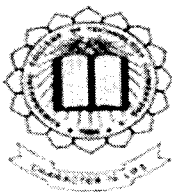

PURIFICATION AND CHARACTERIZATION OF α-GALACTOSIDASE FROM *Penicillium chrysogenum*

A PROJECT REPORT

Submitted by

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Of

BACHELOR OF TECHNOLOGY

in

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KUMARAGURU COLLEGE OF TECHNOLOGY

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Certified that this project report entitled **"Purification and Characterization of α-galactosidase from *Penicillium chrysogenum*"** is the bonafide work of Miss. R. Sangeetha, Miss. K. P. Sivasankari, and Miss. M. Vidya who carried out the project work under my supervision.

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(INTERNAL EXAMINER) (EXTERNAL EXAMINER)

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ABSTRACT

The present study was carried out to purify, characterize and applications of α-galactosidase from *Penicillium chrysogenum*. The enzyme was optimally active at 60°C for the hydrolysis of p-nitrophenyl-α-D-galactopyranoside (PNPG). The optimum pH of the enzyme was found to be 4.5. The metal ions such as Ag^{+} , Hg^{2+} and Cu^{2+} strongly inhibited the enzyme activity. Among the sugars tested on enzyme activity melibiose and lactose showed the maximum inhibitory effect. Among the reagents tested n-bromosuccinimide exhibited maximum enzyme inhibition. The kinetic properties of α-galactosidase of *Penicillim chrysogenum* were investigated in detail using PNPG. K_m and V_{max} for the enzyme was found to be 0.33 mM and 0.005U, respectively. The TLC pattern revealed that stachyose and raffinose in soymilk were completely hydrolyzed by the enzyme in 4 hours at 60°C.

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ABBREVIALIONS	EXPLANATIONS
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
h	Hour
l	Litre
min	Minutes
EDTA	Ethylene diamine tetra SAcetic acid
PNPG	p-Nitrophenyl-α-D-galacto -pyranoside
M	Molar
mM	Millimolar
nm	Nanometer

INTRODUCTION

1. INTRODUCTION :

Enzymes are biocatalyst, which speed up the rates of reactions without undergoing any permanent change. Life on this planet is possible because of the presence of the enzymes, without which many of the reactions would not occur over a period of years. The term enzyme is derived from the Greek meaning 'in yeast' and was first used by Kuhne in 1878 to the extracts or secretions from whole microorganisms. The enzyme activity could be expressed even in the absence of an integrated cellular Buchner demonstrated structure in 1897. Emil Fischer first time proposed the specificity shown by an enzyme for its substrate in 1894. Sumner in 1926 for the first time crystallized the enzyme 'urease' from jack bean extract and has shown that it is protein in nature. X-ray crystallography was used in 1965 to deduce the three-dimensional structure of lysozyme, an enzyme cleaving certain bacterial cell walls. The catalytic activity of certain enzymes is regulated by the binding of small molecules (effectors) in response to changes in physiological conditions and led Monod and his colleagues in 1965 to propose their 'allosteric model' to such enzymes.

1.1 α–Galactosidase [α–D-galactoside galactohydrolase, EC 3.2.1.22]

The disaccharide melibiose was hydrolysed by the crude enzyme preparations (melibiases) from bottom fermenting yeast (Fischer and Lindner, 1885). Weidenhagen (1928) who studied the specificity of action of the melibiose using a number of sugars having non-reducing terminal α–D-galactose residues and coined the name α–galactosidase to melibiase.

1. Specificity:

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

A. Hydrolase activity, and

B. Transgalactosylase activity.

A. Hydrolase activity :

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

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

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

Like other carbohydrates, α–galactosidase can also tolerate at C-6 of the glycosyl moiety of the substarte. Hence α–galactosidase from several sources has the capacity to hydrolyze β-L-arabinosides. However, α–galactosidase from *Streptococcus bovis*, *Diplococcus pneumoniae* and *Calvatia cyathiformis* cannot act on arabinosides (Dey) and Pridham, 1972. Dey and Pridham (1969a) have reported that p-nitrophenyl α–D-fucoside can be hydrolysed by α–galactosidase, because p-nitrophenyl α–D-fucoside has the similar configuration with that of D-galactose. α–Galactosidase from sweet almond and yeast are not able to hydrolyze the α-isomer of D-glycero-D-galactoheptoside.

Dey and Pridham (1969a) have carried out detailed study with respect to V_{max} and K_m on the glycosidic specificity of α–galactosidase from *Vicia faba*. They have shown that hydrolyzability (V_{max}) of glycosides show an apparent random variation. However, the affinity ($1/K_m$) of the enzymes varies largely on the configurational changes in the glycon moiety and follows the order:

α–D-galactoside > α–D-fucoside > β-L-arabinoside

The above arguments strongly suggest that primary alcohol group of the galactose molecule of the substrate is the specific point of attachment to the enzyme. Generally, aglycon group of a substrate have little or no effect on hydrolysis by glycosidase. α–Galactosidases from various plant, animal and microbial sources hydrolyze the following naturally occurring and synthetic α–D-galactosides, which are listed under two heads in Table1.1.

