STUDIES ON EXTRACELLULAR β-GALACTOSIDASE FROM THERMOPHILIC FUNGUS *MUCOR sp.*

A Thesis
Submitted to the
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(In Biochemistry)

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Remain and contemplate awhile

Upon the noble thermophile,

It bears its breast to searing heat

Whilst lesser cells make fast retreat

Despite that stress, it still is able

To maintain its enzymes stable

Through steam and stench, it reigns supreme

In each heated niche of nature's scheme

Whether royal blue of boiling springs

Or biomass of lowly and discarded things

So, salute when you pass the compost pile

Wherein reigns the noble thermophile.....

_ F. Stutzenberger

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Declaration

Certified that the work incorporated in the thesis entitled '**STUDIES ON EXTRACELLULAR β-GALACTOSIDASE FROM THERMOPHILIC FUNGUS *MUCOR* Sp.**' submitted to the University of Pune by Mrs. Shamim A. Shaikh was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

M. I. Khan
(Research Guide)

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Shamim

Shamim

ABSTRACT

β-Galactosidase (β-D-galactohydrolase EC 3.2.1.23) which catalyses the hydrolysis of lactose to glucose and galactose is a commercially important enzyme used in dairy and food industry. Though ubiquitous in distribution, the enzymes of commercial interest are produced from mesophilic yeast and fungi. Since thermophilic organisms grow at high temperatures, their use not only helps to avoid microbial contamination but also permits enzyme production in a shorter time. Therefore, there is a need to look for thermophilic strains capable of producing β-galactosidase. Thermophilic fungi have an added advantage since they produce the enzyme extracellularly. A thermophilic strain of *Rhizomucor* sp. which showed extracellular β-galactosidase activity when grown at pH 6.8 and 45°C was selected for the present studies. Investigation was carried out to a) optimize the medium for maximum enzyme production b) purify and extensively characterize the enzyme to understand its structure-function relationships.

Chapter I: General introduction:

This part comprises of literature survey pertaining to β-galactosidases, their occurrence, production, purification, properties and applications.

Chapter II: Production of β-galactosidase from *Rhizomucor* sp. in submerged and solid state fermentation.

An isolate of *Rhizomucor* sp. which produced extracellular β-galactosidase when grown at pH 6.8 and 45°C, under submerged conditions was selected for the present study. Maximum enzyme level (0.55 U/ml) was observed in 4 days when the organism was grown in a medium containing (w/v) yeast extract, 1 %; lactose, 1 %; K_2HPO_4, 0.1 % and $MgSO_4$, 0.05 %. Organic nitrogen supplements like tryptone, peptone, yeast extract and casamino acids supported growth of the fungus while yeast extract and peptone supported growth and enzyme production. Inorganic nitrogen supplements by themselves could not support the growth of *Rhizomucor* sp. in the absence of organic supplements. Unlike mesophilic fungi,

Rhizomucor sp. did not require divalent metal ions for growth and enzyme production. Solid state fermentation studies at 45°C in a medium containing (w/v) wheat bran, 1.0 %; lactose, 5.0 %; K_2HPO_4, 1.0 % and $MgSO_4$, 0.5 %, yielded 5.05 U/ml of enzyme activity in 5 days. Comparison of submerged and solid state fermentation revealed that the latter yielded higher enzyme levels (5.05 U/ml in solid state compared to 0.55 U/ml in submerged fermentation).

Chapter III: Purification and Characterization of Extracellular β-Galactosidase from *Rhizomucor sp.*

An extracellular β-galactosidase from a thermophilic fungus *Rhizomucor* sp. has been purified to homogeneity by successive DEAE cellulose chromatography followed by gel filtration on Sephacryl S-300. The native molecular mass of the enzyme is 250,000 and it is composed of two identical subunits with molecular mass of 120,000. It is an acidic protein with a pI of 4.2. Purified β-galactosidase is a glycoprotein and contains 8 % neutral sugar. The optimum pH and temperature for enzyme activity are 4.5 and 60°C respectively. The enzyme is stable at 60°C for four hours, has a $t_{1/2}$ of 150 min at 70°C, one of the highest reported for any fungal β-galactosidases. Amino acid composition showed a high content of aromatic amino acids and a low content of cysteine. Substrate specificity studies indicate that the enzyme is specific for β-linked galactose residues with a preference for p-nitrophenyl-β-D-galactopyranoside (pNPG). The K_m and V_{max} values for the synthetic substrates p-nitrophenyl-β-D-galactopyranoside and o-nitrophenyl-β-D-galactopyranoside (oNPG) are 0.66 mM and 1.32 mM; and 22.4 mM min^{-1} mg^{-1} and 4.45 mM min^{-1} mg^{-1} respectively, while that for the natural substrate, lactose is 50.0 mM and 12 mM min^{-1} mg^{-1}. The end product galactose and the substrate analogue isopropyl thiogalactopyranoside (IPTG) inhibited the enzyme with Ki of 2.6 mM and 12.0 mM respectively. The energy of activation for the enzyme using pNPG and oNPG were 27.04 kCal and 9.04 kCal respectively.

Chapter IV: Active Site Characterization and Environment of Tryptophan of β-Galactosidase from *Rhizomucor* sp.

Chemical modification of the purified β-galactosidase of *Rhizomucor* sp. with phenylmethylsulfonyl fluoride, N-acetylimidazole, 5-5'-dithiobisnitrobenzoic acid, 2,3, butanedione, carbodiimide and diethypyrocarbonate did not have any effect on the enzyme activity indicating that serine, tyrosine cysteine, arginine, carboxyl groups and histidine were not involved in the catalytic function of the enzyme. The enzyme lost 70% of its activity on treatment with N-bromosuccinimide and 60% on treatment with trinitrobenzene sulfonic acid indicating that tryptophan and lysine groups have a role in the catalytic function of the enzyme. Presence of the substrate (lactose) protected the enzyme from inactivation with the above reagents. The plots of percent residual activity versus the number of residues modified revealed that a single tryptophan and a single lysine residue are essential for the catalytic activity of the enzyme.

Extensive modification of tryptophan residues with NBS in β-galactosidase led to a 55 % decrease in the intrinsic fluorescence with 12 tryptophan residues per subunit being modified. However, the loss in activity preceded well before loss in intrinsic fluorescence. Effect of various solute quenchers indicated that acrylamide is a better quencher (55 %) as compared to KI (21 %) where as, CsCl was a poor quencher (3 %) under native conditions. Denaturation of the enzyme with guanidine hydrochloride (0 – 6.0 M) was concentration dependent with maximum effects observed at 5.0 M guanidine hydrochloride. There was a significant decrease in the intrinsic fluorescence intensity (~ 60 %) accompanied by a large red shift in the λ_{max} of emission from 337 to 350 nm. Guanidine hydrochloride mediated denaturation led to increased accessibility of the solute quenchers as evidenced by the increase in $f_{(a)eff}$ values with increase in the concentration of guanidine hydrochloride. CsCl was found to be a poor quencher even under denaturating conditions. Quenching studies indicate that more than 1/2 of the tryptophan fluorophores in β-galactosidase are located in a fairly hydrophobic environment and the surface exposed tryptophan residues are in an electropositive environment.

List of Abbreviations

β-ME	β-mercaptoethanol
SmF	Submerged fermentation
SSF	Solid State fermentation
p-NPG	*p*-nitrophenol-β-D-galactopyranoside
o-NPG	*o*-nitrophenol-β-D-galactopyranoside
TCA	trichloroacetic acid
HEPES	(N-[2-Hydroxyethyle]piperazine-N'-[2-ethanesulfonic acid])
MES	(2[N-Morpholino] ethanesulfonic acid)
PEG-8000	polyethylene glycol-8000
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
NEM	N-ethyl maleimide
DTNB	5,5'-dithiobisnitrobenzoic acid
PMSF	phenylmethylsulfonyl fluoride
PHMB	p-hydroxymercurybenzoate
DEP	diethylpyrocarbonate
EADC	1-ethyl-3-(3-diethylaminopropyl) azonia carbodiimide
HNBB	2-hydroxy-5-nitrobenzylbromide
NBS	N-bromosuccinimide
TNBS	2,4,6,-trinitrobenzenesulfonic acid
NAI	N-acetylimidazole
Gdn.HCl	guanidine hydrochloride

CHAPTER I
INTRODUCTION

Glycosidases are a diverse group of enzymes that catalyze the hydrolysis of glycosidic bonds and play a central role, in a number of biological processes of significant interest in biochemistry, medicine and biotechnology. They are broadly classified as:

(i) Exoglycosidases i.e. those enzymes that act on the glycosidic linkage from the non-reducing end of the saccharide chain, and

(ii) Endoglycosidases i.e. those that act on the glycosidic linkage within the saccharide chain (Table 1).

In recent years commercial use of glycosidases in biotechnology industry has also increased and specific enzymes, such as invertase - for production of invert sugars; cellulase - for fruit juice processing and biostoning of denim textiles; xylanases - for bio-bleaching in paper and pulp industry, and in the degradation of biomass to convert solid biomass into liquid fuel and β-galactosidase for lactose reduction are increasingly being used [1].

The diasaccharide lactose, 4-O-β-D-glactopyranosyl-D-glucose or 4 (β-D-galactosido)-D-glucose is the predominant sugar found in milk and is also known as milk sugar. It is present in milk of mammals, such as humans (6.7 %) and cow (4.5 %). Milk at body temperature contains lactose as an equilibrium mixture of 2 parts of α-lactose and 3 parts of β-lactose. Both forms of lactose are useful with the α-form predominating as a nutrient in preparing modified milk and food for infants, in baking mixtures, for lactic acid fermentation, in culture media and in the pharmaceutical industry [2]. Lactose also forms a major part of whey, a predominant byproduct of cheese manufacturing process [3]. The hydrolysis of lactose has been the subject of intense research in the past two decades due to several reasons, of which mainly, the fact that this sugar is scarcely digestible by non-Causasian people, has emphasized the importance of lactose hydrolysis and the enzyme lactase for clinical purposes [4-6]. Another important reason for the hydrolysis of lactose is the environmental pollution caused due to the direct disposal of dairy byproduct (whey) without pretreatment. The whey, contains 6.35% solids, of which lactose forms a major portion (76 %). The presence of whey in effluents causes an increase in the biological oxygen demand (BOD),

Table 1: Action of some of the exo and endo glycosidases

Exogycosidases	*Endoglycosidases*
α-Galactosidase **Gal (α 1)↓R** *β-Galactosidase* **Gal (β 1)↓R** *α-Mannosidase* **Man (α 1)↓R** *β-Mannosidase* **Man (β 1)↓R** *α-N-Acetylgalactosaminidase* **GalNac (α 1)↓R** *β-N-Acetylgalactosaminidase* **GalNac (β 1)↓R** *α-L Fucosidase* **Fuc (α 1)↓** R	*Endo β-galctosidase* GlcNac (β1-3)**Gal [β1↓4(3)]** GlcNac or Glc *Endo β-N-acetylglucosaminidase* R'Man (α1-6) Man (β 1-4) **GlcNac(β1↓4)GlcNac**(β1-N)Asn R'Man (α1-3)

and lactose is the main component that causes the greatest problem of disposal [7]. To deal effectively with this problem, it is necessary to remove the lactose from the dairy waste and convert it to more useful and nonpolluting products. Production of various useful industrial products like lactitol, lactitol palmate, lactosylurea, etc. has been reviewed in depth by Henning [8]. An important and attractive way of utilizing whey is to hydrolyze its lactose to sweet syrup consisting of glucose and galactose, which is 60 % more sweeter than lactose [9]. The sweet syrup prepared from whey by lactose hydrolysis can be used as a source of sugar in bakery, confectionery, soft fruit drinks, ice creams, feed stuff for cattle instead of molasses etc [6]. Production of methane from lactose and other fermentation options to convert lactose and whey into useful products can relieve the burden of whey disposal for cheese manufacturers [8]. Fermentation products of whey have been reviewed by Marth [11] and more recently by Belem [12]. Technologically, lactose crystallizes easily, setting limitations to certain processes in the dairy industry. Therefore it is essential to hydrolyze the milk lactose, which has various advantages over untreated milk. For e.g.

1) Cheese prepared from lactose hydrolyzed milk ripens faster as compared to that from lactose containing milk.

2) Hydrolysis of lactose in the milk leads to increased sweetness of resulting milk thereby avoiding the addition of sweeteners for the preparation of flavored milk products, cheese prepared from lactose hydrolyzed milk ripens faster, etc [13].

Generally two methods have been employed for the hydrolysis of lactose:

(a) Acid hydrolysis coupled with high temperatures (150°C) and

(b) The use of enzymes.

The enzymatic hydrolysis is particularly useful in case of food processing procedures as it avoids protein denaturation, formation of undesirable byproducts as well as the formation of brown colouration usually associated with acid hydrolysis methods [2,5,13]. The enzymatic hydrolysis of lactose is brought about by the enzyme lactase or β-D galactoside galactohydrolase (E.C.3.2.1.23). The enzyme belongs to the family of glycosyl hydrolases and catalyzes the hydrolysis

of terminally linked galactose residues present in simple galactose containing oligosaccharides as well as in complex polysaccharides.

CLASSIFICATION OF β-GALACTOSIDASE

The variation in molecular properties of various β-galactosidases with respect to their quaternary structure, metal ion requirement etc., suggests a strong structural diversity of the β-galactosidases. The widely accepted and practiced IUB Enzyme Nomenclature is based on the reaction catalyzed by the enzyme and its substrate specificity. This classification however was not appropriate for the classification of enzymes showing broad substrate specificity like some of the glycosyl hydrolases, as well as it did not reflect on the structural features of the enzyme. Further, the evidence of a direct relationship, between sequence of enzymes and their folding similarities, led to a systematic comparison of the primary sequences of glycosyl hydrolases, resulting in a new way of classifying enzymes based on their sequence similarities which reflected on the structure as well [14]. Thus classification of the enzyme has recently been based on sequence similarities of the enzyme from various sources and according to this system of classification, β-galactosidases have been classified into glycosyl hydrolase families 1, 2 and 35 [15]. The most well studied β-galactosidase i.e. from *E.coli*, and from other sources like *Clostridium thermosulfurogenes, Streptococcus thermophilus, Kluyveromyces lactis, Lactobacillus delbruekii* and *Leuconostoc lactis* have been assigned to family 2 whereas the enzyme from archaeon *Solfolobous solfactaricus* belongs to family 1 [15]. The β-galactosidases from humans and mice have been assigned to family 35. However the β-galactosidases from *Bacillus stearothermophilus* and the Thermophilic anaerobe NA 10 have been classified under a new family, 42, owing to very low sequence similarity with other family 2 β-galactosidases (~100 residues) [16]. More recently β-galactosidases in general as well as the β-galactosidase from *Saccharopolyspora rectivelgaria* has been classified as a glycosyl hydrolase in view of its wide substrate specificity and associated exoglucosidase activity [17,18].

OCCURRENCE OF THE ENZYME

β-Galactosidases are widely distributed in microorganisms, plants and animals [13,19]. In plants the enzyme is present in almonds [20], peaches [21], apricots [22], apples [23], alfalfa seeds [24], kefir grains [25], tips of wild roses [26], coffee berries [27] etc. In animals, β-galactosidase is found in the intestine of dogs, rabbits, calves, sheep, goat and man [28-30]. Microorganisms are an important source of various enzymes with about 80% of various industrially important enzymes now being obtained from microbial sources [31]. β-Galactosidase from microorganisms are plentiful (Table 2) and among microorganisms, which are considered most suitable for commercial applications, yeast (*Kluyveromyces lactis*, *Kluyveromyces fragils*) and fungi (*Aspergillus oryzae and Aspergillus niger*) have been found to be most suitable and have been widely investigated [13]. Among the bacterial sources, the β-galactosidase from *E. coli* has been studied extensively, especially in gene regulation and induction–repression studies [2,32,33]. In yeasts, the occurrence of β-galactosidase was first observed in *Candida kefyr* and *Torulopsis tyrocola* in 1949 by Beijernek [34]. Besides these well documented microbial sources, the enzyme has also been observed to occur in some of the other bacterial, and fungal species like the anaerobic bacteria *Thermanerobacter* [35], the psychotropic bacterium *Arthobacter* [36], the protozoa *Tritrichomonos foetus* [37], the rumen anaerobic fungi *Neocallimastix frontalis, Saphaeromonos cummunis* and *Piromonos communis* [38] and the entamopathogenic fungi *Beauveria bassiana* [39], *Pediococcus* sp. [40] and *Trichosporon cutaneum* [41]. A novel β-galactosidase from *Arthrobacter* that catalyses β-1-4 or β-1-3 linked galactose residues has also been reported [42].

Thermal stability of enzymes is an important property with respect to industrial processes, therefore enzymes from thermophiles, which exhibit enhanced thermal stability as compared to those derived from mesophilic sources are gaining importance. Thermostable β-galactosidase from mesophilic organisms like *Alternaria alternate* [9], Neurospora *crassa,* [4], *Sclerotina sclerotiorum* [5] have been reported. Extensive studies, on thermophiles has been carried out and a

Some of the microbial sources of β-galactosidase

BACTERIA:	*M. smegmatis*
Aeromonos formicans	*Neisseria catarrhalis*
A. hydrophila	*Paracolobactrum aerogenoides*
Arthobacter sp.	*Pasterurella pestis*
Bacillus anthracis	*P. pseudotuberculosis*
B.coagulans	*Propionibacterium shermanii*
B.megaterium	*Proteus mirabilis*
B.stearothermophilus	*Pseudomonos sp.*
B.subtilis	*Salmonella arisonae*
Bifidobacterium	*S. typhimurium*
Bifidum Citrobacter freudi	*Serratia marscescens*
Corynebacterium simplex	*Shigella dysenteriae*
Enterobacter cloacae	*S. paradysenteriae*
Escherichia coli	*S. sonnei*
Klebsiela aerogenes	*Staphylococcus aureus*
Lactobacillus acidophilus	*Streptococcus cremoris*
L.brevis	*S. durans*
L. bulgaricus	*S. faescalis subsp. liquesfaciens*
L. cellobiosus	*S. lactis ATCC 7962*
L. helveticus	*S. lactis var. tardus*
L. lactis	*S. pneumoniae*
L. leichmanii	*Streptococcus sp. 6646 K*
L. plantarum	*S. thermophilus*
L. salivarius	*Thermus aquaticus*
L. thermophilus	*Morgamella sp.*
Leuconostoc cremoris	*Mycobacterium king*
L. paractitrovorum	*M. minetii*

YEAST	*Kefir yeast*
Candida kefyr	*Kluyveromyces fragilis*
C. pseudotropicalis	*K. lactis*
Candida muscorum	*K. marxianus*
C. utilis	*Lactic acid yeast*
Cryptococcus sp.	*Mtsyr yeast*
Debaryomyces sp.	*Pichia sp.*
Hansenula saturnus	*Torulopsis tyrocola*
Torula lactosa	MOULDS
MOULDS	*Fusarium sp.*
Alternaria sp.	*Helminthosporium sp.*
A. Tenuis	*Humicola grisea var. thermodia*
Aspergillus awamori	*H. languinosa*
A. Flavus	*Macrophomina phaseoli*
A. foetidus	*Malbranches pulchella var. thermodia*
A. nidulans	*Mucor miehei*
A. Niger	*Mucor pusillus*
A. oryzae	*Neurospora crassa*
A. Phoenicus	*Neurospora sp.*
A. Sydowi	*Penicillium cheysogrnum*
A. Terreus	*P. funiculosum*
Cephalosporium sp.	*Penicillium sp.*
Cercospora sp.	*Pestalotia sp.*
Chaetominum thermophile var. corprophile	*Sclerotinia sp.*
	Sclerotinium sclerotiorum
Cladosporium sp.	*Sclerotium tuliparum*
Coniothyrium piricolum	*Sporotrichum sp.*
Coniothyrium sp.	*S. thermophile*
Corticium rolfsii	*Stemphillum sp.*
Curvularia sp.	*Stysanus sp.*
Curvularia inaequalis	

Adapted from Gekas, Lopez - Leiva *et al* [13] and Agarwal S.K *et al* [19]

number of thermophilic microorganisms producing β-galactosidase have been reported. Among the thermophilic bacteria, *B. stearothermophilus* [43], *Thermus aquaticus* [44], *Saccharopolyspora rectivirgula* [45] and thermophilic eubacterium *Rhodothermus maxinus* [46] have been reported to produce β-galactosidase. However β-galactosidase from **thermophilic yeast or fungi** are not common. Among thermostable β-galactosidases from thermophiles, the enzyme from *Solfolobus solfactiricus* has been studied in details and the crystal structure has also been determined [17,47-51].

ASSAY OF THE ENZYME:

The ability of hydrolytic enzymes to hydrolyze natural as well as synthetic substrates has led to the development of a variety of synthetic substrates for assaying their activities. Routine assays of β-galactosidase are generally carried out by following the hydrolysis of chromogenic substrates like *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG), *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG), or fluorogenic substrates like 4-methylumbelliferyl-β-D-galactopyranoside. The liberated *p*- / *o*-nitrophenol (indicated by appearance of a yellow colour) is measured spectrophotometrically, at 410 / 405 nm as the phenolate ion in alkaline pH (>10) [52,53]. When 4-methyl-umbelliferyl substrates are used, the enzyme is assayed on the basis of increase in fluorescence. The 4-methyl-umbelliferone released is determined fluorometrically using an excitation and emission wavelength of 365 nm and 440 nm, respectively [54-56], and it directly corresponds to the amount of galactose released. When lactose is used as the substrate the hydrolytic product, glucose, is determined by the glucose oxidase-peroxidase method [57], or by analyzing the end products by HPLC. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmole of product per min under standard assay conditions. When substrates like X-Gal (5-bromo-4-chloro-3-indol-β-D-galactopyranoside) are hydrolyzed, it causes the release of indoxyl or 5-bromo-4-chloro-3-indoyl chromophore characterized by a blue colour. It is commonly employed for the rapid detection of microorganisms

(bacteria) on agar plates, which show typical blue coloured colonies indicating the intracellular β-galactosidase where as the formation of a blue halo surrounding the secreting colony indicates the presence of extracellular β-galactosidase [58]. As the hydrolysis of synthetic substrates by these enzymes causes considerable increase in the fluorescence and / or absorption of the enzyme substrate mixture, a judicious choice of the synthetic substrates can also be employed for the detection of several reactions simultaneously [59,60].

PRODUCTION OF β-GALACTOSIDASE

Microorganisms have been considered as the most suitable source of enzymes for industrial applications, and the conditions required for the growth of the microorganism may not be the same as that required for the production of the enzyme. The production of β-galactosidase in bacteria, yeast and fungi has been investigated extensively [19], and the enzyme was found to occur intracellularly as well as extracellularly. In yeasts and bacteria, β-galactosidases occur intracellularly while the extracellular β-galactosidases have mostly been confined to fungi. The β-galactosidase is an inducible enzyme, produced in the presence of inducers like lactose [61,62] galactose [63], arabinose [64] or polygalacturonic acid [65]. However the constitutive production of β-galactosidases has also been reported in *A. oryzae* [66] *Bacillus stearothermophilus* [43] and *S.solfatricus* [47]. Addition of metal ions like Mn^{2+}or other stimulators like glycine / glycine methyl ester / glycine ethyl ester / glycenamide enhanced the production as well as release of the enzyme to the exterior of the cell [67].

