement of lysine in the catalytic activity.

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CONCLUSIONS

1. *The thermophilic fungus Humicola sp. (NCIM 1252) produces a thermostable invertase free extracellular α-galactosidase when grown in 5% wheat bran extract and 0.5% (w/v) beef extract medium at 45°C under submerged fermentation.*

2. *Increased yields of the enzyme was obtained in 6 days when Humicola sp. was grown on cheap and economical agricultural residues like soyflour, coconut cake, groundnut cake and wheat bran under solid state fermentation.*

3. *The partially purified enzyme could effectively hydrolyze raffinose and stachyose in soymilk at pH 5.0 and 50°C in 2 h.*

4. *The purified enzyme is a homotetramer with a subunit M_r 87.1 kDa.*

5. *The optimum pH for the enzyme is 5.0 and it is stable in the pH range 4.5-6.0.*

6. *The optimum temperature for enzyme activity is 60°C and it is stable at 60°C for 50 min.*

7. *The enzyme exhibits strict substrate specificity in hydrolyzing α-linked galactosyl residues from the non-reducing end. It showed high affinity for pNPG and oNPG as compared to stachyose, raffinose or melibiose which are the natural substrates of the enzyme.*

8. *Active site studies of the purified enzyme suggested the involvement of a single tryptophan and lysine and two carboxyl residues in the catalytic activity of the enzyme. The active site studies also revealed that, Humicola α-galactosidase is the only enzyme reported so far, exhibiting the involvement of lysine in the catalytic activity.*

List of publications

1. Production of thermostable α-galactosidase from thermophilic fungus *Humicola* sp. ***S.M. Kotwal.***, M.I. Khan and J.M. Khire (1995) *J. Indust. Microbiol.* **15**, 116-120.

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

3. Purification and characterization of an extracellular α-galactosidase from the thermophilic fungus *Humicola* sp. ***S.M. Kotwal.***, J.M. Khire and M.I. Khan (1999) *J. Biochem. Mol. Biol. & Biophys.* **3**, 9-17.

4. Production, purification and characterization of a constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp. ***S. M. Kotwal.***, M. M. Gote., M. I. Khan. and J. M. Khire (1999) *J. Indust. Microbiol. Biotechnol.* **23,** 661-667

5. Chemical modification of α-galactosidase from the thermophilic fungus *Humicola* sp. ***S.M. Kotwal.***, J.M. Khire and M.I. Khan (1999) *J. Biochem. Mol. Biol. & Biophys.* (In press)

Papers / posters presented at seminars/symposium

1. Studies on thermostable α-galactosidase by thermophilic fungus *Humicola* sp. (NCIM 1252). ***S.M. Kotwal.***, J.M. Khire and M.I. Khan Presented at 64th Annual Meeting of Society of Biological Chemists, held at Lucknow between 6-8 October, 1995.

2. Production of constitutive intracellular α-galactosidase by thermophilic fungus *Humicola* sp. NCIM 1252 ***S. M. Kotwal.***, M. M. Gote., M. I. Khan. and J. M. Khire Presented at 37th Annual Conference of Association of Microbiologists of India, held at Madras between 4-6 December 1996.

Patents filed

1. Patent No. 1193/DEL/dtd 23-9-94. A process for the preparation of thermostable α-galactosidase from thermophilic fungus *Humicola* sp. J.M. Khire., ***S.M. Kotwal*** and M.I. Khan (1995)