Table 1.1. List of synthetic α–D-galactosides and oligosaccharides

i) α–D-Galactosides	ii) Oligosaccharides
1. Methyl α–D-galactoside	1. Melibiose
2. Ethyl α–D-galactoside	2. Epimelibiose
3. N-Propyl α–D-galactoside	3. Melibiotol
4. Phenyl α–D-galactoside	4. Melibionic acid
5. O-Cresyl α–D-galactoside	5. Raffinose
6. M-Cresyl α–D-galactoside	6. Umbelliferose
7. P-Cresyl α–D-galactoside	7. Planteose
8. P-Aminophenyl α–D-galactoside	8. Manninotriose
9. m-Cholophenyl α–D-galactoside	9. Manninotritol
10. O-Nitrophenyl α–D-galactoside	10. Stachyose
11. M-Nitrophenyl α–D-galactoside	11. Verbascose
12. P-Nitrophenyl α–D-galactoside	12. Manninotrionic acid
13. 6-Bromo-2-naphthyl α–D-galactoside	13. Lychyose
14. P-Nitrophenyl α–D-lyxopyranoside	

iii) Polysaccharides :

Galactomannans are found as a major component of the endosperm in the seeds of many plants. Galactomannans consists of a backbone of β-(1,4)-linked D-mannosyl residues to which single α–(1,6)-linked D-galactosyl groups are attached. α–D-Galactopyranosyl residues from galactomannan are released during seed germination and serves as energy source for the growing seedling (McCleary et al., 1981). The removal of terminal α–D-galactopyranosyl moieties of blood group-B substances by α–-galactosidase to type-O has been reported for a limited number of enzymes, including coffee bean α–D-galactosidase (Harpez et al., 1975).

B. Transgalactosylase Activity :

Blanchard and Albon (1950) for the first time reported the transferase properties α–galactosidase from yeast. They found that galactose from one melibiose was transferred to a second melibiose acceptor molecule leading to the formation of manninotriose. This transgalactosylation property of α–galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity, donor and acceptor concentration, pH, temperature and the source of enzyme (Dey and Pridham, 1972).

Apart from hydrolase activity, α–galactosidase also exhibits transglycosylase properties, but relatively at high substrate concentrations (Ohtakara et al. 1984; Van Laere et al. 1999). In recent years, transgalactosylation catalyzed by α–galactosidase is receiving a considerable attraction from investigators (Eneyskaya et al. 1998; Van Laere et al., 1999; Spangenberg et al., 1999; Andre et al., 2001). Transgalactosylation occurs in two steps: 1)

transfer of glycogen preferably α–D-galactose, from donor to enzyme, and 2) transfer of glycogen from enzyme to an acceptor. Usually, in step 2, the glycon moiety is transferred to hydroxyl groups other than water. Thus, transgalactosylation reactions lead to the formation of a triple complex, ES'S, where S' is a glycosidic residue from donor and S is a second substrate molecule to which S' is transferred (Eneyskaya et al., 1998). In tranglycosylation reactions, glycogen moiety from donor is accepted by hydroxyl group of methionine present at the active site of α–galactosidase from *Trichoderma reesi* (Eneyskaya et al., 1998). The nature of galactosidic bond [α–(1,3)-, or α–(1,4)-, or α–(1,6)-] in transgalactosylation product(s) depend on the donor and the source from which α–galactosidase is obtained (Mitsutomi and Ohtakara, 1988; Van Laere et al., 1999). Because of competition in transgalactosylation reactions (self-condensation and condensation reactions), the end products are usually complex mixture (Eneyskaya et al., 1988).

Literature in this area of transgalactosylation reveals that hexoses act as galactosyl accepters. The efficiency of various sugars as galactose acceptors can be calaculated in terms of percent transfer phenyl α–D-galactoside as donor (Courtois and Percheron, 1961) is as follows:

$$\frac{\text{Phenol liberated (mole)} - \text{galactose liberated (mole)}}{\text{Phenol liberated (mole)}} \times 100$$

Dey and Pridham (1972) opinioned from the results of transgalactosylation studies that α–galactosidase preferably transfer galactosyl moieties to the primary alcoholic groups of acceptor molecule. Table 1.2 briefly introduces us to the results of some of the transgalactosylation studies with α–galactosidase.