Fungi produce the β-galactosidases extracellularly and can be grown on the surface of liquid media as well as under shaking conditions. Optimum production of enzymes requires the presence of right nutrients and physical conditions. With further developments in strain improvement techniques, efforts were made to improve the fungal cultures to give higher yields of products by use of selection, mutation, hybridization and other genetic techniques [68-70]. Most of the fungi require lactose as an inducer for the production of the enzyme [62,71-73]. There are numerous reports on the influence of environmental conditions on

production of β-galactosidase by moulds [74-76]. Traditionally β-galactosidases from bacteria, yeast and fungi have been obtained by submerged fermentation (in a liquid medium), using a suitable carbon and nitrogen source. In addition metal ions like Mg^{2+} or Mn^{2+} are also required for the production of the enzyme [2,13,19]. Besides these, factors like the initial pH of the medium, temperature for growth, and period of incubation also play a crucial role in the production of β-galactosidase. However it has been observed that many species of moulds produce β-galactosidase when grown on solid substrates like wheat bran, corn grits etc. or in a liquid medium containing suspended solid medium constituents [77,78].

Solid State Fermentation (Solid Substrate Fermentation) [SSF]:

This terminology is generally used to describe any fermentation process in which the substrate is not liquid. The concept of using solid substrate for fermentation is probably the oldest method used by man to make organisms work for him. The process of solid state fermentation has been derived from the Koji process, which involves the growth of moulds on cereals, grains or seeds [79]. The Koji preparations have been used for centuries as a source of exo-enzymes in almost all food fermentation carried out in Asian, African and other developing countries [80]. In recent years SSF has acquired considerable importance for the production of many commercial enzymes like cellulase [81], xylanase [82], amylase [83], polygalacturonase [84], β-glucosidase [85], protease [86], lipase [87], β-galactosidase [88] etc. Solid state fermentation has various advantages [89-91]. For e.g.

i) the medium and apparatus required for SSF is more simple as compared to that used in the conventional Submerged fermentation (SmF) method.

ii) As the moisture content in SSF is low it avoids the possibility of bacterial contamination.

iii) The products can be extracted in smaller volumes allowing in obtaining the products in a concentrated form. Solid state fermentation is of three types i.e.

1) In which the solid substrate is allowed to ferment without agitation.

2) With occasional stirring.

3) With continuos agitation.

The greatest possibilities are offered by solid state fermentation when fungi are used. Unlike other organisms, fungi typically grow in nature on solid substrates like pieces of wood, seeds, stems, roots, leaves etc. (materials that have low moisture content). Solid state conditions mimic this condition and hence are favorable for the propagation of fungi. Solid state fermentation has its limitations as well; e.g. bacteria can grow only when they are in a liquid phase or the nutrient medium must have free water present. For this reason, the bacterial fermentation is usually carried out in liquid media or semi-liquid substrates. Similarly yeast grow in fruit juices or simple sugars made by enzymatic digestion of cereal grains. But with progress in solid state fermentation techniques, the yeast [92] as well as bacteria [93] is also being used in solid state fermentation to obtain higher yields of the desired enzymes.

The best substrates for the SSF process are generally the agro-industrial residues like sugarcane bagasse, bran of wheat / rice / maize / gram, straws of rice / wheat, rice husk, soy hull, sago hampas, grapevine trimming dust, saw dust, corn cobs, coconut coir pith, banana waste, tea waste, cassra waste etc [87,94-97]. However among these wheat bran is the most commonly used substrate for the production of various enzymes by SSF process [98,99]. An ideal substrate is that which supplies all the essential nutrients required by the organism for its optimum growth, should be easily available, and cost effective. Besides these the particle size of the substrate is also an important factor to be considered for SSF process. Small size of the substrate particles provides larger surface area allowing better growth of the microorganism. However too small a substrate size results in substrate accumulation, which affects microbial respiration leading to poor growth. Similarly too large a substrate size helps in better respiration but provides a limited surface area for microbial growth. β-Galactosidases have been produced by SSF process from moulds like *A. fonsecaeus* [100] and *F. moniliforme* [101] using wheat bran as solid substrate. Besides moulds the yeast *K. lactis* has also been used for the production of β-galactosidase by SSF process using wheat bran and corncobs as solid substrates [92,102].

PURIFICATION OF β-GALACTOSIDASE

Since the discovery of the β-galactosidase and its importance in the hydrolysis of lactose for clinical purposes, the enzyme has been known to occur ubiquitously, and has been purified from many sources. The β-galactosidase has been purified by conventional methods using ammonium sulfate precipitation, ion exchange chromatography, gel filtration, isoelectric focusing, affinity chromatography, or using a combination of one or more of the above methods [103-106]. The β-galactosidase from *E. coli* has been purified in a single step by affinity chromatography on PAPTG-Sepharose [107]. More complex methods like HPLC [108] and FPLC [109] have also been used to obtain homogeneous preparations of the enzyme and for the purification of the isoforms.

BIOCHEMICAL PROPERTIES OF β-GALACTOSIDASE

The properties of β-galactosidase depend on its source and the characteristics of the enzyme from plants and animals have been described in various reviews [13,19]. β-Galactosidases from various microbial sources have often been observed to be an enzyme complex, consisting of several subunits and variable molecular weights. The other properties of the enzyme are generally observed to be quite similar, except that they differ characteristically in their pH optima and stability and the requirement of metal ions for the activity.

Optimum pH and temperature of β-galactosidases

It is generally observed that the β-galactosidases from bacteria and yeast have a pH optima in the neutral region (between 6.0-7.0 and 6.5-7.5, respectively) and are stable over a small pH range (6.5-8.0). The enzyme derived from fungi have their pH optima in the acidic range (2.5-5.5) and are stable over a comparatively broad pH range (3.0-8.0) [110]. The optimum temperature for the β-galactosidase from *E. coil* is 28°C, however the β-galactosidases from mesophilic and thermophilic organisms are thermostable and exhibit a much higher temperature optimum (50°-80°C) [111]. Among thermostable β-

galactosidases, those derived from thermophilic bacteria exhibit higher optimum temperature for enzyme activity as compared to those derived from thermophilic fungi, but the bacterial enzyme has a shorter half life (few minutes) [43] at these high temperatures as compared to the fungal β-galactosidases (few hours) [112,113].

Molecular Mass

β-Galactosidases obtained from various sources has been observed to be a complex of several subunits and large variation in the molecular mass has been observed. The *E. coli* β-galactosidase is a tetramer with identical subunits of Mr 116 kDa. Variations in the molecular mass of the *E. coli* β-galactosidase has also observed (540 kDa) [2] whereas the β-galactosidase from *E. coli* ML 308 had a M_r of 747 kDa [114]. The β-galactosidases from other bacterial sources had a molecular mass ranging between 150 kDa – 365 kDa [41,62,115-117].

The β-galactosidase from yeast *Kluyveromyces* sp. has a M_r of ~200 kDa and is a homodimer [118,119]. β-Galactosidases from basidiomycetous yeasts like *Serobasidium magnum* has a M_r of 67 kDa, [120], while that from *Cryptococcus laurentii* and *Sterigmatomyces elviae* are homodimers with subunit M_r 86 kDa and 100 kDa, respectively [72,121].

The β-galactosidases from fungi also show a wide variation in their molecular properties and the subunit composition. The enzyme from *Aspergillus niger* which is of commercial importance, has a M_r of 120 kDa [122], while those obtained from *Aspergillus fonsecaeus* [123], *Sclerotina sclerotium* [113], *Curvularia inaequalis* [124], *Penicillium canescens* [125] have M_r between 115 kDa – 130 kDa. Monomeric as well as dimeric β-galactosidases have been observed in fungi. The β-galactosidase from *Sclerotina sclerotium* (120 kDa) [113], *Mucor pusilius* (131 kDa) [126] and *Ophiostoma novo-ulmi* (135 kDa) [127] are monomeric while the enzyme from *A. oryzae* [66] and *Penicillium citrinium* [128] are dimeric. Among the β-galactosidases from thermophiles,

molecular masses of, 440, 150 and 240 kDa were observed for *Thermus 4-1A* [129], *Thermanaerobacter* [35] *and Solfolobus solfactiricus* [48], respectively.

The β-galactosidases obtained from various sources has been observed to be acidic in nature with a pI between 3.0-5.5 [2,13,19]. Presence of isoenzymes has been observed in case of *Thermanerobacter* [35], *Bacillus subtilis* [109], *B. circulans* [130] *Bifidobacterium* sp. [131] and *B. stearothermophilus* [43]. Four isoenzymes with molecular masses 630, 5540, 41 and 19 kDa were observed in *Kluyveromyces lactis* [132]. Multiple forms of the enzyme have also been reported in *E. coli* [2] as well as *Aspergillus sp.* [133].

Metal Ion

The metal ion requirement of β-galactosidases differs significantly with the source of the enzyme. Most of the β-galactosidases studied so far have been found to require metal ion for the activity and stability of the enzyme [2,134-136]. The activation or inhibition of the enzyme activity from different sources by mono- and divalent metal ions has been well documented [137,138]. The divalent metal ions, Mg^{2+} and Mn^{2+} are important for the activity and for the maintenance of the active site conformation [139,140]. In *E. coli* one Mg^{2+} is bound per subunit and Glu 461 is the ligand to which the metal ion binds. Substitution of Glu 461 with ligands that do not have a negative charge results in a decreased binding of the Mg^{2+} ion with a concomitant decrease in enzyme activity [141]. Besides Mg^{2+} and Mn^{2+}, monovalent ions like Na^{+} and K^{+} are also known to activate the β-galactosidases [2,142-144].

Enhancement of the hydrolytic activity of β-galactosidase in presence of Mg^{2+} was observed in *Lactobacillus delbruekii* subsp. *Bulgaricus* 20056, *Lactobacillus casei* 20094, *Lactococcus lactis* subsp. *lactis* 7962, *Streptococcus thermophilus* TS2, *Pseudococcus pentosaceus* PE39 and *Bifidobacterium bifidum* 1901 [145]. The β-galactosidase of *Lactococcus lactis* subsp. *lactis* 7962 was inactive in the absence of Mg^{2+} [145]. Activation of the β-galactosidase activity in presence of Mg^{2+} was also observed in case of *S. thermophilus* [138,146], *Lactobacilli* [147] and *Bifidobacterium* [148]. Various other metal ions like Ca^{2+}

have also been observed to have a positive effect on the enzyme activity [149], but metal ions like Zn^{2+}, Hg^{2+} or Cd^{2+} were observed to inhibit the enzyme activity [40,105,]. Ca^{2+} was observed to stimulate β-galactosidase activity in *Trichosporon cutaneum* [41], and restored the lost activity in *Enterobacter agglomerans biotype* 5 [134]. In plants, higher β-galactosidase activity was observed in the germinating seeds of *Trigonella foenum graceum* in the presence of Se^{2+} [150]. Although it has been hypothesized that, the activity of β-galactosidase, is affected by inducing conformational changes in the enzyme structure, (due to ionic radius of Na^{+} and K^{+} ions); the specific role played by the monovalent or divalent metal ions in the activity of β-galactosidase still remains to be investigated [151].

In contrast to the known β-galactosidases, the β-galactosidase from *Saccharopolyspora rectivirgula* was observed to be a monomeric enzyme with multimetal binding sites (Ca^{+}, Mg^{2+} and Mn^{2+}), and the presence of Mg^{2+} and Mn^{2+} was essential for the thermal stability of the enzyme for prolonged periods [18]. However it has been observed that the fungal β-galactosidases and the enzyme obtained from thermophilic sources do not require metal ions for activity or stability of the enzyme [152]. The tetrameric β-galactosidase of an archaeon *Sulfolobus solfataricus* [49] and the β-galactosidase from *Thermoanerobacterium thermosulfurigenes* EM1 [152] did not require any metal ion for the activity or stability of the enzyme activity. The sequence comparison of the β-galactosidase from *Thermoanerobacterium thermosulfurigenes* EM1 and *E. coli,* suggested that the non requirement of the metal ions for activity or stability of the β-galactosidase from *Thermoanerobacterium thermosulfurigenes* EM1, could be due to the absence of metal binding sites in *Thermoanerobacterium thermosulfurigenes* EM1 [152].

Substrate Specificity

The main catalytic activity of β-galactosidase is the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides, oligosaccharides,

polysaccharides as well as in glycoproteins and glycolipids [2]. Although lactose is the natural substrate of β-galactosidase, the enzyme also hydrolyzes a wide range of synthetic substrates like methylumbelliferyl linked galactosides [54-56], *para* or *ortho* nitrophenol-β-D-galactopyranoside (*p*NPG, *o*NPG) etc.; with a higher affinity for the synthetic substrates as compared to lactose [2,110,142,153-156]. Many other chromogenic probes have also been used as substrates of β-galactosidase [157-160].

β-Galactosidase is known to have two subsites for the binding of the substrate one for galactose and one for glucose [161,162]. The galactose site has high affinity for galactose, but also binds to galactose linked to hydrophobic sugar groups that are attached via a β- glycosidic bond like IPTG, with a higher affinity. The glucose site in the free enzyme has a poor affinity for glucose, but after cleavage of the glycosidic bond, the affinity for glucose increases after cleavage of the glycosidic bond. Sinnott has suggested a role for conformational changes at the active site, which could be responsible for this increased affinity towards glucose [161,162].

The substrate specificity of β-galactosidase of *E. coli* has been investigated and it exhibits a wide tolerance to structural variations in the glycone moiety [2]. Monosaccharides or alcohols with hydroxyl groups at position 3, 4 and 6, and in the same orientation as that of galactose, binds well to the galactose site. Any alterations in these positions or absence of hydroxyl group at these positions results in decreased binding to the enzyme [163]. Although many substrates bound well to the enzyme, they were not hydrolyzed. The inability of the β-galactosidase of *E. coli* to hydrolyze p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-L-arabinofuranoside, p-nitrophenyl-β-D-xylopyranoside, cellobiose and gentobiose further indicated that the presence of hydroxyls as well as their configuration at positions 3 and 4 is important in catalysis [163].

Besides the natural substrate lactose, galactooligosaccharides and fragments of plant cell wall polysaccharides may also act as biological substrates of some of the β-galactosidase from fungi [164]. The enzyme derived from *Tritrichomonos foetus* hydrolyzed lacto-N-biose 1 (Gal (β-1-3- GlcNAc) and N-

acetyllactosamine (Gal (β-1-4 Glc Nac) [37]. β-Galactosidase from *Sphingomonos* sp. and *A. oryzae* hydrolyzed lactobionic acid [165]. The plant β-galactosidase (*Nasturtium*) hydrolyzed terminal non-reducing β-D-galactopyranosyl residues from xyloglucan but not from seed galactomannan. Besides, the increased β-galactosidase activity (in-vitro) and concomitant decrease in the xyloglucan levels suggests that xyloglucan could be the natural substrate of β-galactosidase in *Nasturtium* [166]. β-Galactosidase from rice shoots hydrolyzed arabinogalactan in an exo-fashion, and weakly hydrolyzed arabinosyl and galactosyl residue-rich polymers of the pectic polysaccharide from cell walls of the rice shoot [167].

The β-galactosidases in some cases exhibit a high specificity for β (1-4) linked galactosidic linkages and for the structure of the glycon moiety of the disaccharide [2,154,155,168,169]. The β-galactosidase from *A. nidulans* was specific for β-galactosides like lactose and *p*NPG while the β-galactosidase from *Arthrobacter* hydrolyzed β (1-4) or β (1-3) linked substrates [36,73]. Associated activities like β-D-glucopyranosidase and α-L-arabinopyranosidase activity along with the β-galactosidase activity are also known to be present in some microbial β-galactosidases [164]. The β-galactosidase from *Paecilomyces varioti* hydrolyzed cellobiose and gentobiose along with *p*NPG [170].

ACTIVE SITE RESIDUES AND 3-D STRUCTURE

Glycosidases play a key role in numerous biological processes and are of importance in clinical and biological applications. The knowledge of catalytically important active site residues is critical in understanding the mechanism of action, classification of the enzyme, and in targeted bioengineering of the enzymes with altered characteristics. Various methods as well as novel strategies have been used for the identification of key active site residues in glycosidases e.g. by 3-D structure analysis, using group specific labels, by derivatization with affinity labels, by using mechanism based inhibitors, specific labeling of the active site nucleophile or tandem mass spectroscopy [171]. Although numerous gene

sequences coding for glycosidase have been determined [172-178], the biochemistry and structure of these enzymes is yet being understood. The catalytic residues can also be predicted on the basis of the gene sequences [179-181]. Among the active site functional groups identified in glycosyl hydrolases, the carboxylate ion is an integral part of the active site of those enzymes in which acid-base type catalytic mechanism is prevalent [182]. The possibility that all glycosidases share a common mechanism of catalysis has been investigated intensively for various glycosidases of which the hen egg white lysozyme is a classic paradigm [183].

Glu and Asp residues are commonly found at the catalytic center in glycosyl hydrolases either as proton donors (in their protonated form) or as nucleophiles or oxocarbonium stabilizing agents (in their charged forms) [182]. In most of the β-galactosidases the amino acids important for catalysis have been identified from their deduced amino acid sequences and generally a single Glu and a single Tyr have been found to be conserved. The carboxylate residue is found to be conserved in almost all of the β-galactosidases sequenced so far [172-178]. Gene homology studies of *Lactobacillus lactis* ssp. *Lactis* ATCC 7962 with other reported β-galactosidases, has indicated that Glu 429 and Tyr 475 are conserved suggesting their in the active site of the enzyme [184].

Despite the early crystallization of the β-galactosidase in 1961 [168], the 3-D crystal structure has been resolved only lately [185], Fig 1a, 2a. The structure of β-galactosidase from *E. coli* (Fig 1) shows that the protein is a tetramer with a 2,2,2, point symmetry. Each monomer is made up of 1,023 amino acid polypeptide that folds into 5 sequential domains, and an extended segment at the amino terminus contributes to the activating interface. The 3-D structure of *E. coli* β-galactosidase shows that active site residues, Glu 461, Met 502, Tyr 503 and Glu 537 are located close together and are at the end of the TIM barrel which is identified as the substrate binding site [185]. This TIM barrel is the third domain and is distorted. Most TIM barrels usually consist of eight α/β repeats, however in the *E. coli* β-galactosidase, the fifth α/β helix is missing. Among homologous β-galactosidases, these residues that form the active site pocket, are found to be

highly conserved. These residues were also previously shown to be important in the catalytic function of the enzyme by various other methods [186-189].

Besides these residues, other residues were also observed to play a role in the catalytic activity of β-galactosidase. Cys, Met and His were observed to be important in the catalytic activity of β-galactosidase from a *Bacillus* sp. that also had transgalactosylation activity [190]. Glu 268 has been identified as the catalytic nucleophile of the human lysosomal β-galactosidase precursor [189].

It was also observed that all the proteins included in clan GH-A glycosyl hydrolases (which includes β-galactosidase) share a similar catalytic domain consisting of a $(\alpha/\beta)_8$ barrel with conserved functional amino acids at the C-terminal of six of the eight strands constituting the barrel [191]. A structural comparison of TIM barrel proteins suggests a functional as well as evolutionary relation between β-galactosidase and other glycosidases. It has been proposed that the β-galactosidase arose from a prototypical single domain α/β barrel with an extended active site cleft. The reduction in size of the active site from a cleft to a pocket could possibly have occurred for better hydrolysis of lactose as well as the to facilitate the production of allolactose, the inducer of lacZ β-galactosidase as proposed by Juers *et al* [192]. It has also been observed that in case of retaining glycosyl hydrolases, the 2 carboxyl groups are close together (4.5-5.5 Å) as compared to the inverting enzymes (9-9.5 Å) [193]. A single point mutation at these residues can convert a retaining glycosidase to an inverting one [194,195].

Besides the *E. coli* β-galactosidase, the X-ray crystal structure of a thermostable β-glycosidase which also hydrolyses X-gal, from a hyperthermophilic archeon *Solfolobus solfataricus* has also been resolved and the features responsible for the thermostability of the enzyme have been suggested (Fig 1b, 2b) [51]. The authors suggest an important role for surface ion-pair and buried solvent networks along with hydrophobic interactions to be responsible for added resistance to denaturation by increasing the kinetic barrier to unfolding [51]. The *Solfolobus solfataricus* β-galactosidase is also a tetramer with a 222 point symmetry, and shows a typical $(\alpha/\beta)_8$ barrel fold which has been predicted

Fig. 1a: 3-D structure of *E. coli* β-galactosidase depicting the interaction between the active dimer and the composition of the activating interface. **(Ref: *Jacobson* et al. Nature (1994), 369, pp. 764)**

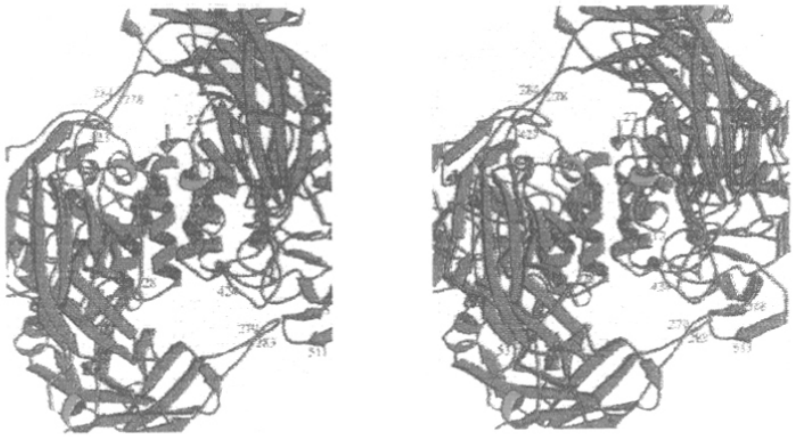


Fig. 1b: Secondary structure of β-glycosidase of *Solfolobus solfactaricus* (222 tetramer) indicating the interaction between the subunits. **(Ref: Aguilar *et al.* J. Mol. Biol. (1997), 271, pp. 792)**

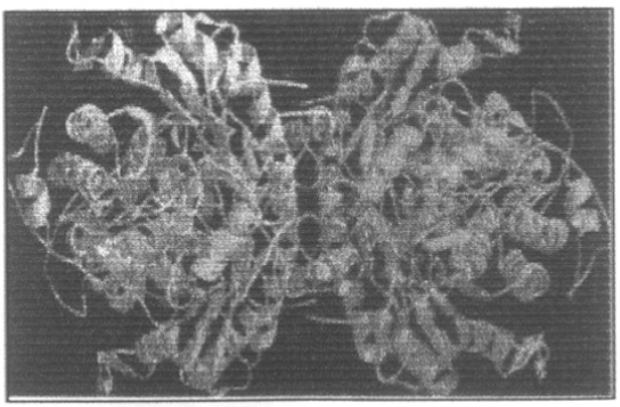


Fig 2a: The active site residues (Glu 461, Glu 537, Tyr 503 of β-galactosidase of *E. coli* important for catalysis seen at a resolution of 3.5 Å. **(Ref: Jacobson *et al.* Nature (1994), 369, pp. 763)**

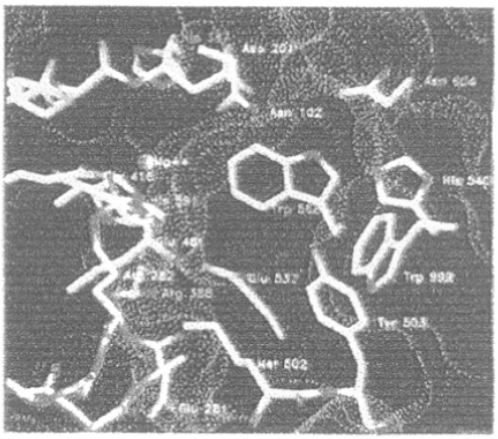
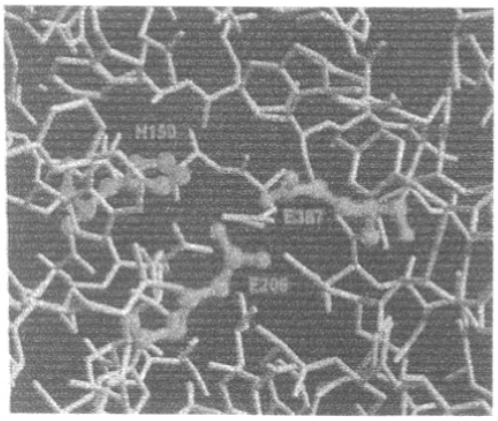


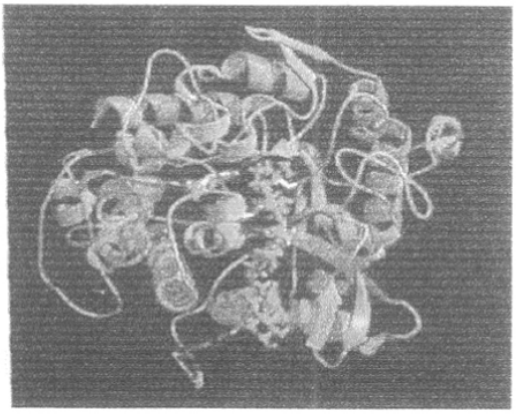
Fig. 2b: i) Active site residues of *Solfolobus solfataricus* β-glycosidase showing the conserved nucleophile (Glu 387) and side chains of Glu 206 and His 150, which participate in catalysis.

ii) The monomer of the *Solfolobus solfataricus* β-glycosidase showing a model of the ligand β-1-4 linked cellohexaose molecule lodged into the substrate binding site.
(Ref: Aguilar *et al.* J. Mol. Biol. (1997), 271, pp. 795)

i)



ii)



for the clan GH-1A family of β-glycosidases [196-198]. The $(\alpha/\beta)_8$ repeat was first observed in case of triose phosphate isomerase [199] and has also been observed in the structures of cyanogenic β-glucosidase from *Trifolium repens* [200] and β-glucosidase from mesophilic bacterium *Lactobacillus lactis* [201]. The active site residues are located at the center of the top face of the barrel and are connected to the surface by radial channel that acts as the substrate binding site [200,201].