Table 1.2: Transgalactosylation properties of α-Galactosidase

Acceptor	Donor	Transfer product	Source of α–galactosidase
Raffinose	Galactinol	Stachyose	*Phaseolus vulgaricus*
Sucrose	Melibiose	Raffinose	*Streptococcus bovis*
Sucrose	Raffinose	Planteose	*Triticum sp.*
Raffinose	Raffinose	Stachyose	*Vicia sativa*
Glucose	PNPG	Melibiose	*Prunus amygdalus*

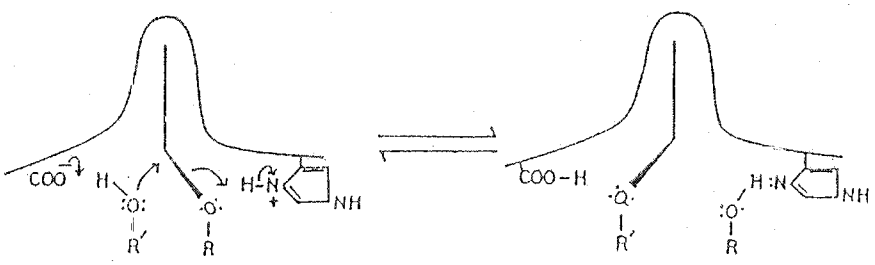
Hara et al., (1994) have reported that coffee bean α–galactosidase transferred a galactosyl residue not only to glucosyl cyclodextrins, but also directly to cyclodextrin rings. They further reported that α–galactosidase from *M.vinacea* transferred a galactosyl residue on side chains of maltosyl cyclodextrins. Van Laere et al., (1999) reported that α–galactosidase from *Bifidobacterium adolescentis* synthesized stachyose in the presence of raffinose, and from stachyose, verbascose was obtained.

1.2 Mechanism of Action of α–Galactosidase :

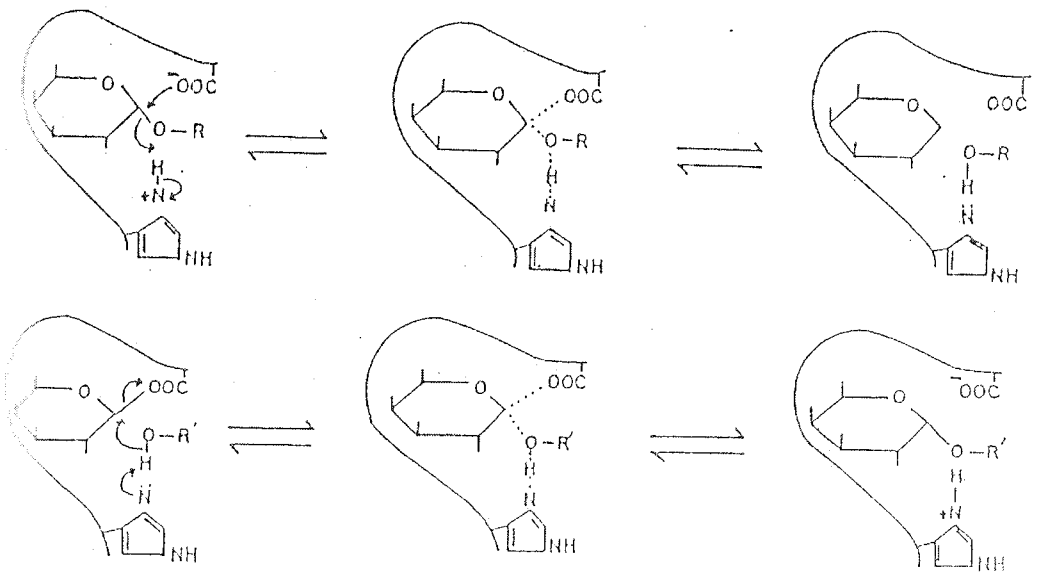
When compared with bond fission by other glycosidases in their substrates α–galactosidase is also believed to cleave the galactose–oxygen bonds of substrates (Dey and Pridham, 1972). Nuclear magnetic resonance and polarimetry studies have clearly indicated that the anomeric configuration of the liberated galactosyl residues has the same configuration as that of its substrates (Dey et al., 1970). Like other glycosidases, in α–D- from sweet almond using aryl α–D-galactosides as substrates indicate that the electronic nature of the aglycon has a noticeable influence on the rate of enzymatic hydrolysis.

A plot of log Vmax versus Hammett constants (σ) results in two sets of straight lines indicating the possibility of substituent in the aromatic ring. Later, Dey and Malhotra (1969) have identified the presence of basic and acidic groups at the active by kinetic studies as carboxyl (deprotonated) and imidazolium (protonated) groups. On the basis of the above results, Dey (1969) has reported two alternative mechanisms for the action of sweet almond α–galactosidase, i.e., two-step mechanism and one-steep mechanism. Mathew and Balasubramaniam (1987a) have proposed a new mechanism of action of α–galactosidase from coconut. (Figure 1.1). The effect of pH on Km and Vmax values reflects the involvement of two ionizing groups with pKa values of 3.5 and 6.5 in catalysis. Chemical modification studies suggest the presence of two carboxyl groups, a tryptophan and a tyrosine, at or near active site of enzyme.

The carboxylate anion would not only help to stabilize the carbonium ion intermediate but also would direct the nucleophilic attack on the carbonium ion from one direction so that the product retains the same configurations the substrate. The galactose molecule can assume the half chair conformation when carbonium ion is formed, the group with a pKa of 6.5 could be perturbed carboxylic group that is present in the protonated form and is involved in the donation of a proton. The carboxyl group is perturbed due to the hydrophobic environment produced by the presence of tryptophan and tyrosine residues in its vicinity. Shabalin et al. (2002) reported that stereochemical course of stachyose hydrolysis by α–galactosidases from *Trichoderma reesei* occurs with retention of anomeric configuration and was assumed to operate via a double displacement mechanism.