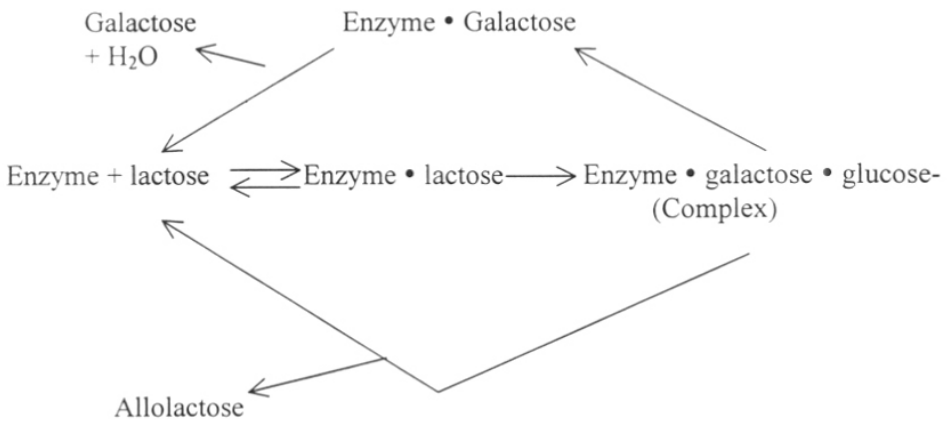
MECHANISM OF ACTION OF β-GALACTOSIDASE

Glycosidases can be separated into two classes depending on the mechanism of action as **i)** Inverting glycosidase and **ii)** retaining glycosidase. All glycosyl hydrolases are thought to act by a general acid catalysis mechanism in which two amino acid residues participate in the direct displacement of the leaving group by water (inverting mechanism), or a double displacement reaction, involving a glycosyl-enzyme complex, resulting in retention of configuration at the anomeric carbon of the hydrolyzed glycoside [161].

β- Galactosidases are also retaining enzymes where the hydrolysis of lactose occurs via formation of enzyme-glycosyl intermediate and the liberation of glucose. This intermediate is further hydrolyzed with involvement of a water molecule to form galactose and free enzyme [201]. The galactosyl-enzyme complex can be intercepted by nucleophiles (acceptors) other than water also [202]. An interesting feature for β-galactosidases is the intramolecular transfer of galactose, which yields allolactose, the natural inducer of Lac operon [2,203]. In the hydrolysis of lactose, the monosaccharides (galactose and glucose) are formed with the release of a water molecule. Whereas in the transfer reaction the sugar molecule formed from hydrolysis (glycone moiety) is transferred to an acceptor molecule containing a hydroxyl group, like alcohol or other sugar molecule present in the reaction mixture leading to the formation of oligosaccharides [3,204].

The figure below represents the accepted scheme for the reaction of β-galactosidase put forth by Huber *et al* [205]. The allolactose formed is a transient product as it is also a substrate of the enzyme. During lactose hydrolysis, about half the lactose molecules are isomerised to allolactose, the natural inducer of the lac operon [2,205].

Hydrolysis reaction



Transfer reaction

The catalytic action of β-galactosidase, involves a mechanism in which non-covalent interactions, a general acid-base catalysis as well as electrostatic forces play an important role [205]. Sinnott and Souchard have suggested that conformational changes (induced by substrate) occur at the active site, which could be of non-covalent nature [206]. This hypothesis is supported by the observation that β-galactosidase shows higher activity with certain substrates in the presence of Mg^{2+}, while with other substrates higher activity was observed in the presence of Mn^{2+}. This difference in activities could be due to the difference

in conformation causing different orientation and therefore different non-covalent interactions [205].

The active site of β-galactosidase from *E. coli* shows the presence of two groups, one with a pKa of 8.0 (in presence of positively charged inhibitors) and the other with a pKa of 9.4 (in presence of neutral inhibitors), indicating that tyrosine could be the acid-base catalyst [207]. A Tyr equivalent to that at position 503 was found to be conserved in every β-galactosidase for which a sequence has been determined [172-178,208,209]. Replacement of Tyr with His resulted in a change of the pH profile of the enzyme and His was observed to act as the base catalyst [205].

Glu 461 was observed to be important for the electrovalent or covalent catalysis due to the presence of a negative charge on it. Replacement of Glu 461 with groups devoid of negative charges, resulted in a loss of enzyme activity [205]. Kinetic studies using substrates with Deuterium in the β-position of the anomeric carbon of galactose indicated the formation of a galactosyl cation which was stabilized by a negative charge indicating the importance of the negative charge in electrostatic catalysis [206].

PHYSIOLOGICAL ROLE OF β-GALCTOSIDASES

The proposed function of β-galactosidase in the development of microorganisms is in mobilizing storage food and as a degradative enzyme in the ultimate breakdown of diasaccharides or oligosaccharides. In majority of the cases the enzyme is intracellular in nature but it is also secreted extracellularly. Induced secretion of β-galactosidase by polygalacturonic acid indicates that the enzyme may also be involved in the utilization of complex carbohydrate substrates [65].

Endogenous plant β- galactosidase have been implicated in the autolysis of cell wall polymers during fruit ripening. [210]. The process of fruit softening results in the modification of cell wall carbohydrates by the action of wall-associated enzymes like polygalacturonase, cellulase and β-galactosidase [211-214] during ripening. An increase in the activities of these enzymes is observed

about 2 weeks prior to fruit maturity [215]. The involvement of β-galactosidase and its isoforms in the modification of cell-wall components during ripening has been established in musk melon [214] *Cicer arietinum* [216], tomato [217], mango [218] and hot peppers [219]. These isoforms may be distributed differently in relation to tissue position and their activity changed differently during ripening [220,221]. The three isoforms of β-galactosidase in the fruit of d'"Anjour" pear (*Pyrus communis L.*) were observed to possess distinct regulatory mechanism during ripening [222]. The ability of β-galactosidase to degrade pectic polymers from the cell wall was observed in case of carrot [223] and coffee bean during ripening [224].

β-Galactosidase also occurs in multiple forms in the cotyledons of germinating seeds of barley (*Hordeum vulgare*) and *V. radiata* and the multiplicity was not as a result of differential glycosylation [225]. The β-galactosidase from Spartan apple fruit was observed to be multifunctional with associated α-L arabinopyranosidase and β-O-fucosidase activities along with β-galactosidase activity [226]. The β-galactosidase has been observed as one of the predominant proteins in the intracellular fluid of tomato leaves infected with Tobacco Mosaic Virus (TMV) [227]. β-Galactosidase having an exo – 1 –4 β- D galactanase activity is also involved in the mobilization of cell wall storage galactan from *Lupinus angustifolious* germinating seeds [228].

The role of β-galactosidase as a putative virulence factor has been investigated very superficially. In humans the deficiency of β-galactosidase results in a number of well defined inherited metabolic disorders. There are four well defined activities, two lysosomal or acid enzymes which are genetically distinct [229], a neutral β-galactosidase and an intestinal membrane-bound enzyme also known as 'lactose phlorizin hydrolase'. Deficiency of one of the lysosomal enzymes causes GM1 gangliosidosis while the other causes Krabbe's disease [230]. The lysosomal GM1 β-galactosidase exists as a complex of β-galactosidase, neuraminidase and a protein, which protects the complex against hydrolysis by lysosomal proteinase [231-235]. The membrane bound β-

galactosidase is responsible for splitting of lactose in the diet into glucose and galactose in adults; and its deficiency causes lactose intolerance [236]. GM1 gangliosidosis is primarily a neurological disorder causing central nervous system manifestations, facial and skeletal abnormalities and viseromegaly. The cDNA coding for the human and rabbit β-galactosidase (cloned and sequenced) shows that the protein has two independent active sites. One of them hydrolyzes lactose while the other splits a wide range of β-glycosides. Both these active sites could be labeled with conduritol B epoxide suggesting that both enzymes could have been evolved by gene duplication, in turn indicating that these two enzymes may have a common precursor [237].

APPLICATIONS OF β-GALACTOSIDASE

β-Galactosidase is one of the most well studied enzymes with respect to its properties, reaction mechanism as well as the 3-D structure. This enzyme is of immense importance mainly because of its application in the preparation of lactose free milk and milk products for Non-Caussian persons. Moreover enzyme immobilization techniques have opened many new and interesting possibilities for its utilization in food processing [13]. Further, the presence of lactose in whey causes environmental pollution problems, which has emphasised the importance of hydrolysis of lactose and in turn the enzyme lactase (β-galactosidase), as enzymatic hydrolysis is preferred over the other methods. The lactose hydrolyzed products have varied uses for e.g. the sweet syrup prepared from whey can be used as a source of sugar in some cases as well as a source of protein in the bakery, in confectionery, in soft fruit drinks, icecreams, feed stuff for cattle instead of molasses or dairy desserts [8,12]. Hydrolyzed and demineralized lactose syrup has been used in ice-cream mixes as a substitute for sucrose [9]. An orange flavored beverage prepared from hydrolyzed and deproteinized cheese whey has been recommended as a shelf stable drink for athletes [9]. The product of whey hydrolysis is also being used to grow baker's yeast [238].

However, only those enzymes produced by microorganisms generally regarded as safe (GRAS) are useful in the food industry. β-Galactosidase obtained

from *Kluyveromyces* (yeast) and *Aspergillus* (fungus) are acceptable for the hydrolysis of milk lactose and other milk products [9,13,239]. Although the yeast and fungi have been exploited for commercial purposes, not all lactase sources are acceptable (GRAS) for the use in food systems. The lactase from *E-coli*, although most investigated is not used in food processing because of its high cost and due to the toxicity problems (enteric) associated with the crude extracts of coliforms [146]. The fungal enzyme, especially *Aspergillus* sp. is commercially important source of the enzyme as it has been accepted for use in the food industry and its extracellular nature is an added advantage for industrial use [66,239].

Galacto-oligosaccharides generated by the transglycosylase activity of exo-β-galactosidase and the hydrolytic activity of endo β-galactosidase serve as growth promoting factors, especially for the genus *Bifidobacterium* (intestinal bacteria) that plays an important role in maintaining human health [240].

As the assay system for β-galactosidase is an easy one that makes use of readily available synthetic substrates it has been employed in the rapid detection of certain essential trace elements like 'Selenium' (Se^{2+}). Se^{2+} is present at the active site of several prokaryotic and eucaryotic proteins called selenoproteins. These selenopoteins are important for their application in human health as well as a new charactersitc of the genetic code, and can be detected on agar plates [241]. Incorporation of Selenium into the proteins has been followed by using recombinant DNA construct in which the expression of Lac Z gene in *E. coli* is proportionally and specifically driven by UGA-directed selenocysteine incorporation [241].

β-Galactosidase gene of *E. coli* has often been used as a marker protein in fusion proteins to detect the expression of various gene products of interest. However the insuitability of the *E. coli* food grade cloning (to meet GRAS safety standards) has been overcome by using the β-galactosidase from lactic acid bacteria (designated GRAS status) which is now being used for the purpose [242].

Objectives of the present investigation

β-Galactosidase (β-D-galactohydrolase E.C. 3.2.1.23) which catalyses the hydrolysis of lactose to glucose and galactose is a commercially important enzyme used in dairy and food industry. Though ubiquitous in distribution, the enzymes of commercial interest are produced from mesophilic yeast and fungi. Since thermophilic organisms grow at high temperatures, their use not only helps to avoid microbial contamination but also permits enzyme production in a shorter time. Therefore, there is a need to look for thermophilic strains capable of producing β-galactosidase. Thermophilic fungi have an added advantage since they produce the enzyme extracellularly. A thermophilic strain of *Rhizomucor* sp. which showed extracellular β-galactosidase activity when grown at pH 6.8 and 45°C was selected for the present studies. Investigation was carried out to a) optimize the medium for maximum enzyme production b) purify and extensively characterize the enzyme to understand its structure-function relationships.

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CHAPTER II

PRODUCTION OF β-GALACTOSIDASE FROM *RHIZOMUCOR* SP.

SUMMARY

An isolate of *Rhizomucor* sp. which produced extracellular β-galactosidase when grown at pH 6.8 and 45°C, under submerged conditions was selected for the present study. Maximum enzyme level (0.55 U/ml) was observed in 4 days when the organism was grown in a medium containing (w/v) yeast extract, 1 %; lactose, 1%; K_2HPO_4, 0.1 % and $MgSO_4$, 0.05 %. Organic nitrogen supplements like tryptone, peptone, yeast extract and casamino acids supported growth of the fungus while yeast extract and peptone supported growth and enzyme production. Inorganic nitrogen supplements by themselves could not support the growth of *Rhizomucor* sp. in the absence of organic supplements. Unlike mesophilic fungi, *Rhizomucor* sp. did not require divalent metal ions for growth and enzyme production. Solid state fermentation studies at 45°C in a medium containing (w/v) wheat bran, 10 %; lactose, 5.0 %; K_2HPO_4, 1 % and $MgSO_4$, 0.5 %, yielded 5.05 U/ml of enzyme activity in 5 days. Comparison of submerged and solid state fermentation revealed that the latter yielded higher enzyme levels (5.05 U/ml in solid state compared to 0.55 U/ml in submerged fermentation).

INTRODUCTION

A variety of plant, animal and microbial sources have been used for the production of industrial enzymes like papain, chymotrypsin, trypsin & β-amylase [1,2]. However, microbial sources are preferred for the production of many of these industrial enzymes due to various advantages such as purity, consistency, economy, higher production yields, ease of process modification, use of cheap and readily available substrates and technology, as well as easier stepping up of production [3]. Among them fungi are favored since they secrete the enzyme extracellularly. Enzymes traditionally obtained from plant and animal sources are being gradually replaced by microbial enzymes to overcome the limited availability of plant and animal tissues and about 80 % of the total enzyme production is now being met by microbial sources [3,4].

β-Galactosidase (β-D-galactohydrolase E.C.3.2.3.23) that catalyses the hydrolysis of β-1-4 linkage in lactose to form glucose and galactose, is a commercially important enzyme with varied applications and its use in the dairy and food industry is well documented [5,6]. The occurrence of the enzyme is diverse i.e. in plants, animals and microorganisms [7]. Most microorganisms produce β-galactosidase intracellularly e.g. *Kluyveromyces lactis* [8] and *Saccharopolyspora rectivirgula* [9], where as the enzyme produced by fungi is generally extracellular e.g. *Penicillium citrinum* [10], *Penicillium notatum* [11], *Fusarium moniliforme* [12], *Mucor* sp. [13] and *Aspergillus oryzae* [14]. β-Galactosidase derived from mesophilic organisms are thermotolerant [15-17], but those derived from thermophilic sources are preferred because of their various advantages due to greater thermostability, e.g. the higher temperatures required for reaction procedures reduces viscosity of reaction mixtures and higher rates of reaction can be achieved [18,19]. Enzymes from microbes are generally produced by submerged fermentation in liquid medium or by solid-state cultivation employing solid materials like wheat bran, corn grits, broken pieces of food grains etc.

In recent years, solid state fermentation (SSF) has acquired importance because of its economical and operational advantages, for example, the low

moisture content prevents bacterial contamination and can be extracted easily in a concentrated form [20-23]. Besides these, another important advantage of solid state fermentation is that, most of the secondary metabolites which are produced in extremely low yields in submerged fermentation can be produced in comparatively large amounts by this method [24,25]. Cultivation of the organism by solid state fermentation imparts certain desirable and unique characteristics to the product, which are usually absent when the product is obtained by submerged fermentation [26]. Moreover, the conditions in which the fungus grows in solid state are similar to its natural growth conditions [24] and very simple equipment is required as compared to the conventional submerged fermentation. β-Galactosidase is being produced mainly by submerged fermentation and only few reports elaborate on semi-solid fermentation and solid state fermentation [12,26]. A system for production of β-galactosidase from yeast (*K. lactis*) by solid state fermentation has been reported [27].

The present chapter describes the studies on the optimization of fermentation parameters for production of β-galactosidase by *Rhizomucor* sp. under submerged and solid state fermentation.

MATERIALS AND METHODS

Yeast extract, malt extract, peptone, tryptone, lactose and liver extract were obtained from Hi-Media Laboratories, India. *p*-Nitrophenol-β-D-galactopyranoside (*p*NPG), *o*-nitrophenol-β-D-galactopyranoside (*o*NPG) and lactose were purchased from Sigma Chemical Company, USA. Wheat bran was obtained locally. All other chemicals used were of analytical grade.

A *Rhizomucor* sp. exhibiting extracellular β-galactosidase activity was selected for this study. The culture was routinely maintained on potato-dextrose-agar (PDA) slants (prepared by suspending extracts from potatoes, 200 g, glucose, 20 g and yeast extract, 1.0 g and agar 20 g in one liter of distilled water). The *Rhizomucor* sp. is deposited with the National Collection of Industrial Microorganisms (NCIM) under the accession no. 1253.

Enzyme production

a) *Preparation of inoculum*

The inoculum was prepared by suspending the spores of *Rhizomucor* sp. from a 7- day old well-sporulated slant maintained on PDA, in 20 ml sterile distilled water. The number of spores was counted using a haemocytometer. The concentration of spores was adjusted to 10^6 spores per ml and one ml of this suspension was used as the inoculum.

b) *Submerged fermentation (SmF)*

The basal medium for submerged fermentation contained (g/L), yeast extract, 10; lactose, 10; K_2HPO_4, 1.0 and $MgSO_4.7H_2O$, 0.5. The medium (50 ml) was dispensed in 250 ml capacity Erlenmeyer flasks and sterilized (121°C, 15 lbs, 20 min). 1.0 ml of the spore suspension (containing 10^6 spores) was used as inoculum per flask. The flasks were incubated on a rotary shaker at 45°C at 200 rpm. After the fermentation period, the mycelium was removed from the culture broth by filtration through Whatman No. 1 filter paper under vacuum and the clear filtrate was used as the crude enzyme.

c) *Solid State Fermentation (SSF)*

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks, each containing, wheat bran, 9.5 g; lactose, 0.5 g; K_2HPO_4, 0.1 g; and $MgSO_4.7H_2O$, 0.05 g moistened with 20 ml distilled water. After sterilization (121°C, 15 lbs., 20 min), each flask was inoculated with 1.0 ml of spore suspension (containing 10^6 spores) and the flasks were incubated at 45°C.

Extraction of enzyme

The enzyme obtained from SSF was extracted by suspending the solid material (from each flask) in 50 ml citrate-phosphate buffer (100 mM, pH 4.5) containing 0.85 % (w/v) NaCl. The suspension was kept shaking (150 rpm) for 90 min at 45°C. The mycelium and other solid material was separated from the

extraction medium by filtering through muslin cloth followed by Whatman No. 1 filter paper. The clear filtrate was used to determine β-galactosidase activity as described below.

Determination of β-galactosidase activity

β-Galactosidase was assayed by incubating 25 µl of suitably diluted enzyme with 50 µl of 6.6 mM *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG) or *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and 925 µl of 20 mM citrate-phosphate buffer, pH 4.5 at 60°C for 30 min. The reaction was terminated by adding 1.0 ml of 0.5 M Na_2CO_3 and the *p*-nitrophenol or the *o*-nitrophenol released was determined by reading the absorbance at 405 / 410 nm respectively. When lactose was used as the substrate, the enzyme activity was assayed using a glucose oxidase-peroxidase kit to determine the amount of glucose liberated [28]. One unit of β-galactosidase activity (U) was expressed as the amount of enzyme that releases 1.0 µ mole of product (*p*-nitrophenol / *o*-nitrophenol / D-glucose) per minute under standard assay conditions.

Determination of protein

The protein in the culture filtrate was determined by mixing 0.5 ml of the culture filtrate with 0.5 ml of 20.0 % trichloroacetic acid (TCA). The mixture was kept in ice for 30 min and the precipitated protein was collected by centrifugation (7,000 **g,** 5 min). The protein was washed with chilled acetone, redissolved in 0.1 N NaOH and the concentration determined by the method of *Lowry* et al [29] using bovine serum albumin (BSA) as standard.

Estimation of biomass in submerged fermentation and solid state fermentation

The mycelium obtained from submerged fermentation was seperated from the fermentation medium by filtering through Whatman No. 1 filter paper. The mycelium was washed with distilled water 3 – 4 times and dried at 60°C till constant weights were obtained and the biomass was essentially hydrolyzed according to the method of Nishio [30]. The dried material (0.5 gm) was suspended

in 5.0 ml 6 N HCl, sealed under vacuum and subjected to hydrolysis at 100°C for 8 hrs. The hydrolyzed sample was centrifuged on cooling and the supernatant was neutralized with NaOH. The glucosamine in the sample was estimated by the method of Blix [31]. The mycelium along with the wheat bran obtained after fermentation, was washed thoroughly with distilled water to remove any remaining lactose or its hydrolyzed end products and dried at 60°C till constant weight was obtained. 0.5 gm of the dried material was hydrolyzed and the glucosamine was determined as described above. Un-inoculated wheat bran treated under similar conditions served as control.

Determination of amino sugars

Amino sugars were estimated by the method of Blix [31]. One ml aqueous solution of hexosamine (0.1-1.0 μ mole) was mixed with 0.2 ml of acetic anhydride and 1.0 ml of sodium borate buffer (100 mM, pH 8.5). The tubes were sealed and incubated in a boiling water bath for 3 min. The mixture was immediately cooled in ice for 5 min. and mixed with 2.0 ml (1.2 %) of *p*-dimethylaminobenzaldehyde (DMAB) and incubated at 37°C for 20 min. The pink colour developed was read at 585 nm.

Scanning Electron Microscopy (SEM)

The growth and morphology of *Rhizomucor sp.* during solid state fermentation was studied by SEM. Samples for SEM were fixed with ultra-violet light and mounted on brass stubs. Specimens were then coated with a thin layer of gold (100° A) in a gold coating unit, model E 5000, Polaron Equipment Ltd., and viewed with a SEM Leica Stereoscan 440, at an accelerating voltage of 10 kV, and beam current 25 Pa. The photographs were recorded by 35 mm camera attached to the high-resolution recording unit.

RESULTS AND DISCUSSIONS

The thermophilic fungus *Rhizomucor* sp. that grew at 45°C, showed, extracellular β-galactosidase activity and the optimum conditions for growth and

production of the enzyme were established. The production of β-galactosidase from molds has been investigated by many workers [32-34] and processes for the production of the enzyme have been patented [35-37]. It has also been observed that the enzyme is inducible [38] and that the conditions required for growth may not be the same, as that required for production of the enzyme [39].

SUBMERGED FERMENTATION

Effect of carbon sources on the production of β-galactosidase: The effect of various carbon sources on the growth and production of β-galactosidase from *Rhizomucor* sp. is shown in Table 1. All the carbon sources examined supported the growth of the fungus. However, β-galactosidase was produced only when lactose was used as the sole carbon source. Glucose, sucrose, maltose, xylose, fructose and mannose did not induce the production of β-galactosidase, while low enzyme activity was observed in presence of galactose. Maximum enzyme activity (0.55 U/ml) was obtained in a medium containing 1.0 % (w/v) lactose and in further experiments the same concentration of lactose was used in the production medium.

β-Galactosidases are produced by most organisms in presence of lactose, however constitutive production of the enzyme has been observed in case of *A. oryzae* [14], S*copulariopsis* sp. [40] and *Bacillus stearothermophilus* [41], where lactose in the medium was utilized during the growth but did not stimulate the enzyme production. Besides lactose, galactose, mellibiose and polygalacturonic acid have also been known to induce the production of the enzyme [42,43]. The production of intracellular β-galactosidase was enhanced in presence of lactose and D-galactose in *K. fragilis* [44] and the yeast like fungus *Aureobasidium pullulans* [45]. However in case of *A. pullulans* and *Sclerotina sclerotium* [46], it was observed that extracellular β-galactosidase was secreted when it was grown in a medium containing polygalacturonic acid.

Effect of nitrogen sources on production of β-galactosidase: The influence of various nitrogen sources on production of β-galactosidase in submerged

fermentation is summarized in Table 2. All the organic nitrogen sources except malt extract supported growth of the *Rhizomucor* sp. Although tryptone, casamino acids and liver extract supported the growth of the microorganism, they did not support enzyme production. β-Galactosidase was obtained in good yields (0.55 U ml^{-1}) in the medium containing either yeast extract (1 % w/v) or peptone (1 % w/v). None of the inorganic nitrogen sources tested, supported the growth of *Rhizomucor* sp. when used in the absence of an organic nitrogen source under submerged fermentation conditions. However in case of *P. notatum* [11], the inorganic N_2 sources ammonium hydrogen phosphate ($NH_4H_2PO_4$) and ammonium sulfate [$(NH4)_2SO_4$] were found to be essential for the production of β-galactosidase, whereas organic nitrogen sources like peptone and urea inhibited the enzyme production. Sodium nitrate ($NaNO_3$) was observed to be the best nitrogen source for the production of β-galactosidase from *A. pullulans* [45]. Wheat bran extract when used as nitrogen source along with the supplementation of 1.0 % (w/v) lactose, supported the growth of the organism as well as production of the enzyme, in smaller amounts as compared to yeast extract or peptone in *Rhizomucor* sp. The production of β-galactosidase in wheat bran medium has been reported in case of *A. oryzae* [14] and *Scopulariopsis* sp.[40].

Effect of metal ions on growth and production of β-galactosidase: The effect of various divalent metal ions (2 mM) on the production of β-galactosidase from *Rhizomucor* sp. is shown in Table 3. The growth of the fungus was completely inhibited in presence of Co^{2+}. The production of the enzyme was considerably inhibited in presence of Mn^{2+} and` $Zn^{2+}$, although they supported the growth of the fungus. $Ca^{2+}$, $Cu^{2+}$ and $Fe^{2+}$ also inhibited enzyme production but to a lesser extent as compared to $Mn^{2+}$ and` Zn^{2+}, whereas Mg^{2+} did not have any effect on the production of the enzyme. This observation is in contrast to the obligatory requirement of Mg^{2+}, observed in many of the microorganisms for the production of β-galactosidase. Besides Mg^{2+}, presence of Mn^{2+} has also been reported to stimulate the production of β-galactosidase [47].

Effect of initial pH on the production of β-galactosidase: The effect of initial pH on the growth of *Rhizomucor* sp. was studied by varying the pH of the medium (4.0–10.0), using HCl or NaOH in submerged fermentation studies (Table 4). Although the fungus grew over a wide pH range (4.0-10.0), the enzyme was produced in a medium of initial pH between 7.0–8.0. It was observed that maximum enzyme activity was detected in medium of initial pH 7.0, where as the *Rhizomucor* sp. did not grow at pH 3.0. The pH of the growth medium has a great influence on the production of β-galactosidase from *P. notatum*, where it was observed that the fungus grew over a narrow pH range (2.0 – 4.0) and optimum enzyme production was at pH 2.5 [11]. Similarly the optimum pH for the production of β-galactosidase from *Trichoderma reesei* was 5.0 [48] while that for the phytopathogenic fungus *Ophiostoma novo-ulmi* was 5.8 [49]. The pH conditions required by bacteria and yeast for the production of β-galactosidase are between 6.0 – 7.0 [7,34]. But in case of the thermophilic bacteria *Sulfolobus solfataricus*, it was observed that the enzyme was produced at pH 3.0 [50].

Effect of temperature on production of β-galactosidase: Influence of temperature on β-galactosidase production revealed that *Rhizomucor* sp. could grow and produce the enzyme between 37-55°C under submerged fermentation conditions. However the enzyme secretion was maximum at 45°C and the fungus did not grow at 60°C (Fig. 1).

Time course for production of β-galactosidase: The time course for the production of the β-galactosidase from *Rhizomucor* sp. under submerged conditions is shown in Fig.2a. Maximum enzyme activity (0.55 U/ml) was obtained in 4 days. The enzyme activity remained unchanged on the fifth day after which a gradual decline in the enzyme levels was observed.

Table 1: Effect of carbon sources on production of β-galactosidase activity under submerged fermentation conditions.

Carbon source 1% (w/v)	Final pH	Enzyme activity U ml^{-1}	GlcNAc (mg g^{-1} dry mass)
Glucose`	8.10	0.003	0.38
Sucrose	8.10	0.002	0.21
Maltose	8.10	0.004	0.20
Xylose	8.10	0.003	0.21
Galactose	8.10	0.014	0.39
Wheat Bran	8.10	0.034	0.10
Fructose	8.10	0.003	0.17
Mannose	8.10	0.003	0.38
Lactose at			
0.5 % (w/v)	8.10	0.053	0.38
1.0 % "	8.10	0.550	0.62
2.0 % "	8.10	0.093	0.67
5.0 % "	8.10	0.053	1.40

The effect of various carbon sources was studied at a concentration of 1 % (w/v). The *Rhizomucor* sp. was grown in a basal medium containing (w/v) lactose, 1 %; yeast extract, 1 %; $MgSO_4$, 0.05 %; K_2HPO_4, 0.1 %. Lactose was replaced by other sources.

Table 2: Effect of nitrogen sources on production of β-galactosidase under submerged fermentation conditions.

Nitrogen Source 1 % (w/v)	Final pH	Enzyme (U ml^{-1})	GlcNAc (mg g^{-1} dry mass)
Yeast extract	8.3	0.55	0.37
Tryptone	7.1	0.05	0.24
Malt extract	6.0	0.03	0.009
Casamino acids	5.1	0.04	0.27
Peptone	8.1	0.50	0.47
Liver extract	7.1		0.22
Yeast extract			
0.5 % (w/v)	8.3	0.31	0.27
1.0 % "	8.3	0.55	0.40
1.5 % "	8.3	0.40	0.56
2.0% "	8.3	0.36	0.60
Wheat bran			
1 % (w/v)	8.4	0.05	0.17
5% "	8.4	0.15	0.28
7% "	8.4	0.12	0.21
10% "	8.4	0.08	0.16

The effect of above nitrogen sources was studied at a concentration of 1 % (w/v). The *Rhizomucor* sp. was grown in a basal medium containing (w/v) lactose 1%; $MgSO_4$ 0.05 %; K_2HPO_4 0.1 % and yeast extract, 0.5%. The yeast extract was replaced by the above nitrogen sources. The effect of varying concentrations of yeast extract and wheat bran on the production of β-galactosidase was also studied.

Table 3: Effect of metal ions on β-galactosidase production in submerged fermentation.

Metal ion (2mM)	Relative Activity (%)
None*	89.0
Mg^{2+}	100.0
Ca^{2+}	62.0
Cu^{2+}	60.0
Zn^{2+}	14.0
Fe^{2+}	35.0
Mn^{2+}	10.0
Co^{2+}	0.0

The basal medium contained (w/v) lactose 1%; $MgSO_4$ 0.05 %; K_2HPO_4 0.1 % and yeast extract, 0.5%.

***No additional metal ions were added to the basal medium.**

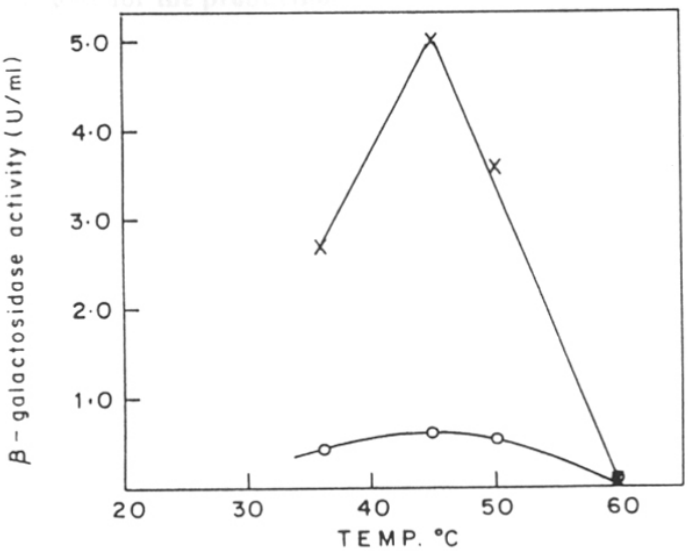
The fungus was grown with above metal ions (2mM) in the fermentation medium at 45°C in submerged conditions as described in materials and methods with Mg^{2+} being replaced by other metal ions.

Table 4: Effect of initial pH on production of β-galactosidase in submerged fermentation.

Initial pH	% Relative activity
3.0	0.0
4.0	24.0
5.0	30.0
6.0	38.0
7.0	100.0
8.0	75.0
9.0	51.0
10.0	42

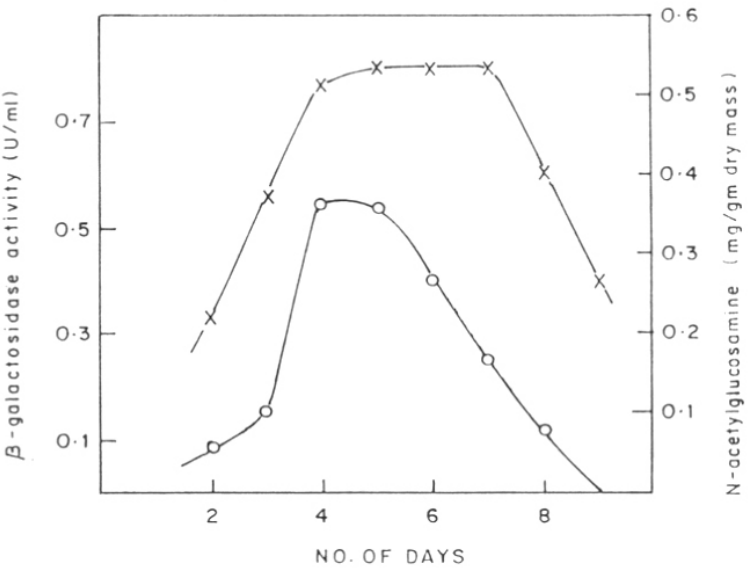
Desired pH of the medium was obtained by using HCl and NaOH.

Fig 1: Effect of temperature on the growth of *Rhizomucor* sp. and the production of β-galactosidase in SmF and SSF.



The effect of temperature on the production of β-galactosidase from *Rhizomuor* sp. was studied under submerged and solid state conditions. The fungus was grown in an enriched medium as described in Materials and Methods and the flasks were incubated at various temperatures over a period of time. (×-×) indicates the production of β-galactosidase in SSF and (o-o) indicates the production of β-galactosidase in SmF

Fig 2a: Time course for the production of β-galactosidase in SmF.



The time course for growth and enzyme production was carried out in an enriched medium containing (w/v) lactose (1 %) yeast extract (1 %); K_2HPO_4 (0.1 %) and $MgSO_4$ (0.05 %). (o-o) indicates the amount of β-galactosidase secreted (U ml^{-1}) as a function of time (days), (×-×) indicates the biomass produced (mg of NAG) as a function of time (days).

SOLID STATE FERMENTATION

Effect of carbon sources on production of β-galactosidase: The effect of lactose and whey on the production of β-galactosidase by solid state fermentation is shown in Table 5. Maximum enzyme was produced in presence of 5 % (w/v) lactose. Comparable enzyme activity was obtained when the fungus was grown on whey, pH (5.0 and 7.0). Solid state fermentation has been used for the production of β-galactosidase from *A. oryzae* [40], *F. moniliforme* [12], and *Scopulariopsis* sp. using wheat bran as the solid substrate [20]. Various other enzymes like glucoamylase, cellulase, lipase, α-galactosidase, catalase, xylanase, β-glucosidase, phytase, invertase, phenol oxidase have been produced by solid state fermentation [20].

Effect of initial pH of production of β-galactosidase: To study the effect of initial pH on the growth and production of β-galactosidase in solid state conditions, the water used for moistening the wheat bran was adjusted to required pH with HCl / NaOH. It was observed that the organism grew over a wide pH range i.e. 4.0-10.0, and the production of the enzyme was maximum between pH 5.0 - 7.0. At pH 8.0, 75 % of the optimum enzyme production was observed (Table 6). The organism was unable to grow at and above pH 11.0 under solid state fermentation conditions.

Effect of metal ions on growth and enzyme production: The presence or absence of supplementary metal ions did not affect the growth or production of enzyme in solid state fermentation.

Effect of temperature on production of β-galactosidase: The effect of temperature on the production of β-galactosidase and the growth of the fungus was studied over a temperature range of 28 - 60°C. The *Rhizomucor* sp. grew over a temperature range of 28 - 50°C as also observed in submerged conditions. It was observed that under conditions of solid state as well as submerged fermentation, the fungus did not grow at 60°C and maximum enzyme production was observed at 45°C (Fig 1).

Time course for production of β-galactosidase: The time course for enzyme production in solid state fermentation studies is shown in Fig 2b. Maximum enzyme was produced on the fifth day of fermentation at 45°C, after which the enzyme activity decreased gradually. The enzyme production was growth associated, as increase in the biomass was accompanied by an increase in the enzyme levels.

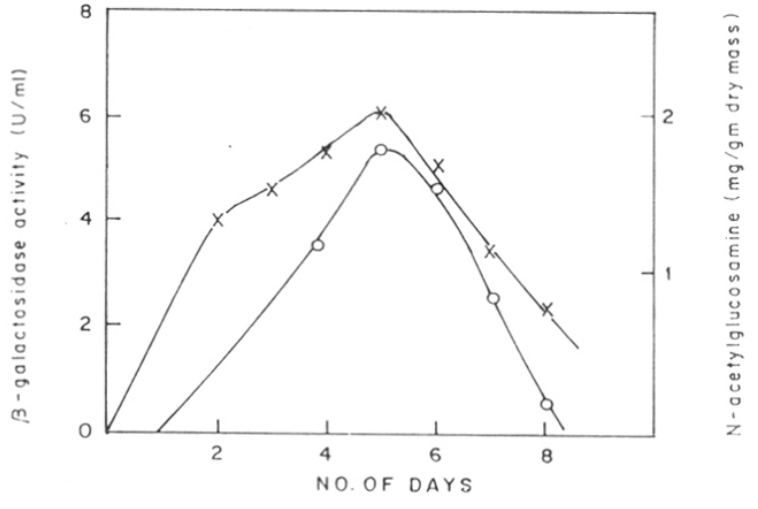
Scanning Electron Microscopy (SEM): Scanning electron microscopy was carried out to study the nature of growth of *Rhizomucor* sp. on wheat bran (Fig 3a,3b,3c). Samples of *Rhizomucor* sp. grown in a medium containing wheat bran (10 %, w/v) lactose 5.0 % (w/v); K_2HPO_4 1 % (w/v) and $MgSO_4$ 0.5 % (w/v), were examined over the incubation period. 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 solid substrate was covered completely with the fungal mycelium on the third day (Fig 3b). On the sixth day it was observed that the solid substrate was totally invisible with the fungal mycelium forming a thick mat and penetrating the solid substrate. Development of the fruiting bodies as well as formation of zygospores was also seen on the sixth day (Fig 4c). SEM has been used to study the ultrastructure of fungal cells [51-53]. It has also been observed that the assimilation of solid substrates involves the penetration of fungal hyphae into the substrate particles, and the ability to soften the substrate by mechanically pushing the cells apart in conjunction with enzymatic degradation. The degree of substrate transformation depends on the ability of the fungal mycelia to penetrate the intercellular and intracellular spaces of the solid substrate particles [54].

Various parameters like initial pH, temperature, moisture content i.e. buffer to solid substrate ratio play a crucial role in the production of desired products in SSF [55]. A critical balance between the ratio of buffer to wheat bran, incubation temperature and initial pH helped in the production of large amounts of α-amylase without the co-production of protease and cellulase in the bacterium *Bacillus megaterium* 16 M [56]. The regulation of enzyme synthesis by end products is known in some fermentation processes and it is of critical importance to overcome

this in industrial application for achieving higher productivity and economics. The production of α-amylase by submerged fermentation suffered due to catabolic repression, which completely inhibited the enzyme production. However this problem was overcome by using solid state fermentation [57].

The study of the various fermentation parameters for the production of β-galactosidase in submerged (SmF) and solid state fermentation (SSF) indicates that the optimum temperature required for the production in both cases is the same. Maximum levels of β-galactosidase were produced in 4 and 5 days in SmF and SSF respectively. The *Rhizomucor* sp. grew over a wide pH range in SSF as compared to SmF. In submerged fermentation lowered β-galactosidase activity was observed with an increase in lactose concentration, (with an optimum enzyme production in a medium containing 1% (w/v) lactose); which probably is a result of catabolite repression. However this catabolite repression was overcome by carrying out the fermentation in solid state, with an optimum level of β-galactosidase being produced in a medium containing 5 % (w/v) lactose, with a nine fold increase in the β-galactosidase production. Similar high yields of enzymes like cellulase [58], lipase [59], α-amylase [60] and α-galactosidase [61] have been obtained by using the solid state fermentation technique.

Fig 2b: Time course for the production of β-galactosidase in SSF.



The time course for growth of *Rhizomucor* sp. and β-galactosidase production was carried out in a medium containing wheat bran 9.5 g; lactose 0.5 g; $MgSO_4$ 0.05 g and K_2HPO_4 0.1 g in each 250 ml Erlenmeyer flask (o-o) indicates the amount of β-galactosidase secreted (U / ml) as a function of time (days); (×-×) indicates the biomass produced (mg of NAG) as a function of time (days).

Table 5: Effect of carbon sources on production of β-galactosidase by solid state fermentation

Carbon source	Final pH	Activity ($U\ ml^{-1}$)	GlcNAc ($mg\ g^{-1}$ dry mass)
Lactose (w/v)			
1% "	8.40	1.2	1.14
2 % "	8.40	3.2	2.08
5 % "	8.40	5.0	1.97
10 % "	8.40	2.1	2.67
Milk whey			
Initial pH 5.0	8.40	4.8	2.18
Initial pH 7.0	8.40	5.0	2.30

The effect of carbon sources on production of β-galactosidase from *Rhizomucor* sp. in solid state fermentation was carried out in 250 ml Erlenmeyer flasks containing wheat bran 9.5 g; $MgSO_4$ 0.05 g; K_2HPO_4 0.1 g and 20 ml water. The concentration of lactose was varied as indicated above. Milk whey was used instead of lactose, as the carbon source and no water was used to moisten the wheat bran.

Table 6: Effect of initial pH on enzyme production in solid state fermentation.

Initial pH	% Relative activity
3.0	24.0
4.0	75.0
5.0	100.0
6.0	100.0
7.0	100.0
8.0	75.0
9.0	52.0
10.0	45.0

Desired pH of the medium was obtained by using HCl and NaOH.

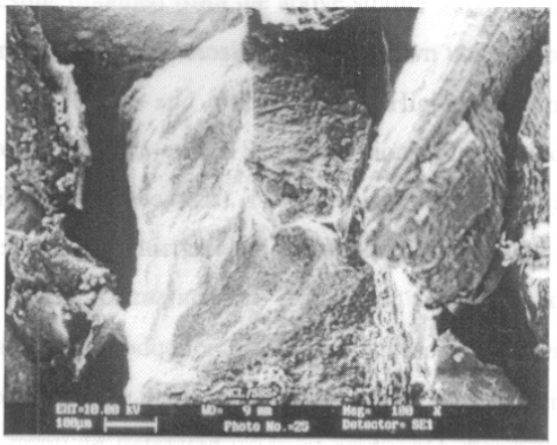
Fig 3: Scanning Electron micrographs of *Rhizomucor* sp. grown on wheat bran during solid state fermentation (magnification × 250).

a) Control (Uninoculated) wheat bran.

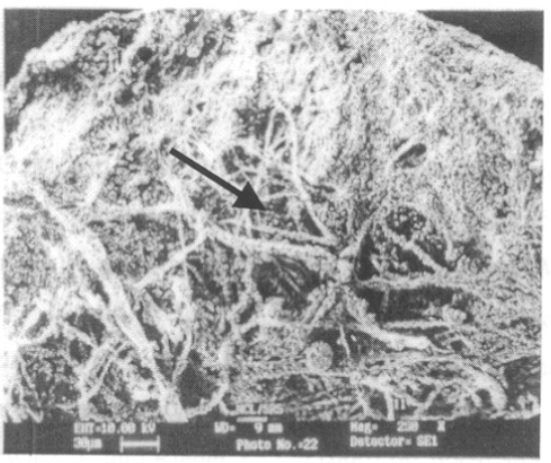
b) *Rhizomucor* sp. after 3 days of fermentation, showing the spreading of the fungal mycelia on the solid substrate (indicated by an arrow).

c) Growth of *Rhizomucor* sp. after 6 days of fermentation, showing the fruiting bodies (indicated by a circle and arrow).