One-step mechanism



Two-step mechanism

Figure 1.2 Mechanism of action of α-galactosidase

A. One step mechanism

B. Two step mechanism

1.3 Raffinose - Family Sugars :

The oligosaccharides belong to raffinose-family sugars are raffinose, stachyose and verbascose (Figure 1.2). These sugars contain one, two or three galactose units joined to sucrose by α–1,6-linkages. The raffinose-family sugars have been identified as one of the contributors of flatus (gas production) inhuman and experimental animals (Cristafaro et al., 1973).

1.3.1. Raffinose :

Raffinose[O-α–D-galactopyranosyl-(1→6)-α–D-glucopyranosyl-(1→2)-β-D-fructofuranoside] is the first member of a homologous series of oligosaccharides, generally termed the raffinose-family, in which successive D-galactosyl groups are joined by O-α–D-galactopyranosyl-(1-6)-linkages. Raffinose was first isolated in a crystalline form from *Eucalyptus mann* (Johnston, 1843). Raffinose and related oligosaccharides accumulates during seed maturation (Holl and Vose, 1980). Raffinose-family oligosaccharides are important sugar translocators in several plant species, such as cucurbits. The accumulation of raffinose is often linked to frost-hardiness of plants and their levels can be regulated by both temperature and photoperiod (Kandler and Hopf, 1982).

1.3.2. Stachyose :

Stachyose[O-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D-glucopyranosyl-(1→2)-β-D-fructofuranoside] was first isolated from the rhizomes of *Stachys tubifera* (Dey, 1985). The kinetics of $^{14}CO_2$ in leaves of *Catalpa buddleia,* demonstrated that

stachyose is synthesized at the expense of the pool of raffinose and galactinol. Immunolocalization of galactinol synthetase and ^{14}C-labelling studies have shown that the complete pathway of Stachyose synthesis is present in both mesophyll and intermediary cells of *Cucurbito pepo* leaves (Beebe and Turgeon, 1992; Flora and Madore, 1993).

In storage organs, for example tubers of *Stachys steboldi*, vacuoles are the site of storage of stachyose. The mechanisms of uptake of oligosaccharides and sucrose are quite similar. A steep concentration gradient of stachyose from cytosol to vacuole exists and the transport is antiport manner (Keller, 1992; Greuterrt and Keller, 1993).

1.3.3. Verbascose :

Verbascose[O-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D-galactopyranosyl-(1→6)-α–D- glucopyranosyl-(1→2)-β-D-fructofuranoside] was first isolated from the roots of *Verbascum thapus* by Bourquelot and Bridel (1910). *In vitro* experiment using an enzyme preparation from the mature seeds of *Vicia faba* demonstrated that the transfer of galactinol to stachyose yielding verbascose. When raffinose was the galactosyl acceptor, stachyose is formed. The syntheis of verbascose is inhibited to a greater extent by raffinose. The enzyme preparations from *Pisum sativum* and *Vicia sativa* are able to synthesize verbascose when it is used as galactosyl acceptor.

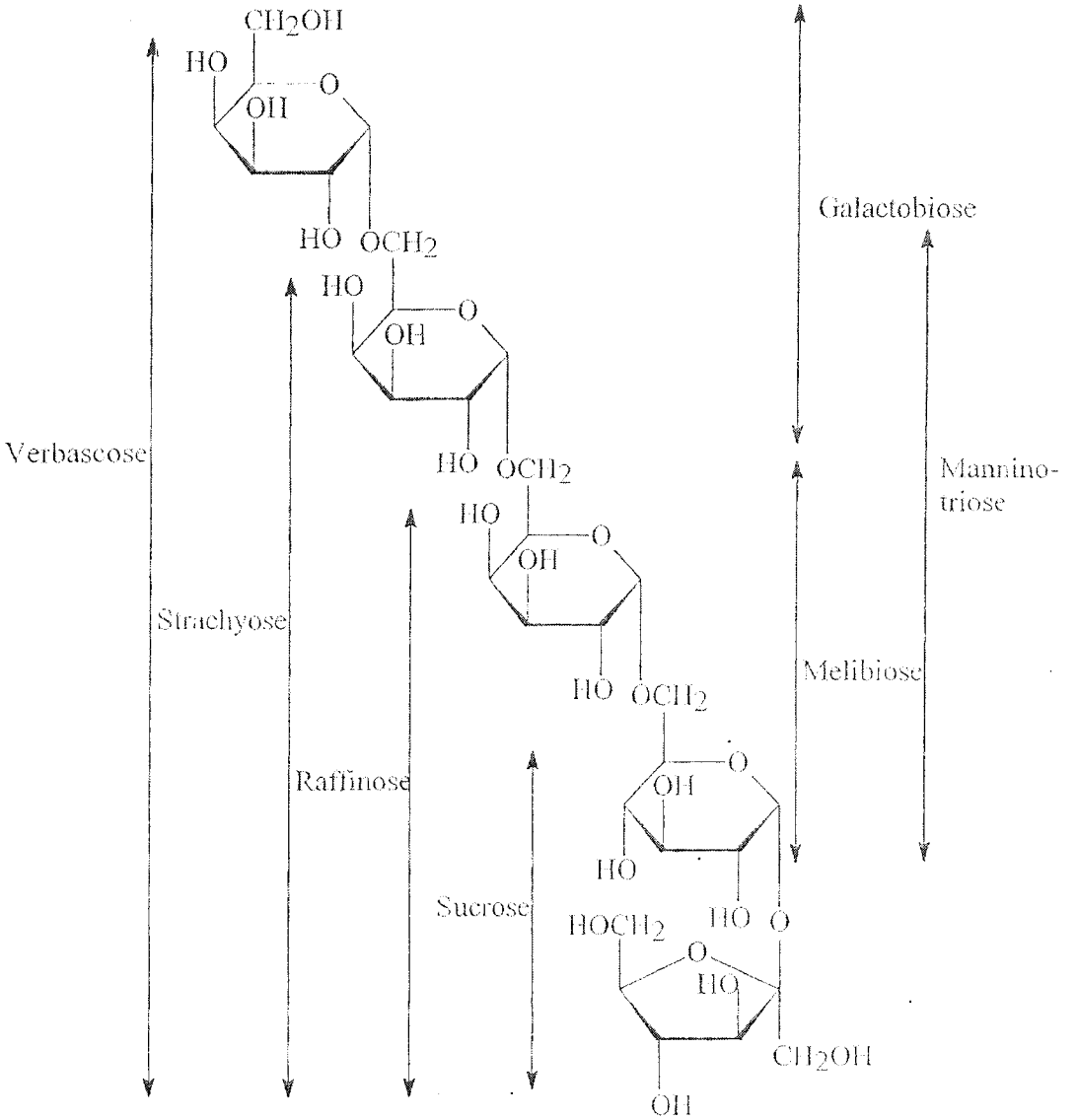


Figure 1.3: Structural relationship of the raffinose family sugars.

1.4. Flatulence :

Leguminosiae is the third largest family of flowering plants with approximately 650 genera and 1800 species. Legume seeds (also called beans, grain legumes or pulses) are second to cereals as source of human and animal food. Nutritionally they are richer in protein than cereal grains. Dry beans are good source of proteins of reasonable quality and they also contain up to 60% carbohydrates (Reddy et al., 1984).

These carbohydrates include monosaccharides and disaccharides, starch and polysaccharides. Starch is the most abundant legume carbohydrate and total sugars (mono- and oligosaccharides) represent only a small percentage of total carbohydrates of the raffinose-family sugars (raffinose, stachyose, verbascose and ajugose) are predominant in kost legumes and account for a significant percentage (31-70%) of total sugars (Hyomowitz et al., 1972; Akpapunam and Markakis, 1979; Fleming, 1981).

Certain legumes such as mung bean, black gram and chickpea contain higher amounts of raffinose-family oligosaccharide oligosaccharides than others. The predominance of a particular oligosaccharide seems to depend on the type of legume. For example, verbascose is the major of oligosaccharide in black gram, Bengal gram (chick pea), red gram and mung beans, whereas stachyose is the major oligosaccharide in California small white beans, navy beans, soybeans, cowpeas and lupine seeds. Ajugose is the other higher molecular weight oligosaccharide raffinose-family sugar, and is present in small amounts in smooth and wrinkled peas and lupin seeds. Raffinose is present in moderate to low amounts in most legumes (Reddy et al. 1984)

Flatulence (gas-production) is one of the constraints limiting consumption of legume seeds by humans and animals (Cristafaro et al., 1973; Steggerda et al., 1966). Man does not digest members of raffinose-family sugars because the intestinal mucosa lack the hydrolytic enzyme α–galactosidase (Gitzelmann and Aurricchio, 1965) and the raffinose-family sugars themselves are unable to pass through the intestinal wall (Rackis, 1975). In other words, when raffinose-family sugars are ingested by humans, two enzymes (invertase and α–galactosidase) are required for complete hydrolysis of these sugars. Because the human gastrointestinal tract does not possess α–galactosidase enzyme and mammalian invertase is α–glucosidase, the metabolic fate of raffinose-family sugars is uncertain (Rackis, 1975).

Many studies have shown that in man and animals these oligosaccharides are involved in flatulence production, which is characterized by the production of large amounts of carbon-di-oxide, hydrogen, and small amounts of methane, the pH also lowered (Rackis, 1975). The gases so produced are responsible for characteristics features of flatulence, namely nausea, cramps, diarrhea, abdominal rumbling and so on (Figure 1.4). Richard et al., (1966) proposed that *Clostridium* was responsible for gas production in the ileal and colonic region of the intestine. Later on it was concluded that not only *Clostridium* but also other microorganisms' inhabitating the lower part of intestine plat an important role in intestinal gas production (Richard et al., 1966).