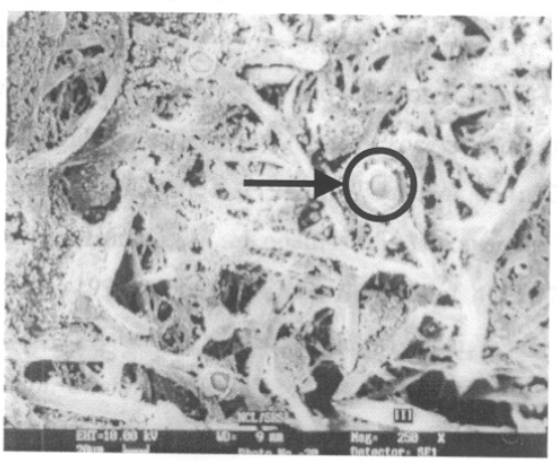
Fig 3a



3b



3c



Conclusions

The results obtained from the above studies suggest that the *Rhizomucor* sp. produced extracellular β-galactosidase when grown under submerged conditions in the presence of lactose and yeast extract. Higher enzyme levels (0.55U/ml-5.05U/ml) were obtained under SSF conditions due to increase in growth (as indicated by increase in biomass). The organism does not require metal ions for its growth and enzyme production and grows over a wide pH range in submerged fermentation. In SSF *Rhizomucor sp.* grows even at pH 3.5 and 9.0.

The increased production of β-galactosidase by *Rhizomucor* sp. observed in SSF can be exploited for the commercial production of this enzyme. The ability of the fungus to grow on untreated whey at acidic pH shows that the fungus can be exploited for the treatment of unutilized whey.

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CHAPTER III

PURIFICATION AND CHARACTERIZATION OF β-GALACTOSIDASE FROM *RHIZOMUCOR SP.*

SUMMARY

An extracellular β-galactosidase from a thermophilic fungus *Rhizomucor* sp. has been purified to homogeneity by successive DEAE- cellulose chromatography followed by gel filtration on Sephacryl S-300. The molecular mass of the native enzyme is 250,000 and it is composed of two identical subunits with molecular mass of 120,000. It is an acidic protein with a pI of 4.2. Purified β-galactosidase is a glycoprotein and contains 8 % neutral sugar. The optimum pH and temperature for enzyme activity are 4.5 and 60°C, respectively. The enzyme is stable at 60°C for four hours and has a $t_{1/2}$ of 150 min at 70°C, one of the highest reported for any fungal β-galactosidases. Amino acid composition showed a high content of aromatic amino acids and a low content of cysteine. Substrate specificity studies indicate that the enzyme is specific for β-linked galactose residues with a preference for *p*-nitrophenyl-β-D-galactopyranoside (*p*NPG). The K_m and V_{max} values for the synthetic substrates *p*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) are 0.66 mM and 1.32 mM; and 22.4 mmoles min^{-1} mg^{-1} and 4.45 mmoles min^{-1} mg^{-1}, respectively, while that for the natural substrate, lactose is 50.0 mM and 12 mmoles min^{-1} mg^{-1}. The end product galactose and the substrate analogue isopropyl thiogalactopyranoside (IPTG) inhibited the enzyme with K_i of 2.6 mM and 12.0 mM, respectively. The energy of activation for the enzyme using *p*NPG and *o*NPG were 27.04 kCal and 9.04 kCal, respectively.

INTRODUCTION

β-Galactosidase is an industrially important enzyme with wide applicability in food and dairy industry [1]. It is also useful for preparation of lactose free milk, for individuals and infants suffering from lactose intolerance [2,3]. For various biotechnological applications of the enzyme, it is important to choose the β-galactosidase with desired properties, to be favorable and economical for the required process. For example a lactase showing pH optima near neutrality will be suitable for the treatment of milk lactose to avoid spoilage of the milk, whereas a lactase having an acidic pH optima may be more useful for the treatment of whey, which is generally acidic. The β-galactosidases found in nature, vary in properties, molecular size and function depending on the sources from which it has been obtained [4,5]. Among the microorganisms, fungal β-galactosidases are more important as they can be obtained in high yields owing to their extracellular nature, thus, making their use for industrial purposes more economical as compared to the enzyme obtained either from yeast or bacteria [6]. β-Galactosidases from various filamentous fungi like *Fusarium moniliforme* [7], *Aspergillus oryzae* [8], *Aspergillus foncecases* [9] *and Mucor pusillus* [10] have been purified and characterized.

Thermostability of the enzyme is an important property with regards to the operational benefits and reaction rates in case of industrial applications. Many of the enzymes derived from mesophilic microorganisms exhibit considerable thermostability (upto 50°C), but those derived from thermophilic sources demonstrate better thermostability [11,12]. Thus a thermophilic fungus with higher productivity and thermostability would be a useful alternative to the existing sources of the fungal enzyme, to be used for industrial purpose.

In the preceding chapter, the optimum conditions for the production of β-galactosidase from thermophilic fungus *Rhizomucor* sp. had been described. The purification and extensive characterization of the β-galactosidase is presented in this chapter.

MATERIALS AND METHODS

p-Nitrophenyl-β-D-galactopyranoside (*p*NPG), *o*-Nitrophenyl-β-D-galactopyranoside (*o*NPG), lactose, *p*NP-β-D-*N*-acetylglucosaminide, *p*NP-α-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-β-D-mannopyranoside, HEPES, MES, polyethylene glycol-8000 and ampholines (pH 3-10) were obtained from Sigma (St. Louis, MO, USA). Sephacryl S-300 and DEAE-Cellulose were obtained from Pharmacia (Uppsala, Sweden). All other reagents used were of the highest purity available from commercial source.

Enzyme production

The cultivation of the *Rhizomucor* sp. and the preparation of the crude enzyme both by SmF and SSF (submerged and solid state fermentation) was carried out as described in Chapter II.

Protein determination

The protein from the culture filtrate was determined as described in Chapter II. Protein estimations of column effluents were monitored by absorbance at 280 nm. Total protein estimation of pooled fractions during purification steps was monitored according to the method of Lowry *et al* [13].

Enzyme assay

β-Galactosidase was assayed by incubating 25 µl of suitably diluted enzyme with 50 µl of 6.6 mM *p*NPG or *o*NPG and 925 µl citrate-phosphate buffer, pH 4.5, at 60°C for 30 min. The reaction was terminated by adding 1.0 ml of 0.5 M Na_2CO_3 and the *p* - nitrophenol or *o* - nitrophenol released was determined by reading the absorbence at 405 / 410 nm, respectively. When lactose was used as the substrate, the enzyme activity was assayed using a glucose oxidase-peroxidase kit to determine the amount of glucose liberated [14]. One unit of β-galactosidase activity (U) was expressed as the amount of enzyme that releases 1.0 µmole of product (*p*-nitrophenol / *o*-nitrophenol / glucose) /min under standard assay conditions.

PURIFICATION OF β-GALACTOSIDASE

All purification steps were carried out at 4°C unless otherwise mentioned. Routine assays of β-galactosidase during purification steps were based on the hydrolysis of *p*NP-β-D-galactopyranoside. The purification procedure followed to obtain a homogeneous preparation of β-galactosidase, obtained either by SmF or SSF was essentially identical.

Ammonium sulfate precipitation: The crude enzyme extract (obtained from SmF and SSF) was brought to 90% saturation by adding solid ammonium sulfate under stirring, and kept standing overnight at 4°C. The precipitated protein was collected by centrifugation (8000g, 15 min.), dissolved in minimum amount of distilled water and dialyzed exhaustively against phosphate buffer (20 mM, pH 7.2). Any insoluble material was removed by centrifugation (5000 **g**, 10 min) and the clear supernatant was used for further purification.

DEAE-Cellulose chromatography: The crude enzyme preparation from the above step was applied to a DEAE- cellulose column (2.5 cm x 25 cm), pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.2 at a rate of 12 ml per hour. The column was washed with the same buffer, containing 50 mM NaCl till flow through fractions showed absorbance below 0.05 at 280 nm, and the washings were free of β-galactosidase activity. The bound enzyme was eluted using a step gradient of NaCl (in increasing steps of 50 mM NaCl, and a total volume of 100 ml for each step) at a rate of 15 ml / h. Fractions of 5.0 ml (for each step) were collected and those fractions showing maximum enzyme activity were pooled, dialyzed extensively against 20 mM potassium phosphate buffer, pH 7.2, lyophilized and rechromatographed on a fresh DEAE-cellulose column. The bound enzyme was then eluted with 0.2 M NaCl in 20 mM potassium phosphate buffer, pH 7.2. The active fractions were pooled, dialyzed exhaustively and concentrated by lyophilization for further purification.

Polyethylene glycol treatment (PEG): In order to remove the coloured impurities associated with DEAE-cellulose eluted β-galactosidase, the enzyme was treated with polyethylene glycol - 8000 (10 % w/v) and sodium citrate (30 % w/v) under mild agitation. The mixture was left at room temperature for 30 min, and then centrifuged at 2000 **g** for 10 min. The colour free protein (lower layer) was collected and dialyzed extensively against distilled water followed by dialysis with 20 mM citrate-phosphate buffer, pH 4.5. The dialysate was concentrated by lyophilization and used for gel filtration.

Gel filtration: The enzyme (8.0 mg in 750 µl) was loaded on to a Sephacryl S-300 column (1.2 cm × 160 cm), pre-equilibrated with 20 mM citrate-phosphate buffer, pH 4.5 at a rate of 8.0 ml / h. Fractions of 1 ml were collected at a rate of 6.0 ml/h, and those fractions showing β-galactosidase activity were pooled, concentrated by lyophilization, dialyzed and rechromatographed on Sephacryl S-300 under identical conditions. The active fractions were then pooled, dialyzed, concentrated and stored at -20°C till further use. No loss of enzyme activity was observed when the purified enzyme was stored under these conditions.

Electrophoresis: Native polyacrylamide gel electrophoresis (PAGE) was carried out in 7.5% (w/v) acrylamide gels in Tris-Glycine system at pH 8.9 [15] and the gels were visualized by staining with silver nitrate [16]. Isoelectric focusing was done in 7.5% (w/v) acrylamide gels according to the method of Vesterberg [17] using ampholines in the pH range 3-10.

CHARACTERIZATION OF THE ENZYME

Determination of M_r: The native molecular mass of the enzyme was determined by gel filtration on Sephacryl S-300 column (100 × 0.7 cm). The column was equilibrated with 100 mM citrate phosphate buffer pH 4.5 and calibrated using blue dextran (2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa). The subunit molecular mass was determined (in presence and absence of β-mercaptoethanol) by

sodium dodecyl sulfate (SDS)-PAGE [18], in 10% (w/v) acrylamide gel using high molecular weight markers (Sigma SDS-6H). The gels were stained with Comassie Blue R-250.

Amino acid analysis: Amino acid composition of the purified β-galactosidase was determined on an automated liquid phase amino acid analyzer (Hewlett Packard Ti series 1050, with HP fluorescence detector). Prior to analysis the samples were hydrolyzed in 200 µl of 6 N HCl at 110 °C for 20 h. Total tryptophan content was determined spectrophotometrically by the method of Spande and Witkop [19] and Edelhoc [20]. The total cysteine was determined by the method of Habeeb [21].

Carbohydrate content: Total neutral sugar content of the enzyme was determined by the phenol-sulphuric acid method of Dubois *et al* [22] using mannose as standard.

Other glycosidase activity

The enzyme was assayed for other glycosidase activities (α-galactosidase, β-*N*-acetylglucosaminidase, β-glucosidase, α and β-mannosidase, β-L-fucosidase, β-D-xylosidase) using their respective *p*NP- substrates.

Inhibition studies with glucose, galactose, sucrose and isopropyl thio-galactopyranoside was carried out by pre-incubating the enzyme with the respective sugar (5-50 mM) for 15 minutes. The substrate *p*NPG was subsequently added to determine the enzyme activity. The inhibition constant (K_i) was determined using a minimum of three different concentrations (2 mM, 5 mM & 10 mM) for glucose and (20 mM, 60 mM & 80 mM) for IPTG, respectively.

Effect of pH on β-galactosidase:

The optimum pH for enzyme activity was determined by carrying out the enzyme assay in universal buffer (pH 3.0 – 10.0, 100 mM) [23] under standard conditions. The stability of the enzyme at various pH was checked by incubating

the enzyme in universal buffer (pH 3.0 - 10.0) for 24 hrs. at 28°C. The enzyme was then assayed for β-galactosidase activity under standard assay conditions.

Effect of temperature on β-galactosidase:

Temperature dependence was studied by monitoring the enzyme activity on *p*NPG in 100 mM citrate-phosphate buffer (pH 4.5), at various temperatures (25 - 75°C). Stability studies were carried out by incubating the enzyme at different temperatures (25 -75°C) in absence of substrate and by monitoring the activity at definite time intervals (0-240 min) using *p*NPG.

To determine the energy of activation of the enzyme, the kinetic rate constants, K_m and V_{max} of β-galactosidase were determined at various temperatures (37°-70C) with *o*NPG and *p*NPG. Suitably diluted enzyme (2 Uml^{-1}) was incubated with varying concentrations of the substrates *o*NPG / *p*NPG (50 μg - 350 μg). Ln V_{max} was plotted versus reciprocal of absolute temperature to obtain an Arrhenius plot for both substrates. Energy of activation was calculated using the equation:

$$\text{slope} = -0.219x\ E_{act} \text{ in cals mol}^{-1}.$$

RESULTS AND DISCUSSION

Purification of β-galactosidase from *Rhizomucor* sp.: The results of a typical procedure for the purification of β-galactosidase from *Rhizomucor* sp. obtained by SmF and SSF is given in Table 1a and 1b, respectively. The enzyme obtained by submerged fermentation was purified 273.9 fold with an overall yield of 36 % and specific activity of 200 U mg^{-1}. The final yield of the pure protein obtained was 0.8 mg from 800 ml of culture filtrate. The elution profile from Sephacryl S-300 is shown in Fig. 1a and the electrophoretic profile at different stages of purification is shown in Fig. 1b.

The β-galactosidase obtained by SSF was purified 60 fold by adsorption on DEAE-cellulose and successive gel filtration on Sephacryl S-300 with an overall yield of 32 % and specific activity of 200 U mg^{-1}. Although most of the colouring impurities and other contaminating proteins were bound to DEAE- cellulose, it was

observed that the enzyme eluted from DEAE- cellulose still contained considerable amounts of coloured impurities. These coloured impurities could be removed by treatment with PEG prior to the gel filtration. The specific activity of purified β-galactosidase from *Rhizomucor* sp. (200 U mg^{-1}) was found to be higher than the commercially available fungal β-galactosidase obtained from the *Aspergillus* sp. (106 Umg^{-1}) [8] and S*accharopolyspora recttivirgula* (23 U mg^{-1}) [24]. However high specific activity has been observed in case of β-galactosidase from *Streptococcus thermophilus* (1032 U mg^{-1}) [25]. The yield of purified β-galactosidase in solid state fermentation was eight times more (8.0 mg / lit of extract) than that obtained in submerged fermentation (1 mg / lit of culture filtrate). Similar enhancement in yields by using SSF technique has been observed in case of β-galactosidase from *F. moniliforme* [7] and α-galactosidase from *Humicola* [26]. The enzyme moved as a single band on electrophoresis and focussed at pH 4.2 on isoelectric focussing and was found to be a glycoprotein with 8 % neutral sugar. The β-galactosidase from *Rhizomucor* sp. shows an acidic pI like most other fungal β-galactosidases and a comparable carbohydrate content except in case of the β-galactosidase from *K. lactis* which has 45 % neutral sugar content [27].

Molecular mass: The molecular mass of the enzyme was found to be 250 kDa as determined by gel filtration (Fig 2). The enzyme moved as a single protein band both in presence and in absence of β-mercaptoethanol on SDS-PAGE indicating that the enzyme was a dimer of identical subunits with a relative subunit molecular mass (M_r) of 120,000 devoid of inter-subunit disulfide bridges (Fig.3a). Considerable variations in the M_r and molecular nature of the β-galactosidases from different sources have been observed. The enzyme from yeast (*K. lactis*) was reported to have 9 - 10 subunits and an average M_r of 201 kDa [27]. In case of the β-galactosidase of bacterial and fungal origin, molecular masses ranging between 150 kDa to 365 kDa has been reported [5].

Among fungi, dimeric β-galactosidase with identical subunits has been reported in case of *Penicillin citrinum* (M_r 60 kDa) [28], and A. *oryzae* (M_r 110

kDa) [8]. Monomeric β-galactosidases have been reported from *Mucor* sp. (M_r 131 kDa) [10], the pathogenic fungus *Sclerotina sclerotium* (M_r 120 kDa) [29] and *Ophiostoma novo-ulmi* (M_r 135 kDa) [30]. However the β-galactosidase from *E.coli* was a homotetramer of M_r 116 kDa [31]. Different molecular forms of the enzyme have been reported in *A. niger* [32].

The amino acid composition (Table 2) showed a high content of aromatic amino acids (11 % of the total residues) and a low content of cysteine residues (0.3% of the total residues). The *Rhizomucor* sp. β-galactosidase displays some of the characteristics indicative of thermophilic proteins [33], such as low cysteine content (3 residues per subunit versus 16) and lower Arg / Lys ratio (0.5 versus 2.7) as compared to *E. coli* lac Z β-galactosidase, indicating that the *Rhizomucor* sp. β-galactosidase could also be thermostable enzyme.

Effect of pH and temperature on β-galactosidase: The optimum pH for the enzyme activity was 4.5 and it shows 80 % of its optimum activity at pH 6.0 and 60% of the optimum activity at pH 7.0. The enzyme was stable over a pH range of 3.5 – 7.5 (>90 %) [Fig 4]. The pH optima and pH stability patterns observed for β–galactosidase from *Rhizomucor* sp. was similar to that of other reported fungal β-galactosidases. The bacterial β-galactosidases have an optimum pH of 6.0 while the enzyme obtained from yeast is optimally active at pH 7.0. Hence the bacterial β-galactosidase is used for the processing of milk lactose while the enzyme derived from fungi are used for the hydrolysis of lactose from whey which is often acidic [4]. The pH stability profile and acidic pH optima suggests the potential use of the β-galactosidase from *Rhizomucor* sp. for hydrolysis of whey as well as in the treatment of milk lactose.

The optimum temperature for the β-galactosidase from *Rhizomucor* sp. was 60°C (Fig 5a). When assayed at 50°C and 70°C, the enzyme showed 90 % and 80 % of its optimum activity, respectively. At its optimum temperature (60°C), the enzyme was stable for four hours. The $t_{1/2}$ of the enzyme at 70°C was 2.5 h and the thermal stability of the enzyme (Fig 5b) was found to be superior to that of other

fungal β-galactosidases like *F. monoliforme* (half-life of 6 hrs. at 50°C) and *A. oryzae* (60°C for two hours) [34]. The enzyme from bacterial sources viz. *Bacillus stearothermophilus* (less than 10 min, 60°C) [35], and from mesophilic fungus *Sclerotinia sclerotium* ($t_{1/2}$ of 30 min at 65°C) [29] were also less thermostable.

The Arrhenius plot of β-galactosidase from *Rhizomucor* sp. exhibited a straight line for the synthetic substrates, *o*NPG and *p*NPG (Fig 6a and 6b, respectively). The energy of activation (E_{act}) was calculated to be 27.04 kCals for *p*NPG and 9.04 kCals for *o*NPG, respectively. Linear Arrhenius plots were observed in case of the β-galactosidase from *F. moniliforme* which had an energy of activation of 8.5 kCals for *o*NPG [7]. Biphasic or discontinuous Arrhenius plots were seen in case of *B. stereothermophilus* [35], which shows energy of activation of 24 kCal below 47°C and 16 kCal above 47°C.

The substrate specificity studies indicated that the enzyme hydrolyzed *p*NPG and *o*NPG besides the natural substrate lactose. The K_m and V_{max} for *p*NPG and *o*NPG were 0.66 mM and 1.32 mM; and 22.5 mmoles min^{-1} mg^{-1} and 4.45 mmoles min^{-1} mg^{-1}, respectively. The K_m for lactose was observed to be 50 mM indicating that the enzyme shows a higher affinity for the synthetic substrates (Table 3). Similar preference for synthetic substrates has been observed in case of the enzyme from A. *fonsecaeus* and *M. pusillus* [9,10] The K_m values were also comparable to those reported in case of β-galactosidase from other fungal sources [36,37].

A comparative study of various β-galactosidases from fungi and yeast, towards the hydrolysis of a series of β-1-4 linked galactooligosaccharides indicated that, galactooligosaccharides and fragments of plant cell wall polysaccharides could also act as biological substrates of some of the microbial β-galactosidases [38]. The β-galactosidase from the cell wall of *Sirobasidium magnum* showed associated β-D-fucopyranosidase, β-D-glucopyranosidase and α-L-arabinopyranosidase activity along with the β-galactosidase activity [39]. The β-galactosidase from *Tritrichomonos foetus* [40] could hydrolyze lacto-N-biose 1 (Gal β 1-3- GlcNAc) and *N*-acetyllactosamine (Gal β 1-4 Glc Nac). However, the purified β-

galactosidase from *Rhizomucor* sp. did not possess any other glycosidase activity as tested against *p*NP-α-D-GlcNAc, *p*NP-β-D-Glc, *p*NP-β-D-Man and *p*NP-β-D-Xyl.

The β-galactosidase from *Rhizomucor* sp. was inhibited by galactose and IPTG, and the inhibition was observed to be competitive with galactose (K_i 2.6 mM); as well as IPTG (K_i 12 mM) (Fig. 7a and 7b). It is generally observed that galactose is a competitive inhibitor of β-galactosidase [34,35,41], but noncompetitive inhibition with galactose has been observed in case of the β-galactosidase from *A. oryzae* [42]. Galactose also showed a mixed type of inhibition towards β-galactosidase from an alkalophilic and thermophilic *Bacillus* sp. TA-11 [43].

Effect of metal ions on β-galactosidase activity: The β-galactosidase from *Rhizomucor* sp. does not require alkaline or divalent metal ions for its activity. No inactivation was observed as a result of extensive dialysis of the enzyme against buffers containing EDTA or by adding chelating agents directly to the assay mixture indicating that it is not a metal requiring enzyme nor a metalloprotein. Divalent metal ions like Hg^{++} and Cu^{++} inhibited the enzyme, while none of the other metal ions had any significant effect on the enzyme activity. It is known that Mg^{++} is required for enzyme activity in most β-galactosidase [4,5,31], but those derived from fungi, especially, the thermostable enzymes do not seem to require metal ions for their activity [44].

Table 1a: Purification of β-galactosidase from *Rhizomucor* sp. obtained by submerged fermentation.

Step	Protein mg	Activity Units	Sp Activity units/mg	Fold purification	Recovery (%)
Culture filtrate *	500	440	0.88	-	100
$(NH_4)_2SO_4$ precipitation	305	400	1.31	1.8	90.90
DEAE-cellulose-I	27	280	10.37	14.2	63.0
DEAE-cellulose-II	10	230	23.0	31.5	52.2
Sephacryl S-300-I	1.2	180	150	205	40.90
Sephacryl-S-300-II	0.8	160	200	273.9	36.0

* From 800 ml of culture filtrate.