Many attempts have been made by several investigators to decrease the flatulence causing factors for food legumes. Murphy (1973) reviewed the nutritional improvement of food legumes for the elimination of flatulence by genetic selection. The addition of antibiotics (penicillin and streptomycin) along with the leguminous seed preparations inhibited the flatus production under *in vitro* conditions. However, the addition of these

compounds to beans may change their organoleptic properties and make them unacceptable (Reddy et al., 1984).

Raffinose-family sugars are soluble in water. Several investigators reported a reduction in raffinose-family sugars fro beans by soaking, cooking, soaking followed by cooking, soaking followed by germination, various extraction methods, membrane filtration techniques and enzymatic processing (Reddy et al., 1984). Among the methods described above, fermentation is used to prepare legume based foods (Reddy et al., 1984). Fermentation improves organoleptic properties and nutritional quality of the legume-based food and also to subsequent reduction in the flatulence-causing raffinose-family sugars.

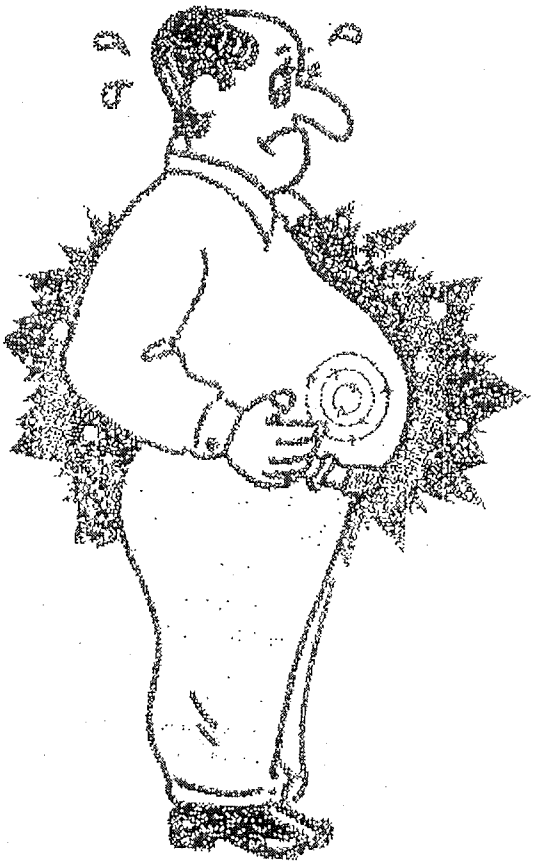


Figure 1.4: Intestinal flatulence (gas production) in human

1.5. Biotechnological Applications of α–Galactosidase :

α–Galactosidase is widely distributed in nature. α–Galactosidases are a group of exotype carbohydrases, which release α–D-galactose from melibiose, raffinose, stachyose, verbascose, galactomannans glycoproteins, ceramide trihexosides and the higher homologous as well as derivatives (Dey and Pridham, 1972; Spangenberg et al., 1999; Andre et al., 2001) α–Galactosidase has the following biotechnological applications:

A. Beet sugar industry,

B. Pulp and paper industry,

C. Food processing industry,

D. Medical application, and

E. Hydraulic fracturing of oil and gas wells.

1.5.1. Applications of α–galactosidase in beet sugar industry :

Raffinose is widely distributed in the plant kingdom. In western countries, the table sugar (sucrose) is manufactured from sugar beet. The content of raffinose in the sugar beet is gradually increased during storage and usually it comes to 0.15%. In the beet sugar industry raffinose is known as an obstacle substance for the normal crystallization of beet sugar. When the content of raffinose in beet molasses is gradually increased and it comes to the range of 6% to 10%, the crystallization of bet sugar is abandoned and the molasses is discarded because of the preventive action of raffinose. Especially, at the later half period of the beet sugar manufacture, a large amount of molasses is discarded, because the content of raffinose in the beet is increased during storage. If the raffinose in beet juice or beet molasses

can be removed or decomposed by some methods, it is possible that the crystallization of beet sugar from molasses is further improved and consequently the yield of beet sugar is increased.The only alternative before the beet sugar industry is the use of crude α-galactosidase (Puchart et al., 2000). Crude α-galactosidase from microbial sources is used to hydrolyze raffinose into galactose and sucrose. This method improves the crystallization efficiency and the yield of sucrose. For the aforesaid purpose microorganism exhibiting an appreciable α-galactosidase activity but with only a slight or without invertase activity is preferred for the decomposition of raffinose. At present crude α-galactosidase from *M. vinacea* or *Monascus sp.* is used for the degradation of raffinose in the beet sugar industry (Linden, 1982).