Table 1b: Purification of β-galactosidase from *Rhizomucor* sp. obtained by solid state fermentation

Step	Protein (mg)	Activity (Units)	Sp. Activity (units/mg)	Fold purification	Recovery (%)
Culture filtrate *	750	2500	3.3	-	100
$(NH_4)_2SO_4$ precipitation	209	2200	10.5	3.18	88.0
DEAE-cellulose-I	70	1800	25.71	7.8	72
DEAE-cellulose-II	50	1650	33.0	10.0	66.0
Sephacryl S-300-I	4.6	900	195	59.0	36
Sephacryl-S-300-II	4.0	800	200	60.0	32.0

*From an extract of 500 ml.

Fig 1a: Elution profile of β-galactosidase from *Rhizomucor* sp. from Sephacryl S-300 (o-o) O.D $_{280\ nm}$ and (•-•) β-galactosidase activity.

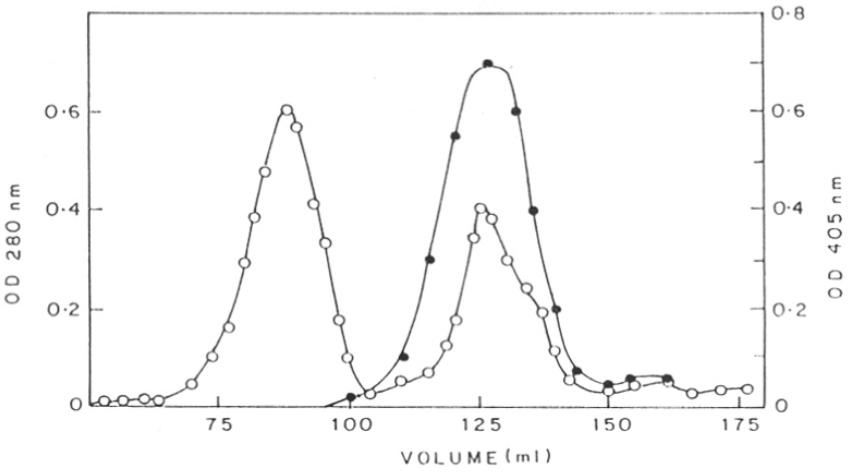


Fig 1b: Electrophoretic profile at different stages of purification of β-galactosidase from *Rhizomucor* sp. on native PAGE (7.5 %, w/v, pH 8.9).

Lane **1**: Ammonium sulfate precipitate, Lane **2**: Enzyme eluted from DEAE-Cellulose step II, Lane **3**: β-Galactosidase after Sephacryl S-300 II

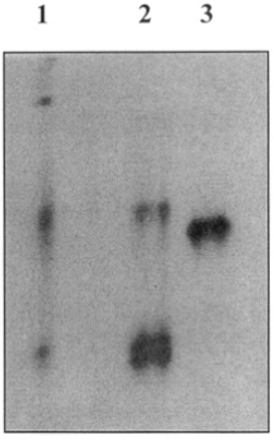


Fig 2: Molecular weight determination of *Rhizomucor* sp. β-galactosidase by gel filtration.

A Sephacryl S-300 column (100 x 0.7 cm) was equilibrated with 100 mM citrate phosphate buffer, pH 4.5 and calibrated with **a)** Thyroglobulin (669,000), **b)** Apoferritin (443,000), **c)** β-amylase (200,000), **d)** Alcohol dehydrogenase (150,000), **e)** Bovine Serum Albumin (BSA) (66,000). V_0, Void volume and V_e, elution volume.

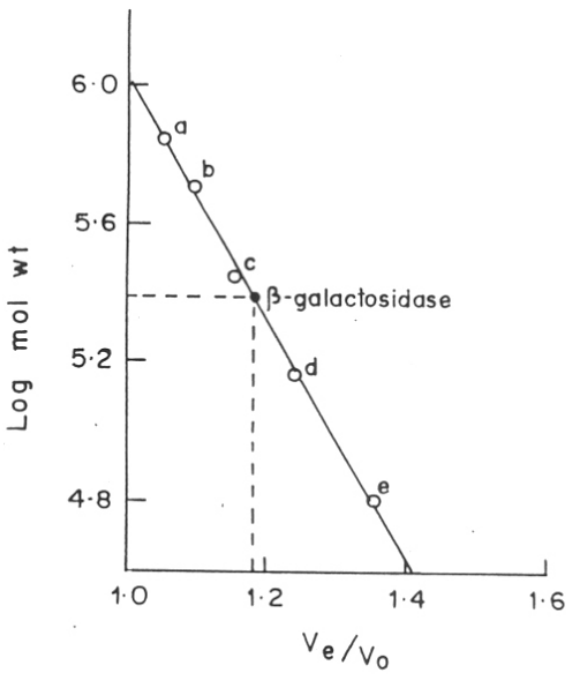


Fig. 3:

a) SDS-PAGE of β-galactosidase from *Rhizomucor* sp.

Purified β-galactosidase (50 μg) subjected to SDS-PAGE in presence and absence of β-ME in 10 % w/v acrylamide gels. The molecular mass of marker proteins is indicated alongside the gel. Lane **1**, purified β-galactosidase; Lane **2**, marker proteins (Sigma SDS-6H), **a**) myosin (205,000), **b**) β-galactosidase (*E. coli*) (116,000) **c**) phosphorylase b (97,400), **d**) bovine serum albumin (66,000), **e**) egg albumin (45,000), and Lane **3**, purified β-galactosidase in presence of β-ME

b) Plot of R_f versus log molecular weight

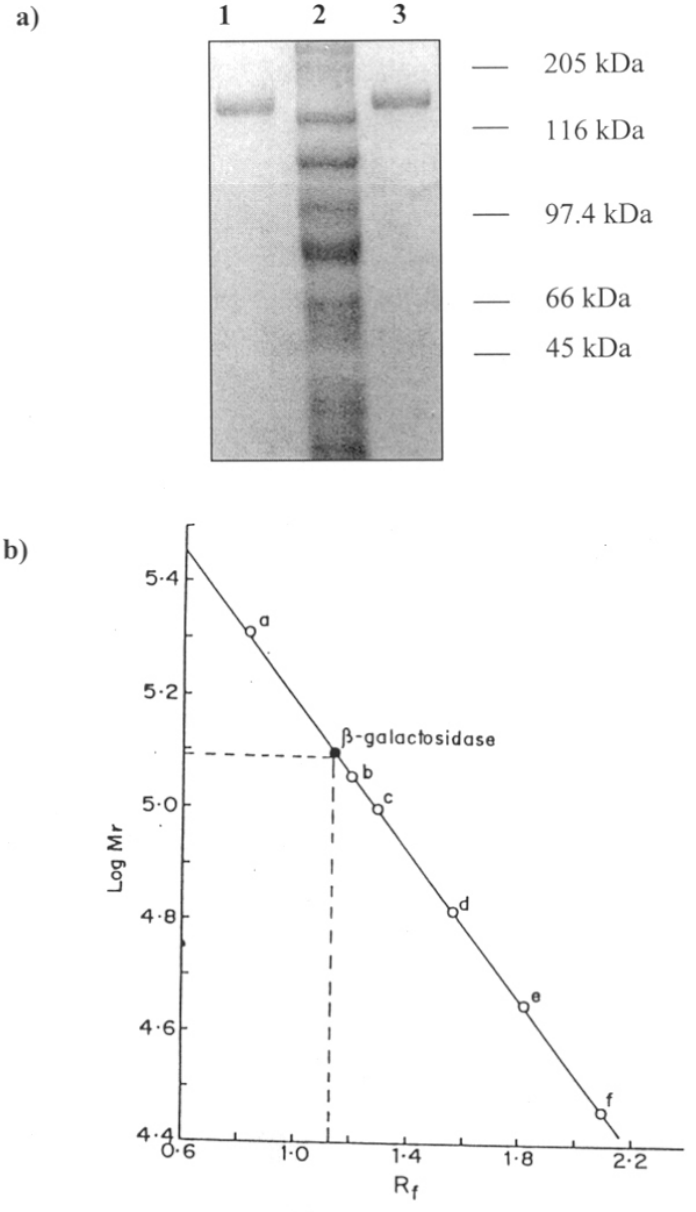


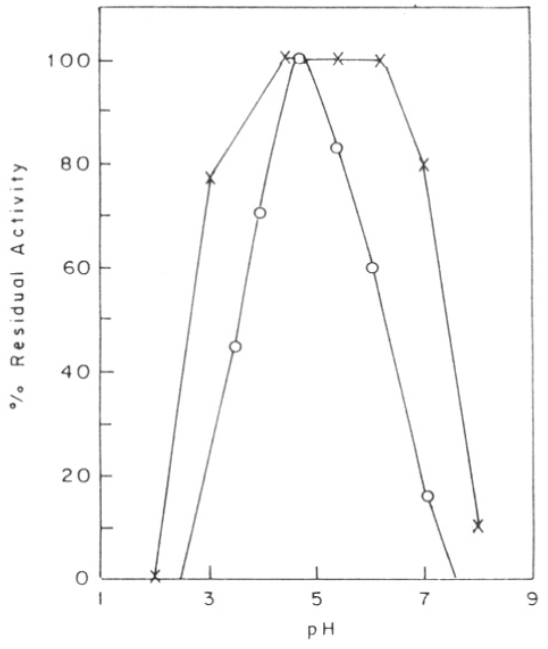
Table 2: Amino acid composition of β-galactosidase from *Rhizomucor* sp.

Amino Acid	No. of residues mol^{-1}
Gly	241
Ser	153
Cys[a]	06
Ala	246
Pro	107
Val	155
As (x)	305
Thr	150
Gl (x)	177
Ile	105
Leu	148
His	21
Lys	106
Met	33
Phe	132
Tyr	107
Arg	50
Trp[b]	54

[a] ***Total cysteine was determined spectrophotometrically by the method of Habeeb [21]***

[b] ***Total tryptophan was determined by the method of Spande and Witkop [19]***

Fig 4: Effect of pH on the activity and stability of β-galactosidase from *Rhizomucor* sp.



Purified β-galactosidase (0.5 U) was assayed at different pH values (2.0-10.0), in universal buffer, at 60°C as described in Methods. The relative activities are expressed as a percent of the β-galactosidase activity at pH 4.5. pH stability studies were carried out by incubating the enzyme (100 μg) at various pH values in universal buffers (2.0-10.0) for 24 h and 25°C. The enzyme was brought to pH 4.5 thereafter and assayed for β-galactosidase activity under standard assay conditions. (o-o) Optimum pH and (x-x) pH stability.

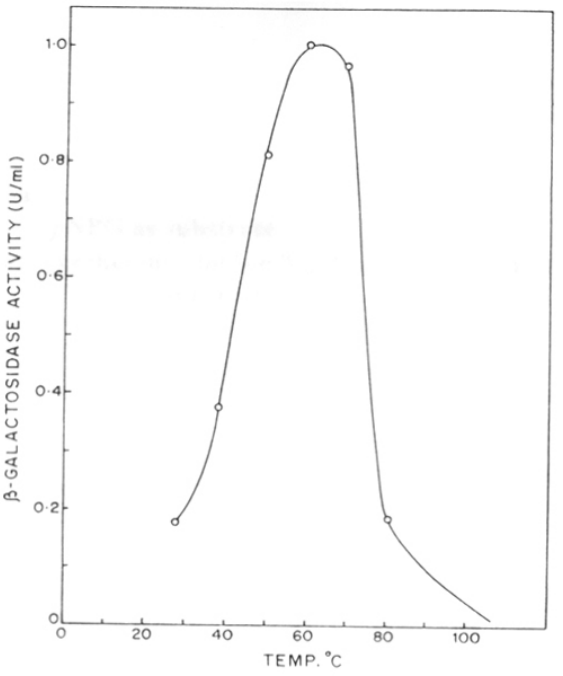
Figure 5a: Optimum temperature for the *Rhizomucor* sp. β-galactosidase

Purified β-galactosidase (0.5U) was assayed at various temperatures (20-80°C) at pH 4.5, as described in Methods. The relative activities are expressed as a percent of the activity at 60°C.

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

Purified β-galactosidase (500 μg / ml) was incubated in Na-citrate-phosphate buffer, pH 4.5, at different temperatures (60°C-80°C) and aliquots withdrawn at definite time intervals (0-240 min). The aliquots were immediately quenched in an ice bath. Subsequently, the substrate pNP-β-D-galactopyranoside was added and the enzyme assayed under standard assay conditions.

5a)


β-GALACTOSIDASE ACTIVITY (U/ml)
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
TEMP. °C

5 b)

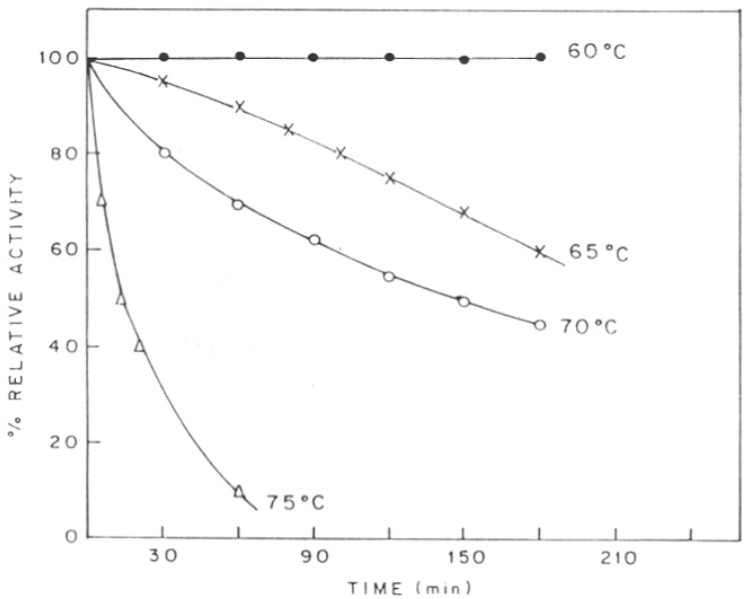
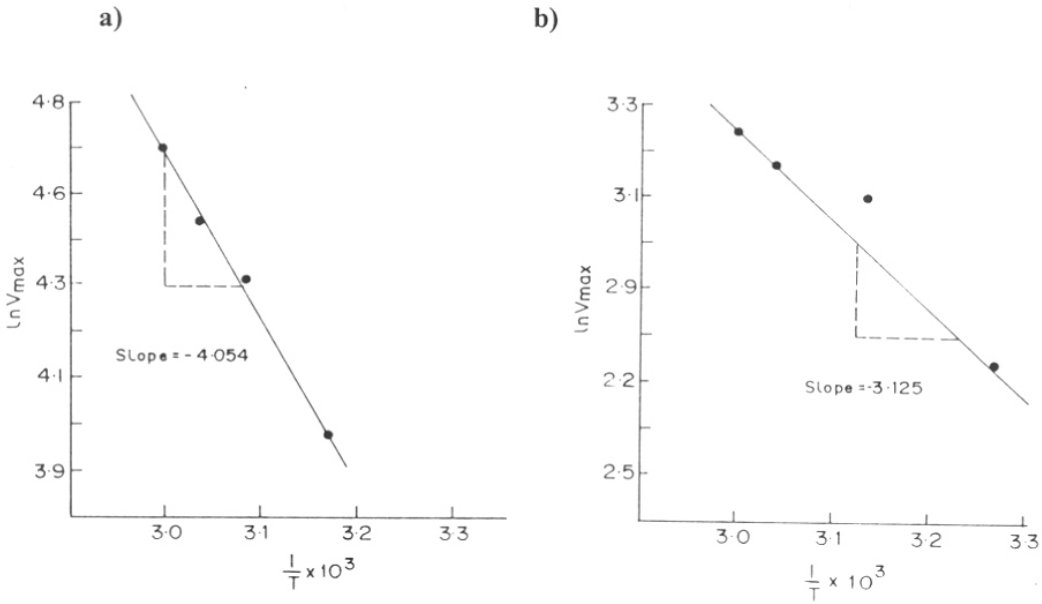

% RELATIVE ACTIVITY
100
80
60
40
20
0
60 °C
65 °C
70 °C
75 °C
30
90
150
210
TIME (min)

Figure 6a: Arrhenius plot for β-galactosidase from *Rhizomucor* sp. using *p*NPG as substrate.

Figure 6b: Arrhenius plot for β-galactosidase from *Rhizomucor* sp. using *o*NPG as substrate.



The reaction rate constant (V_{max}) for β-galactosidase was determined at different temperatures (30°C-60°C) in 100 mM citrate-phosphate buffer, pH 4.5, using saturating concentrations of the substrate pNPG (25 μg - 350 μg). ~ 5μg of the purified β-galactosidase was used for each reaction point and was carried out in duplicates. The activation energies were calculated from the slopes of each linear segment. They are 27.04 k Cal / mol for *p*NPG and 9.04 k Cal / mol for *o*NPG, respectively.

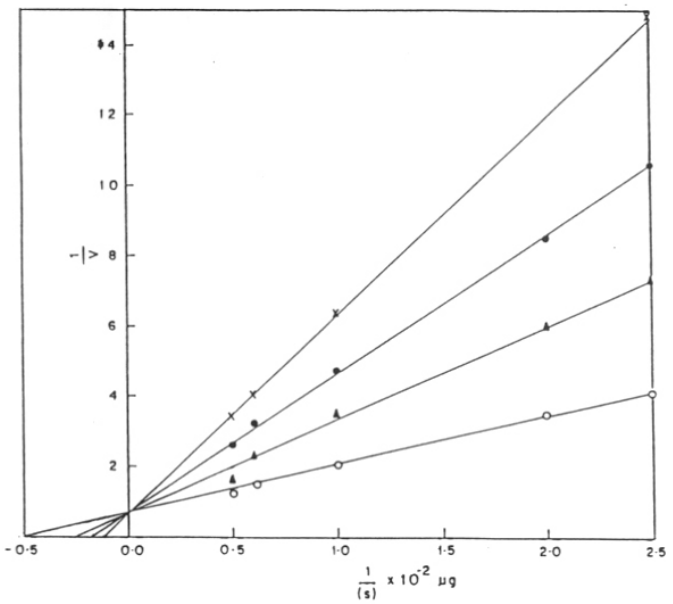
Table 3: Substrate specificity of β-galactosidase from *Rhizomucor* sp.

Substrate	Km mM	V_{max} (m moles $min^{-1}mg^{-1}$)
*p*NPG	0.66	22.14
*o*NPG	1.32	4.45
Lactose	50.0	12.0

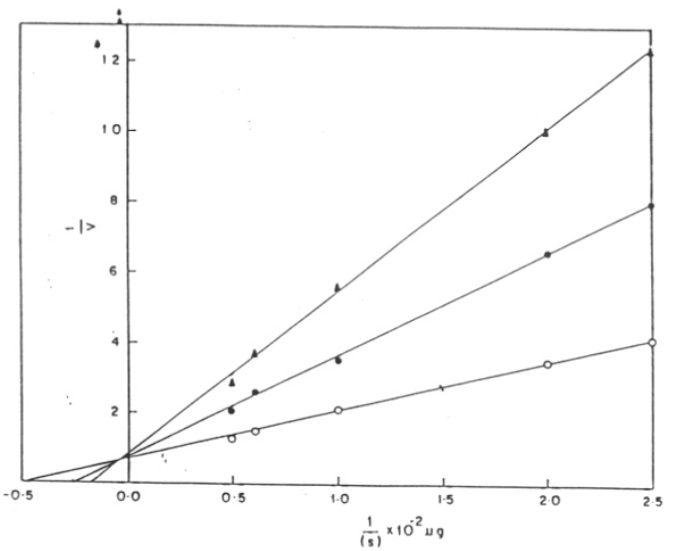
Figure 7: Double reciprocal plots of the inhibition of β-galactosidase from *Rhizomucor* sp. by
a) Galactose and
b) isopropyl-thio-β-D-galactopyranoside (IPTG) for the pNP-β-D-galactopyranoside hydrolysis catalyzed by β-galactosidase of *Rhizomucor* sp.

Reactions were performed as described in Materials and Methods. The concentration of galactose in the reaction was (i) 0, (ii) 2 mM, (iii) 5 mM, (iv) 10 mM. The concentration of IPTG was (i) 0, (ii) 60 mM and (iii) 80 mM.

7a)


14
12
10
8
6
4
2
-0·5
0·0
0·5
1·0
1·5
2·0
2·5
$\frac{1}{v}$
$\frac{1}{(s)}$ x 10^{-2} µg

7b)


12
10
8
6
4
2
-0·5
0·0
0·5
1·0
1·5
2·0
2·5
$\frac{1}{v}$
$\frac{1}{(s)}$ x 10^{-2} µg

Conclusions

The results obtained, indicate that the extracellular β-galactosidase from *Rhizomucor* sp. has an acidic pH optima and is highly thermostable with a half life of 150 min at 70 °C. The β-galactosidase does not require metal ions for its activity or stability. The enzyme is observed to possess no other associated glycosidase activities and is specific towards β linked galactose residues. The pH stability over a wide range suggests the potential use of the enzyme for the hydrolysis of milk lactose as well as the hydrolysis of whey.

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CHAPTER IV

CHARACTERIZATION OF ACTIVE SITE AND ENVIRONMENT OF TRYPTOPHAN RESIDUES OF β-GALCTOSIDASE FROM *RHIZOMUCOR SP.*

SUMMARY

Chemical modification of the purified β-galactosidase of *Rhizomucor* sp. with phenylmethylsulfonyl fluoride, N-acetylimidazole, 5-5'-dithiobisnitrobenzoic acid, 2,3, butanedione, carbodiimide and diethypyrocarbonate did not have any effect on the enzyme activity indicating that serine, tyrosine cysteine, arginine, carboxyl groups and histidine were not involved in the catalytic function of the enzyme. The enzyme lost 70% of its activity on treatment with N-bromosuccinimide and 60% on treatment with trinitrobenzene sulfonic acid indicating that tryptophan and lysine groups have a role in the catalytic function of the enzyme. Presence of the substrate (lactose) protected the enzyme from inactivation with the above reagents. The plots of percent residual activity versus the number of residues modified revealed that a single tryptophan and a single lysine residue are essential for the catalytic activity of the enzyme.

Extensive modification of tryptophan residues with NBS in β-galactosidase led to a 55 % decrease in the intrinsic fluorescence with 12 tryptophan residues per subunit being modified. However, the loss in activity preceded well before loss in intrinsic fluorescence. Effect of various solute quenchers indicated that acrylamide is a better quencher (55 %) as compared to KI (21 %) where as, CsCl was a poor quencher (3 %) under native conditions. Denaturation of the enzyme with guanidine hydrochloride (0 – 6.0 M) was concentration dependent with maximum effects observed above 5.0 M guanidine hydrochloride. There was a significant decrease in the intrinsic fluorescence intensity (~ 60 %) accompanied by a large red shift in the λ_{max} of emission from 337 to 350 nm. Guanidine hydrochloride mediated denaturation led to increased accessibility of tryptophan residues to the solute quenchers as evidenced by the increase in $f_{(a)eff}$ values with increase in the concentration of guanidine hydrochloride. CsCl was found to be a poor quencher even under denaturating conditions. Quenching studies indicate that more than 1/2 of the tryptophan fluorophores in β-galactosidase are located in a fairly hydrophobic environment and the surface exposed tryptophan residues are in an electropositive environment.