1.5.2. Use of α-galactosidase in pulp and paper industry :

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature. The major constituents of hemicellulases are hetero-1,4-β-D-xylans and the hetero-1,4- β-D-mannans (galactoglucomannans and glucomannans). Heteroxylans are most abundant in softwoods (Sjortrom, 1981). In the case of galactomannans (softwood), enzymatic hydrolysis occurs with the concerted action of the following hydrolytic enzymes: endo-β-1,4-mannases [EC 3.2.1.78], α-galactosidase [3.2.1.22], and β-glucosidase [3.2.1.21]. In the soft wood pulp bleaching, microorganisms, which could produce enzymes β-mannases and α-galactosidase without cellulose, are preferred to avoid the degradation of cellulose (Zeilinger etal., 1993).

1.5.3. Application of α-galactosidase in food processing industry :

α-Galactosidase is potentially important in the hydrolysis of raffinose-family of

oligosaccharides (raffinose, stachyose, verbascose and ajugose) in pulses. Pulses, defined as seeds of leguminous plants provide major portions of the protein requirement of the daily diet in India and for these reason proteins of these seeds and their nutritive value has been the subject of extensive investigation. Habitual Indian diets contain these pulses and average per capital consumption is around 100 g (Reddy et al. 1984). While pulses are good sources of protein, many common varieties of pulses are known to contain anti-nutritional factors including oligosaccharides of the raffinose-family, in which galactose is present in α-linkage are present in mature seeds (Shallenberger and Moyer, 1961 and Rackis, 1975). Raffinose and stachyose present in soymilk are responsible for intestinal discomfort and flatulence (Steggerda et al., 1966).

The potential soymilk as substitute for cow's milk has been emphasized over the years especially for infants' children who have a very low level of lactase in the intestine. Furthermore, soymilk can be used as an economical protein beverage (Mulimani et al., 2000). Nutritionally soymilk is quite comparable to that of cow's milk (Kay et al., 1960). A few microbial commercial preparations of α-galactosidases have been used to hydrolyze raffinose and stachyose present in soymilk (Thananunkul et al., 1976; Cruz and Park, 1982; Garro et al., 1993; Kotwal et al., 1998).

A variety of microorganisms have been reported to produce α-galactosidase. α-Galactosidase could be used in the food industry and also as a biochemical tool in various structural studies (Zaprometova and Ulezlo, 1988). Galactomannan is a polysaccharide with β-1,4-linked mannan backbone substituted by α-1,6-linked single galactose units.

Galactomannan is widely used in paper, food, cosmetic and other industries.

Galactomannans are also good promoters of gelling when mixed with gelling when polysaccharide such as carrageenan, agar and xanthan. Galactomannan from *Ceratonia siliqua* (locust-bean gum) is extensively used in food industry because of its good gel promoter properties. This unique property of locust-bean gum is by virtue of galactose/mannose (G/M, 1:4) ratio. There is an increased demand for galactomannan from locust-bean gum in food industry. However, the supply of galactomannan from locust-bean galactomannan is by virtue of galactose/mannose (G/M,1:4) ratio. There is an increased demand for the galactomannan from locust-bean gum in food industry. However, the supply of galactomannan from locust-bean is restricted by the following reason

(a) Locust- bean gum is an annual crop and can be grown in semi-arid conditions, Carob (Locust-bean) trees may take 20 years to reach full maturity and are grown in highly intensive farmed Mediterranean, and

(b) Significantly high cost.

(c) Composition from other crops and tourism guar gum more readily available and significantly cheaper than locust-bean gum (Bulpin et al., 1990) However, the galactose/mannose(G/M) ratio of galactomannan from guar gum is nearly 1:2, which is less effective gel promoter than locust-bean gum (G/M,1:4). The extended galactose-deficient regions of the galactomannan are believed to interact with the gelling polysaccharides. Subsequent stabilization of the secondary structure generates a mesh with enhanced water-retaining properties (Dea and Morrision,1985; Grant-Reid et al., 1992).

1.5.5 Medical applications of α-galactosidase :

Knowledge of human blood groups is essential in clinical medicine for the safe practice of blood transfusion, in addition to its outstanding value in fundamental genetical and anthropological studies, red blood cells have some 100 known blood group determinants (epitopes) that comprise 15 genetically distinct blood group system and rheus (Rh) blood-group have major clinical importance. The A,B and O antigens differ in sugar residues at their non-reducing ends (Figure 1.3). The precipitation inhibition experiments of Kabat and Leskowitz (1955) first time indicated the role of a α-galactosyl group in blood group type B specifically.

The α-galactosidase is capable of hydrolyzing the non-reducing terminal α-D-galactopyranosyl residue of blood group–B erythrocytes, thus leading to blood group O - erythrocytes. α-Galactosidase from a number of plant and microbial sources is able to release D-galactose from blood group-B. However, hydrolysis of the terminal α-galactopyranosyl moieties of the blood group-B substances has been reported for a limited number of α-galactosidases, including those from coffee beans, soybeans, *Streptomyces sp.* and *Trichoderma foetus*. Zhu et al., (1995) cloned a cDNA for α-galactosidase from coffee beans into a vector for expression in methylotrophic yeast, *Pichia pastoris*. Harmening (1994) for the first time reported that only rare individuals produce clinically significant antibodies to be sero-converted O antigen and therefore, type O red blood cells are universally compatible and in great demand. Fabry disease of humans is due to a deficiency of thermolabile lysosomal α-galactosidase A (Ulezlo and Zaprometova, 1982). α-Galactosidase may be used in the near future for such medical purposes as enzyme therapy.