INTRODUCTION

Identification of specific amino acid residues involved in the catalytic function of enzymes and the specific role played by them is important in understanding the mechanism of action and structure-function co-relation. Several approaches like site directed mutagenesis, chemical modification, affinity labeling etc have been used to determine the residues that are involved in the catalytic function of enzymes [1,2]. Chemical modification using group specific reagents along with kinetic measurements of the inactivation process have been used in the study of many enzymes [3,4]. β-Galactosidases catalyze the hydrolysis of β-1-4 glycosidic linkages and have been classified into glycosyl hydrolase family 1, 2, 35 and 42 in view of its substrate specificity and sequence homology [5,6]. These enzymes have been extensively studied as regards their functional properties and industrial application [7,8].

The mechanism of action of glycosyl hydrolases is of two types: **a)** one that proceeds with an inversion of anomeric configuration and **b)** the other with a retention of the anomeric configuration. Despite the difference in the mechanism, these enzymes have been proposed to perform the catalysis via two carboxyl groups [9]. The active site and catalytic mechanism of β-galactosidase has been studied in detail in case of *E coli* β-galactosidase and Tyr 503, [10] Glu 461 [11] and Glu 537 [12] were observed to be important for the catalytic activity of β-galactosidase of *E. coli*. Recent X-ray crystal structure elucidates the involvement of Glu 461, Met 502, Tyr 503 and Glu 537 in the catalytic function of the enzyme [13]. However few reports exist on the active site characterization of fungal β-galactosidases [14].

Quenching studies provide valuable information about the exposure of protein bound intrinsic / extrinsic fluorophores. Tryptophan being an intrinsic fluorophore of proteins is sensitive to any change in the local environment which is reflected by a change in the wavelength and / or intensity of fluorescence. Details of the microenvironment of tryptophan residues can be obtained by comparing the quenching profiles for a protein using different quenchers [15]. The involvement of

tryptophan residues at the active site of various glycosyl hydrolases in the stacking of substrate has been documented earlier [16].

The present chapter discusses the results of chemical modification studies on the β-galactosidase of thermophilic fungus *Rhizomucor* sp. and the identification of amino acid residues involved in the catalytic function of the enzyme. The microenvironment of tryptophan residues and its accessibility in β-galactosidase from *Rhizomucor* sp., was accessed by comparing the quenching profiles of different solute quenchers in the absence and presence of denaturant.

MATERIALS AND METHODS

Materials

p-Nitrophenyl-β-D-galactopyranoside (*p*NPG), N-ethyl maleimide (NEM), 5,5'-dithiobisnitrobenzoic acid (DTNB), phenylmethylsulfonyl fluoride (PMSF), *p*-hydroxymercurybenzoate (PHMB), diethylpyrocarbonate (DEP), 3-nitro-L-tyrosine ethylester (NTEE), 1-ethyl-3-(3-dimethylaminopropyl) azonia carbodiimide (EDAC), 2-hydroxy-5-nitrobenzylbromide (HNBB), N-bromosuccinimide (NBS), iodoacetamide, 2,4,6,-trinitrobenzenesulphonic acid (TNBS), succinic anhydride, acetic anhydride, N-acetylimidazole, 2,3, butanedione (diacetyl), trichloroacetic acid, HEPES, MES, guanidine hydrochloride, acrylamide, KI and CsCl were obtained from Sigma Chemicals Co. (St. Louis, MO,USA). L-tryptophan, L-ascorbic acid, immidazole and hydroxylamine were purchased from Sisco Research Laboratory, India. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). All other reagents used were of analytical grade.

METHODS

Organism and culture conditions

Growth and maintenance of *Rhizomucor* sp., and the production and purification of was carried out as described in Chapter II and III, respectively.

Enzyme assay

β-Galactosidase was assayed as described in Chapter II. The *p*-nitrophenol or *o*-nitrophenol released on hydrolysis (pH 4.5, 60°C) was determined by reading the absorbance at 405 / 410 nm, respectively. One unit of enzyme activity was expressed as the amount of enzyme that releases 1μmole of product (*p*-nitrophenol / *o*-nitrophenol) per minute under standard assay conditions.

Protein determination

The protein concentration of purified β-galactosidase was determined by the method of Lowry *et al* [17] with bovine serum albumin (BSA) as standard.

Chemical modification studies

Modification of Tryptophan

Reaction with N-bromosuccinimide (NBS): The enzyme solution (2 μM, 1 ml) in 100 mM, sodium-acetate buffer, pH 5.5 was titrated with increasing concentrations of freshly prepared NBS (5-100 μM). The reagent was added in ten additions of 10 μl each. After each addition an aliquot was removed and quenched with 20 μl L-tryptophan (25 mM) and residual activity was determined, under standard assay condition. Enzyme samples incubated in the absence of NBS served as control. The NBS mediated inactivation was also monitored spectrophotometrically, by measuring the decrease in absorbance at 280 nm. The number of tryptophan residues modified was determined using a molar extinction coefficient of 5500 M^{-1} cm^{-1} [18]. Enzyme samples in the absence of reagent served as control.

Reaction with 2-hydroxy-5-nitrobenzylbromide (HNBB): HNBB was prepared freshly in dry acetone. Modification of the tryptophan residues of β-galactosidase was carried out by incubating separate aliquots of 2 μM of enzyme (500 μl) with varying concentrations of HNBB. The reaction mixture was rapidly mixed and allowed to react for 10-20 min. After the incubation period, the reaction was terminated by addition of a ten-fold excess of L-tryptophan (50 mM stock). The reaction mixture was passed through Sephadex G-25 (0.5 x 10 cm) to remove the

excess reagent and the residual activity was measured under standard assay condition. Enzyme samples incubated in the absence of reagent served as control. The number of tryptophan residues modified was determined spectrophotometrically at 410 nm, using a molar absorption coefficient of 18450 M^{-1} cm^{1} [19]. The acetone concentration in the reaction mixture did not exceed 5% (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. Enzyme samples incubated in the absence of reagent served as control.

Modification of lysine residues

Reaction with Succinic anhydride: This was carried out according the method of Habeeb et al. [20]. Purified β-galactosidase (2μM, 1 ml) in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentrations of succinic anhydride (0.5-3.0 M) prepared in dry dioxane and the residual activity was determined as described above. Enzyme samples in absence of reagent served as control.

Reaction with acetic anhydride: Acetylation was performed as described by Fraenkel-Conrat [21]. To 2 μM purified β-galactosidase in 1 ml saturated sodium acetate, six aliquots of acetic anhydride (0.1-1.0 mM) were added over a course of 1h. After each addition, an aliquot was removed and assayed for β-galactosidase activity under standard assay conditions.

Reaction with 2,4,6 trinitrobenzenesulfonic acid (TNBS): To the purified enzyme (2μM, 500 μl), 0.5 ml of 4 % sodium bicarbonate pH 8.0 and 0.5 ml of TNBS (0.1-1.0 %) was added. The reaction mixture was incubated in dark at 37°C for 2 h. The reaction was terminated by adjusting the pH to 4.5 and the residual activity was determined under standard assay conditions. Enzyme samples incubated in absence of TNBS served as control. The number of amino groups modified was quantitated spectrophotometrically, using a molar absorption coefficient of 9950 $M^{-1}cm^{-1}$ at

335 nm [22] for the trinitrophenylated lysine. β-Galactosidase incubated in the absence of reagent served as control.

Modification of Carboxylate groups

Modification with 1-ethyl-3-(3-dimethylaminopropyl) azonia carbodiimide (EDAC): 1.5 μM of enzyme solution (1 ml) in 100 mm HEPS / MES buffer (75:25 v/v) pH 6.0 was incubated with 50 mM EDAC and 30 mM NTEE at 30°C. Samples (5 μl) were removed and the reaction quenched with by addition of equal amount of 1 M, Na-acetate buffer, pH 4.5 and the residual activity was determined under standard assay conditions. To determine the number of nitrotyrosyl groups incorporated, the EDAC / NTEE treated enzyme samples were precipitated by the rapid addition of an equal volume of 10 % TCA and the mixture was left at 4°C for 30 min. The precipitate was washed thoroughly with chilled acetone, and air dried. The precipitated protein was collected by centrifugation (5000 **g** x 5 min.), washed extensively with chilled acetone and reconstituted in 1.0 ml of 100 mM NaOH. The number of nitrotyrosyl groups incorporated was determined by reading the absorbance at 430 nm, using a molar extinction coefficient of 46,00 M^{-1} cm^{-1} [23]. Enzyme in the absence of reagent served as the control.

Modification of Cysteine

Reaction with p-hydroxymercurybenzoate (PHMB): Reaction with *p*-hydroxy mercury benzoate (PHMB), was carried out in acetate buffer pH 5.5 by incubating varying concentration of PHMB (2-10μM) at 25°C, with 1.5μM enzyme (1 ml) [24]. Aliquots were withdrawn at different time intervals and passed through sephadex G-25 and the residual activity was determined under standard assay conditions. Enzyme in absence of the reagent served as the control.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB): β-Galactosidase (1.5μM, 1 ml) in 100 mm phosphate buffer, pH 8.0 was incubated with 0.5 mm DTNB (effective conc.) at 30°C for 1 hr. Aliquots were withdrawn to determine residual activity under standard assay conditions. The modification was monitored

by the absorbance at 412 mm and the number of residues modified was calculated by using a molar extinction coefficient of 13,600M^{-1} cm^{-1} [25]. Enzyme incubated in the absence of reagent served as control.

Modification of Arginine

Reaction with 2,3 butanedione (diacetal): The purified enzyme (1.5 μM, 500 μl) was incubated with varying concentration of the reagent (10-50 μM) in presence of 100 mM borate buffer pH 7.5 for 1 h at 30°C [26]. Aliquots were taken out to determine residual activity after removing the excess reagent by passing through Sephadex G-25. The enzyme in absence of reagent under identical conditions served as the control.

Modification of Tyrosine residues:

Reaction with N-acetylaimidazole (NAI): 1.5 μM of enzyme (1ml) in 100mM sodium borate buffer, pH 7.5, was incubated with 10 mM NAI for 30 min. at 28°C [27]. Aliquots were withdrawn and the residual activity was determined under standard assay conditions. Enzyme in absence of NAI served as control.

Modification of Histidine

Reaction with diethylpyrocarbonate (DEPC): An aliquot of DEPC was diluted with absolute alcohol. The concentration of this DEPC solution was determined by quenching an aliquot in 3 ml immidazole buffer (10mM, pH 7.0), and reading the absorbance at 230 mm. The amount of N-carbethoxyimidazole formed was calculated using a molar absorption coefficient 3300 M^{-1} cm^{-1} [28]. The concentration of DEPC was adjusted to 20 mM and used for the modification of β-galactosidase. 2 μM, (1 ml) of the enzyme in 100 mM MES buffer pH 6.0 was titrated with increasing concentrations of DEPC and the reaction was monitored by the increase in absorbance at 240 nm. Aliquots were withdrawn and the reaction was quenched by the addition of an equal volume of 100 mM imidazole buffer, pH 6.8. Subsequently the residual activity was determined under standard assay conditions. The addition of DEPC was continued till there was no change in the

absorbance at 240 mm. The number of residues modified was calculated as described by *Ovadi et al* [29]. Enzyme in the absence of DEPC serves as the control.

Modification of Serine residues

Reaction with phenylmethylsulfonyl fluoride (PMSF): The enzyme (1.5 μM, 1ml) in 50 mM sodium phosphate buffer, pH 7.5, was incubated with 10 mM of PMSF at 30°C for 2 h [30]. Aliquots were removed at different time intervals and the residual activity was determined under standard assay conditions.

Substrate protection studies

In all the chemical modification studies the effect of substrate was studied incubating the enzyme with excess amounts of lactose (50mM) followed by treatment with various modifying reagents.

FLUORESCENCE STUDIES

Preparation of enzyme solution

For all fluorescence studies, the enzyme solution was dialyzed against 20 mM Na-acetate buffer, 20 mM, pH 5.5 or Na-citrate-phosphate buffer, 20 mM, pH 5.5, for 24 h, and filtered through 0.45 micron filter and the protein concentration determined.

Fluorescence measurements

Fluorescence measurements were made on a Perkin Elmer LS 5B Spectrofluorimeter at 25°C, using an excitation and emission slit width of 5 nm and excitation wavelength of 295 nm. The spectra were recorded in the range of 300-400 nm. Influence of N-bromosuccinimide (NBS) on the intrinsic fluorescence and activity of β-galactosidase was studied by titrating the native enzyme (2 μM, 2 ml) with increasing concentration of NBS (1 μM – 50 μM) in 20 mM Na-acetate buffer, pH 5.5 at 25 ± 1°C. In a parallel experiment, the number of tryptophan

residues oxidized per subunit of enzyme was determined spectrophotometrically according to *Spande* and *Witkop* [18].

Solute quenching

The β-galactosidase (2 μM, 2.0 ml in 20 mM Na-citrate buffer, pH 4.5) was titrated with acrylamide, KI and CsCl in absence and presence of guanidine hydrochloride (0 – 6M). A stock solution of acrylamide (8M), KI (5.0 M) and CsCl (5.0 M) was prepared in Na-citrate buffer, 20 mM, pH 4.5 and 20 aliquots of 5-10μl were added to the protein solution. The stock solution of KI was routinely made in 0.1 mM $Na_2S_2O_3$ to eliminate the small amount of I_3^-. An excitation wavelength of 295 nm was used to selectively follow the tryptophan fluorescence of the protein and the relative fluorescence intensity of the protein was recorded in the range 300 – 400 nm. The quenching data was analyzed by the Stern–Volmer and modified Stern-Volmer equation using micro cal origin 4.0.

$$F_0/F=1+Ksv[Q]$$

$$F_0/\delta F = \{ 1/(f_a K_{sv})1/Q + 1/fa \}$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher, f_a is the effective fraction accessible fluorescence, K_{sv} is the effective quenching or Stern-Volmer constant, [Q] is the quencher concentration and δF is the difference in the fluorescence intensity in the absence and presence of quencher.

RESULTS AND DISCUSSION

Chemical modification

Involvement of tyrosine, histidine, methionine and carboxylate residues at the active site of the most well studied β-galactosidase from *E. coli* has been shown [13]. However, few reports exist on the characterization of the active site of fungal β-galactosidase [14]. Hence the effect of various chemical modifiers on β-galactosidase activity from *Rhizomucor* sp. was studied and the results are shown in

Table 1. Treatment of the enzyme with NAI, EDAC, phenylglyoxal, DEPC and DTNB did not result in any loss of enzyme activity indicating that tyrosine, carboxyl groups, arginine, histidine or sulfhydryl groups are not involved in the catalytic function of the enzyme. But modification of tryptophan residues by NBS / HNBB and lysine residues by acetic anhydride, succinic anhydride and TNBS led to total loss of enzyme activity indicating the presence of these residues at the catalytic site of the enzyme.

Modification of tryptophan: Purified β-galactosidase on incubation with a maximum of 20 μM NBS at pH 5.5 and 30°C lost all of its activity. However, no loss of activity was observed in the control samples. NBS mediated inactivation of the enzyme was concentration dependant and was accompanied by a decrease in absorption of the modified enzyme at 280 nm. Based on a molar absorption coefficient at 280 nm to be 5500 M^{-1} cm^{1} [18] and subunit M_r of 120 kDa, the total number of tryptophan residues modified was found to be eight. However the plot of percent residual activity versus the number of residues modified showed that the loss in activity resulted from the modification of a single tryptophan residue per monomer of the enzyme (Fig.1A). Similarly, modification of the purified enzyme with HNBB resulted in approximately 70 % loss of its initial activity and the inactivation was concentration dependent. The plot of percent residual activity against the number of tryptophan residues modified revealed that the loss of activity occurred due to the modification of one tryptophan residue per monomer (Fig 1B). NBS as well as HNBB mediated inactivation of β-galactosidase could be prevented significantly (75 %) by incubating the enzyme with excess amounts of the natural substrate, lactose (50 mM), prior to the modification reaction. Difference in the number of tryptophan residues modified by NBS / HNBB in the absence and presence of lactose gave a value of 1 (calculated on the basis of subunit M_r of 120 kDa) suggesting the involvement of one tryptophan per monomer, in the catalytic activity of the enzyme (Table 2).

Modification of lysine: Modification of purified β-galactosidase with succinic anhydride and acetic anhydride resulted in 80 % and 83 % inactivation of the enzyme activity, respectively and the inactivation was observed to be concentration dependent. Inactivation with succinic anhydride and acetic anhydride could be prevented to a significant extent (80 %) by preincubating the enzyme with lactose (50 mM). Purified β-galactosidase when incubated with 1 mM TNBS at pH 8.0 and 37°C lost 60 % of its initial activity and the inactivation was concentration dependent. No loss in activity was observed in the control samples. The plot of number of amino groups modified versus the percent residual activity revealed that the loss in activity resulted from the modification of one residue per monomer of the enzyme (Fig.2). The loss in enzyme activity by TNBS was prevented to a significant extent by incubating the enzyme with excess lactose (50 mM) prior to the modification reaction. Determination of the number of lysine residues modified by TNBS [22], in the absence and presence of lactose, gave a value of 8 and 7 , respectively suggesting the involvement of one lysine per monomer in the catalytic activity of the enzyme (Table 3).

In the absence of crystallographic insights, the amino acid residues important in catalysis can be identified through specific labeling along with kinetic analysis of the enzyme [31-33]. Site directed mutagenesis as well as sequence alignment has been used to identify the amino acid residues involved in the catalytic function of β-galactosidase [34]. The chemical modification studies on the β-galactosidase from *Bacillus* sp. revealed the involvement of one cysteine, one histidine and two methionine residues in the catalytic function of the enzyme [35]. Reversible inactivation (with mercaptoethanol) due to modification of methionine using N-bromoacetyl-β-D-galactopyranosylamine, iodoacetamide and bromoacetamide was observed in *E. coli* β-galactosidase [36,37]. Methionine-500 in β-galactosidase of *E. coli* was identified as an active site residue using an affinity label, β-D-galactopyranosyl methyl-*p*-nitrophenyl triazene (β-GalMNT). β-GalMNT also inactivated the β-galactosidase of *Aspergillus oryzae* [14]. However no inactivation of enzyme activity was observed on treatment of the β-

galactosidase of *Rhizomucor* sp. with iodoacetamide, indicating that methionine does not have a role in the catalytic function of the enzyme.

In most of the β-galactosidases the amino acids important for catalysis have been identified from the deduced amino acid sequences compared with that of the *E. coil β*-galactosidase, and generally a single Glu, and a single Tyr is found to be conserved [38]. Conduritol C epoxide proved to be effective in the identification of Glu 461 as the nucleophile [11] in the *E.coli* β-galactosidase however, site directed mutagenesis revealed the involvement of Glu 537 as the nucleophile [12]. The crystal structure of the enzyme has confirmed the presence of both these residues in the active site of the enzyme [13]. Participation of carboxyl groups has been reported in case of various glycosyl hydrolases like lysozyme [39], cellulose [2], cellobiohydrolase [40], glucoamylase [41], α-galactosidase [42], α-amylase [43], cyclodextrin glycosyl transferase [44] and xylanase [45]. Glu 268 has been identified as the catalytic nucleophile of the human lysosomal β-galactosidase precursor using a slow substrate 2,4-dinitrophenyl-2deoxy-2-fluoro-β-D-galactopyranoside. The glycosyl enzyme intermediate was trapped and the tryptic digest was analyzed by mass spectroscopy. This glutamic acid was conserved in the family 35 enzymes [46]. The carboxylate ion is an integral part of the active site of those enzymes in which acid-base type catalytic mechanism is prevalent. The possibility that all glycosidases share a common mechanism of catalysis has been investigated intensively for various glycosidases of which the hen egg white lysozyme is a classic paradigm. The carboxylate residue is found to be conserved in almost all of the β-galactosidases sequenced so far [47]. The inability of EDAC to inactivate the β-galactosidase in *Rhizomucor* sp. indicates that probably the carboxylate groups important for activity are protected and inaccessible to the reagent.

The importance of Tyr-503 in the catalytic function of β-galactosidase was established by multiple replacement, where Tyr was replaced either by Phe, His, Cys, or Lys [10]. Tyrosine is also one of the conserved residues of the active site of β-galactosidases. The β-galactosidase of *Lactococcus lactis* sp. showed a high

Table 1: Effect of different chemical modifying reagents on the activity of galactosidase from *Rhizomucor* sp.: Enzyme (2μM, 1 ml) was incubated with various reagents at room temperature, the reaction was terminated and the residual activity was determined under standard assay conditions.

Chemical reagent	Concentration	% Residual activity	Reaction buffer	No. of residues modified/subunit
None	-	-	-	-
NBS	10μM	0	Na-acetate, pH 5.5, 100 mM	8
HNBBr	50mM	0	Na-acetate, pH 5.0, 100 mM	6
Succinic anhydride	10mM	10	Na-bicarbonate, pH 8.0, 100 mM	N.D.
Acetic anhydride	10 mM	10	Na-bicarbonate, pH 8.0, 100 mM	N.D.
TNBS	50mM	0	Na-bicarbonate, pH 8.0, 100 mM	7
DEPC	50μM	80	Cit-phosphate, pH 6.0, 100 mM	10
WRK	5mM	100	Cit-phosphate, pH 6.0, 100 mM	8
EDC	50mM	100	MES/HEPS, pH 6.0, 50 mM	9
Butanedione	50 mM	85	Na-borate, pH 8.5, 50 mM	-
NAI	10 mM	100	Na-phosphate, pH 7.0, 100 mM	N.D.
TNM	50μM	100	Tris-HCl, pH 8.0, 100 mM	9
PMSF	50 mM	100	K-phosphate, pH 7.2, 100 mM	N.D.
DTNB	1 mM	100	Na-phosphate, pH 8.0, 50 mM	4

N.D.: Not determined

Table 2: Influence of modification tryptophan with NBS and HNBB on activity of β-galactosidase from *Rhizomucor* sp.

Incubation mixture	Residual activity (%)	Number of residues modified
Control	100	
Enzyme + NBS (5μM)	30	4
Enzyme +lactose (50 mM) + NBS	73	3
Enzyme + HNBB (5 mM)	30	3
Enzyme +lactose (50 mM) + HNBB	75	2

Fig 1: Plot of percent residual activity versus the number of residues modified
A: Modification of β-galactosidase of *Rhizomucor* sp. with NBS.
B: Modification of β-galactosidase of *Rhizomucor* sp. with HNBB.

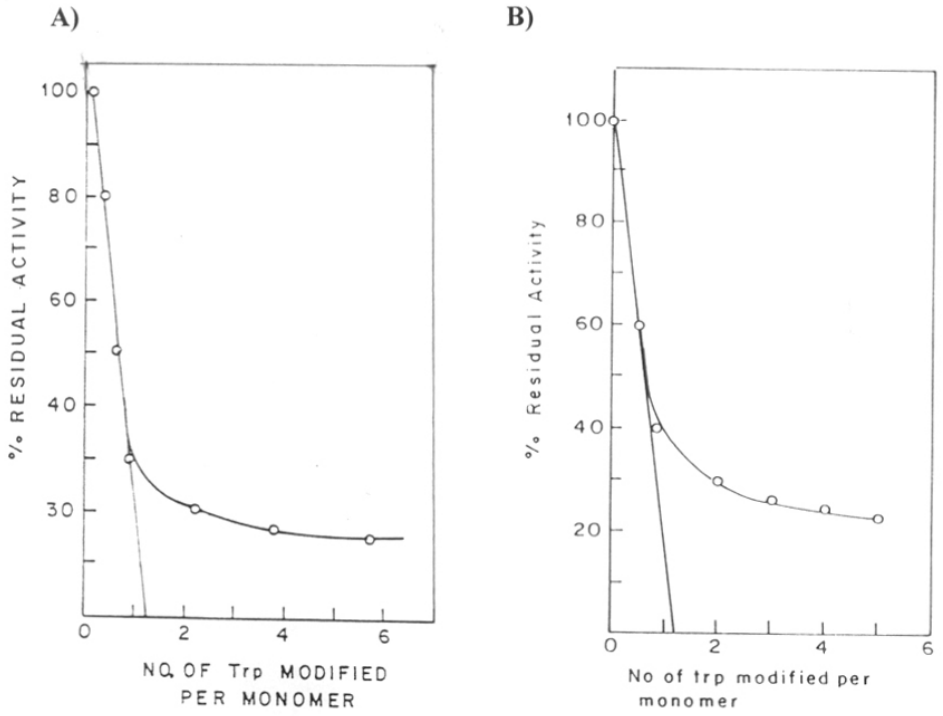
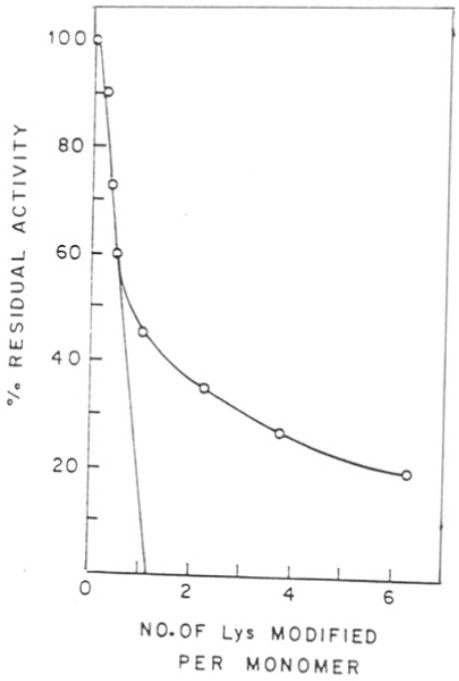


Table 3: Influence of modification of lysine on activity of β-galactosidase from *Rhizomucor* sp.

Incubation mixture	Residual activity (%)	Number of residues modified
Control	100	
Enzyme + TNBS (3μM)	25	8
Enzyme +lactose (50 mM) + TNBS	87	7
Enzyme + Succinic anhydride	20	N.D.
Enzyme + Acetic anhydride	17	N.D.

N.D. Not determined

Fig 2: Plot of percent residual activity versus the no. of residues modified using TNBS



degree of sequence homology to the *E. coli* β-galactosidase and the putative active site residues Tyr 475 and Glu 429 were conserved [48]. Tyr 503 and Glu 537 were also found to be the conserved residues in the swine pathogen *Actinobacillus pleuropneumoniae* [49].

Besides these residues, chemical modification studies have shown the involvement of other residues like cysteine, histidine [50,35] in the catalytic function of the enzyme. Presence of an essential thiol in the active site was observed in the β-galactosidase from *Camellia* tealeaf [51] and the NBS modification indicated the presence of a tryptophan near the galactose binding region in the β-galactosidase from *Macrophomina phaseoli* [52].

FLUORESCENCE STUDIES

Influence of NBS on fluorescence and activity of β-galactosidase

The β-galactosidase of *Rhizomucor* sp. exhibited a fluorescence maximum at 337 nm on excitation at either 280 or 295 nm. The amino acid analysis of the β-galactosidase indicated the presence of 27 tryptophan residues per monomer of the enzyme (Chapter III) and chemical modification studies revealed the involvement of a single tryptophan residue in the active site of the enzyme. Oxidation of tryptophan residues by NBS resulted in a decrease in the intrinsic fluorescence (Fig.3). About 55 % of the original fluorescence was abolished on extensive titration of the β-galactosidase with NBS (molar ratio of enzyme : NBS, 1: 20). Further addition of NBS led to a spectral blue shift from 337 to 332 nm, as well as an increase in A_{280} indicating cleavage of the polypeptide [18]. It was observed that β-galactosidase lost 60 % of its initial activity when only 25 % of the intrinsic fluorescence was quenched by NBS (molar ratio of NBS : enzyme 5 : 1) suggesting that only a few tryptophan residues are involved in the enzyme activity. Extensive titration of β-galactosidase with NBS (molar ratio of enzyme : NBS, 1: 20) revealed that 12 residues could be modified with NBS in its native state. This suggests that more than 50 % of the protein fluorescence in the native state is from these 12 tryptophan residues. However the energy transfer between the non-fluorescent tryptophan residues cannot be ruled out [53]. The NBS quenching data also

indicates that about 15 of the tryptophan residues of the β-galactosidase of *Rhizomucor* sp. are buried in a hydrophobic core of the enzyme.

Solute quenching

Small molecules like acrylamide, CsCl and KI are known to quench the protein fluorophors in proteins. The microenvironment of tryptophan fluorophors in the native β-galactosidase was accessed with these quenchers and it was observed that acrylamide was a better quencher (55 %) as compared to KI (21%) or CsCl (3 %). Although the neutral quencher acrylamide should ideally quench all the tryptophan fluorophors, the 3-D structure as well as the local environment around the tryptophan fluorophors play a major role in the accessibility towards the quencher. The 55 % quenching observed for acrylamide along with the 55 % quenching of the tryptophan fluorophors with NBS indicates that more than half of the tryptophan fluorophors are buried in a hydrophobic core, inaccessible even to the neutral quencher, acrylamide. The ionic quenchers KI and CsCl are able to quench surface exposed tryptophan fluorophors which are either in an electropositive or an electronegative environment. The 21 % quenching of the fluorophors with KI indicates that only few of the surface exposed tryptophan fluorophors are accessible to KI, and are in an electropositive environment. The poor quenching by the anionic quencher CsCl (3 %) further indicates that the tryptophan residues are in an electropositive environment.

The direct Stern-Volmer plots were linear in case of acrylamide and downward curving in case of KI (Fig.4A). This indicates that all the tryptophans that are surface exposed are not accessible to quenching with KI and those that are quenched by KI are in an electropositive environment. The deviation of the Stern-Volmer plot for KI towards the concentration axis indicates the non-permeability of the quencher to the interior of the protein, and a variation in the accessible fluorophores [54]. The modified Stern-Volmer plots were linear for acrylamide and KI (Fig.4B). At higher concentrations of the quencher, all the accessible residues are quenched and the remaining fluorescence is from inaccessible residues, whose fluorescence is independent of the concentration of the quencher. Although an

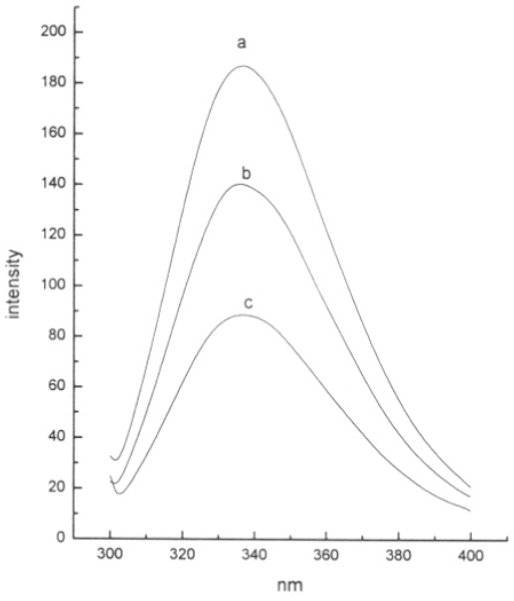
emission maximum at 337 nm indicates that the tryptophan residues are present in a moderately hydrophobic environment, the above quenching studies along with the NBS mediated oxidation of the tryptophan residues indicate that more than half of the tryptophan residues are located in a fairly hydrophobic environment.

As the percentage quenching in case of the neutral quencher acrylamide, in the native state of β-galactosidase was observed to be only 55 %, the protein was treated to varying concentrations of the denaturant, guanidine hydrochloride (3.0-6.0 M), and its effect on the structure and solute quenching parameters was studied. The extent of denaturation of β-galactosidase with Gdn.HCl was dependent on the concentration of the denaturant and maximum effects were observed at and above 5.0 M Gdn.HCl (Fig.5). Denaturation in the presence of Gdn.HCl causes extensive changes in the fluorescence emission, indicated by a large red shift from 337 to 350 nm in the denatured state. The shift in the λ_{max} of emission suggests that the fluorescent tryptophan residues in the native enzyme are in a fairly hydrophobic environment. A 36 % decrease in the intensity of fluorescence was observed in the denatured state of the enzyme as compared to that of the native state. The localization of the emission maxima at 350 nm on denaturation indicates the exposure of the tryptophan side chains to the aqueous environment [55]. Increased fluorescence intensities and quantum yields have been observed in many multi-tryptophan proteins on treatment with denaturing agents [56]. However, in case of the β-galactosidase of *Rhizomucor* sp. a decrease in fluorescence intensities on treatment with Gdn.HCl was observed. This could be due to the shielding effect by certain amino acids lying close to the tryptophan fluorophores which react reversibly with Gdn.HCl [57].

Denaturation of the protein with 3.0 M Gdn.HCl led to increased percentage quenching as well as an increase in the fraction of the accessible fluorescence and exhibited linear Stern-Volmer plots for all the solute quenchers (Table 4). In presence of 5 M Gdn.HCl, linear Stern-Volmer plots were observed in all quenchers with increased quenching efficiencies (Fig.6A). Acrylamide showed almost 100% quenching at 6 M Gdn.HCl indicating that all tryptophan fluorophors are exposed on denaturation and accessible to the neutral quencher. Although an

increase in the percentage quenching was observed for the charged solutes, only 65 % quenching was observed for KI, where as CsCl was observed to be a poor quencher even in the denatured state of the enzyme, with only 18 % of the fluorescence being quenched at 6 M Gdn.HCl. The ionic quenchers KI and CsCl have similar hydrated ionic radii, however, the difference in their quenching is a result of the difference in the charge distribution around the tryptophan fluorophors [58]. At 6 M Gdn.HCl most of the exposed tryptophan residues are in an electropositive environment and therefore accessible to KI indicated by increased $f_{a(eff)}$ (0.77), whereas complete accessibility to CsCl is not achieved even under denaturing conditions.

Fig. 3: Quenching of fluorescence of native β-galactosidase on titration with NBS



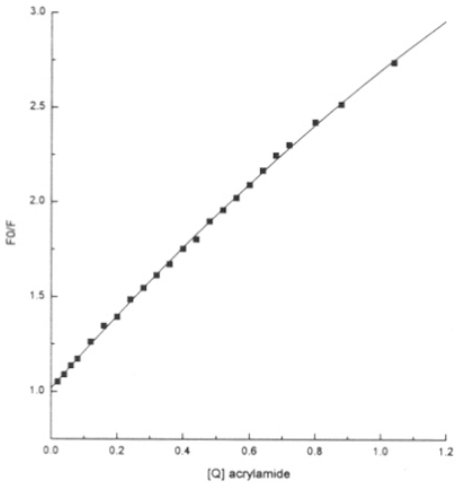
The emission spectra were recorded from 300 - 400 nm after excitation of the enzyme (2 μM, 2.0 ml) at 295 nm. The molar ratios of NBS to β-galctosidase are **a)** native enzyme, λ_{em} 337nm **b)** [NBS] : [β-galactosidase] : : 5 :1, λ_{em} 336 nm **c)** [NBS] : [β-galactosidase] : : 20 :1, λ_{em} 336 nm.

Fig 4A: Stern-Volmer plots for quenching of fluorescence of native β-galactosidase from *Rhizomucor* sp.

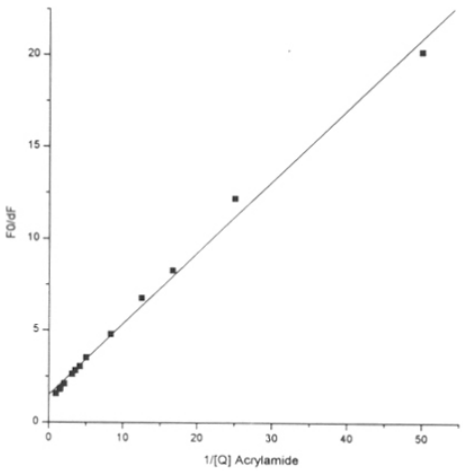
Fig 4B: Modified Stern-Volmer plots for the quenching of native β-galactosidase from *Rhizomucor* sp.

i) Acrylamide ii) KI

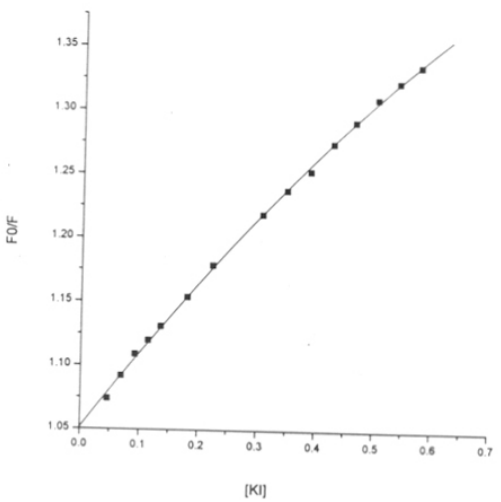
A i)



B i)



ii)



ii)

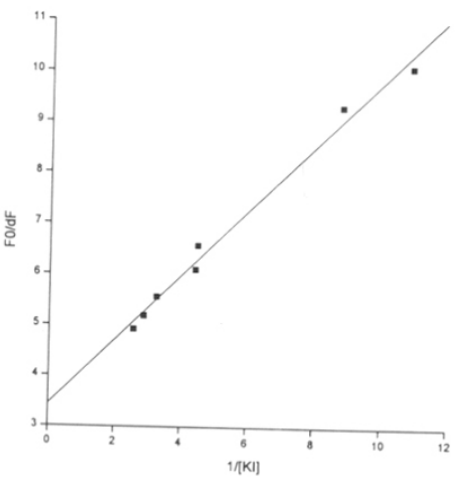


Fig 5: Effect of guanidine hydrochloride (Gdn.HCl) on the fluorescence emission spectrum of β-galactosidase from *Rhizomucor* sp.

β-Galactosidase (2 μM, 2.0 ml, 20 mM Na-citrate buffer pH 5.5) was treated with varying concentrations of Gdn.HCl at 4°C for 48 h. the treated proteins were excited at 295 nm and the emission recorded in the range 300 - 400 nm at 25 ± 1°C. (A) native enzyme, (B) 3 M, (C) 4 M and (D) 5M

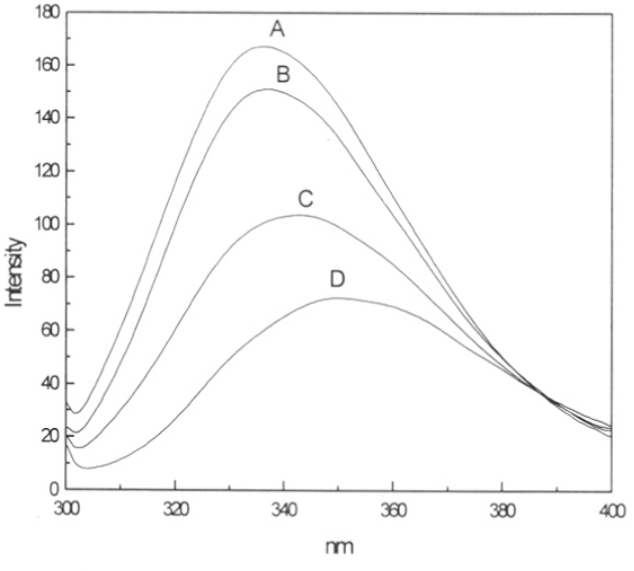
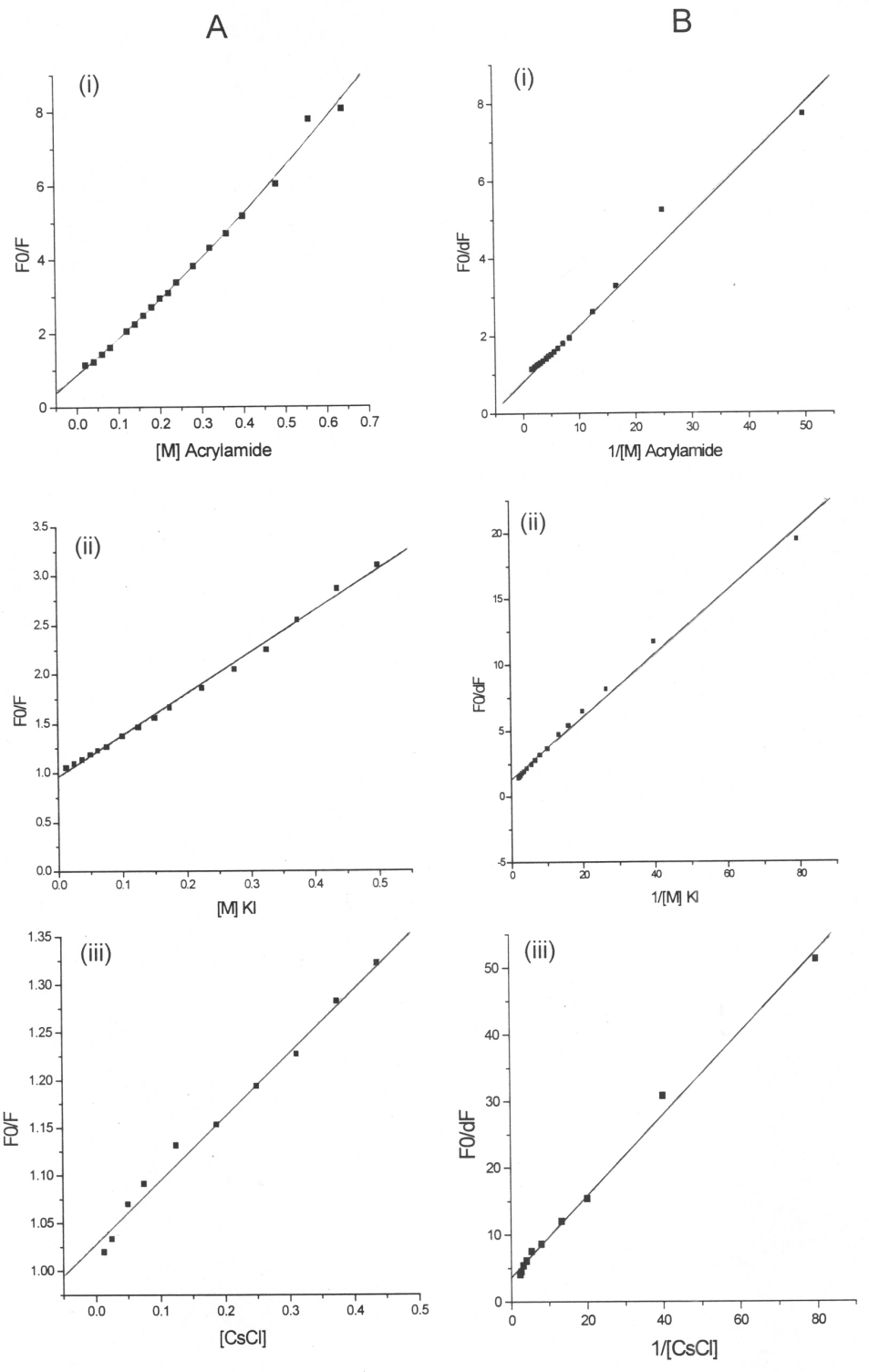


Table 4: Quenching parameters of neutral and ionic quenchers on the fluorescence of native and denatured β-galactosidase from *Rhizomucor* sp. The protein (2 μM in 2.0 ml of Na-citrate buffer, pH 5.5) was titrated with various solute quenchers using an excitation and emission wavelength of 295 nm and the emission recorded in the range 300 - 400 nm.

Quencher	Conc. Gdn.HCL(M)	$F_{a(eff)}$	$K_{(SV)eff}$	% Quenching
Acrylamide	0	0.78	3.43	55
	3	0.60	13.0	65
	4	0.71	7.0	66
	5	0.88	6.66	75
	6	1.15	6.08	97
KI	0	0.29	5.41	21
	3	0.33	5.56	29
	4	0.42	6.66	33
	5	0.54	5.51	55
	6	0.77	5.41	65

Fig 6A: Stern-Volmer plots for the quenching of fluorescence of denatured β-galactosidase (6 M gdn.HCl) from *Rhizomucor* sp.

Fig 6A: Modified Stern-Volmer plots for the quenching of fluorescence of denatured β-galactosidase (6 M Gdn.HCl) from *Rhizomucor* sp.
i) Acrylamide ii) KI iii) CsCl


A
B
(i)
F0/F
[M] Acrylamide
(i)
F0/dF
1/[M] Acrylamide
(ii)
F0/F
[M] KI
(ii)
F0/dF
1/[M] KI
(iii)
F0/F
[CsCl]
(iii)
F0/dF
1/[CsCl]

Conclusion

The chemical modification studies of the β-galactosidase from *Rhizomucor* sp. shows the involvement of a single tryptophan and a single lysine residue in the catalytic function of the enzyme. At least 1/2 of the total tryptophan residues in the native enzyme are in a relatively rigid hydrophobic environment. Analysis of quenching characteristics with various solute quenchers indicates variations in the class of accessible tryptophan fluorophores. In the native enzyme acrylamide is a better quencher than KI, where as CsCl is an inefficient quencher indicating that the tryptophan fluorophors in the native enzyme are in an electropositive environment.

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CONCLUSIONS

1. The thermophilic fungus *Rhizomucor* sp. produces extracellular β-galactosidase (E. C. 3.2.1.23) when grown under submerged fermentation conditions.

2. Increased yields of the extracellular enzyme were obtained under solid state fermentation conditions.

3. The optimum temperature for enzyme activity is 60°C, and the enzyme has a $t_{1/2}$ of 150 min at 70 °C, which is one of the highest reported for any fungal β-galactosidase.

4. The β-galactosidase is specific for β-linked galactosides with a preference for synthetic substrates *p*NPG and *o*NPG.

5. The enzyme is stable over a pH range of 3.5 - 7.5, indicating its potential for application in the hydrolysis of whey hydrolysis as well as in the hydrolysis of milk lactose.

6. The active site characterization indicates the participation of a single tryptophan and a single lysine in the catalytic function of the enzyme.

7. Fluorescence studies indicate that at least 1/2 of the Trp residues in β-galactosidase are in a rigid hydrophobic environment.

8. The solute quenching studies indicate that the exposed tryptophan residues of *Rhizomucor* sp. β-galactosidase are in an electropositive environment.

LIST OF PUBLICATIONS

1. Production of β-galactosidase from thermophilic fungus *Rhizomucor* sp. **Shaikh, S. A.**, Khire, J. M. and Khan, M. I. ***J. Ind. Microbiol. Biotechnol.*** (1997) **19,** 239-245.
2. Characterization of extracellular β-galactosidase from thermophilic fungus *Rhizomucor* sp. **Shaikh, S. A.**, Khire, J. M. and Khan, M. I. ***Biochim. Biophys. Acta*** (1999) In Press.