1.5.6 Hydraulic fracturing of oil and gas wells :

In hydraulic fracturing applications, the polymer solution that is added to the wellbore contains particles (proppants) that are added to hold open crevices generated by applying high levels of hydrostatic pressure in the flooded well. To allow gas or oil to flow to the wellbore viscosity of the fracturing fluid must be subsequently reduced or broken *in situ*, either by chemical oxidation or enzymatic hydrolysis of the polymer structure.

The low viscosity fluid can be pumped out, after which oil or gas can be produced. Well stimulation is achieved by flooding the wellbore with viscous, water –based fracturing fluid (Clark, 1949; King,1989). The fluid is a water–soluble solution containg guar gum, a natural product derived from the leguminous plant , *Cyamopsis tetragonaglobus.* Chemical oxidizers (persulfates) have beeen used to break the fracturing fluid as have mixtures of hemicelluloses. However, because temperature can exceed 120°C in the deeper reaches of the wellbore, the use of currently available chemical and enzymatic methods for viscosity before sufficient stimulation is accomplished. As such, more thermostable and thermally activated versions of existing hemicellulases (α-1,6-galactosidases and β-1,4-mannanases are highly desirable (McCutchen et al., 1996).

Currently used enzymatic breakers are mixtures of hemicellulases produced by *A.niger* (Tjon-Joo-Pin, 1993). McCutchen et al., (1996) have reported that use of extremely thermostable hemicellulases (α-1,6-galactosidases and β-1,4-mannanases) from the hyperthermophilic bacterium *Thermotogo neapolitana* for the hydrolysis of guar gum in the hydraulic fracturing of oil and gas wells.

LITERATURE REVIEW

Penicilllium chrysogenum is a mold that is widely distributed in nature, and is often found living on foods and in indoor environments.

Microorganisms secrete a variety of enzymes, which are either constitutive or inducible. Constitutive enzymes are produced irrespective of the presence of their substrates sby the addition of enzymes substrate to the medium in which the cell is growing. The substrate is called an inducer. Among eukaryotic microorganisms, most of the studies on induction and regulation is confined to yeast, because of the availability of temperature-sensitive secretory mutants (Rios et al., 1993). During growth filamentous fungus secrete several hydrolytic enzymes of industrial importance, including α-galactosidase. The mechanism of protein routing and secretion is not well described in such organisms (Rios et al., 1993). To our knowledge, there are no reports on induction and regulation of α-galactosidase from *P.chrysogenum.* There are a large number of reports on the purification and characterization of fungal α-galactosidases from *Mortierella vinacea* (Suzuki et al., 1970), *Corticum rolfsii* (Kaji and Yoshara, 1972, *A. niger* (Adya and Elbein, 1977), *P. cinnabrinus* (Ohtakara et al., 1984), *Cephalosporium acremonium* (Zaprometova and Ulezlo, 1988), *P.ochrochloron* (Dey et al., 1993), *T. reesi* (Zeilinger et al., 1993). Computer searches concerning on induction, purification and characterization of α-galactosidase in *P.chrysogenum* showed that it has not reported.

The present study reports the purification and characterization of α-galactosidase from *P.chrysogenum.*

2.1 α-Galactosidase:

The dissacharide melibiose was hydrolysed by the crude enzyme preparation (melibiases) from bottom fermenting yeast (Fischer and Linder,1885). Weidenhagern (1928) who studied the specificity of action of the melibiose using a number of sugars having non_reducing terminal α-D-galactosyl residues and coined the name α-galactosidase to melibiase.

α-galactosidase exhibited a broad range of specificity and removes the terminal α-D-Galactose residues attached by α-1-2, α-1-3, α-1-4, and α-1-6 linkages (Dey and Pridham, 1972; Varbarnets et al.,2001).

Synthetic substrates PNPG had lower k_m and higher k_{cat} as compare to natural substrates, melibiose, raffinose and stachyose (Gote et al., 2006).

Heat stability of the enzyme decreased as purity increased. This trend was partially reversed by addition of 2-mercaptoethanol, NADH, cystein, and/or bovine serum albumin to reaction mixture (Timothy Durance & Brent Skura, 1984)

The alkaline α-gal genes show very high sequence homology with a family of undefined 'seed inhibitor proteins' (SIPs) which are present in a wide range of plant families(Carmi, Nir et al., 2003).

Screening of variables to find their relative effect on α-galactosidase production was done using Plackett – Burman design. Out of seven factors screened, salinity, magnesium sulphate and temperature were found to influence the enzyme production significantly (G.S. Anisha et al., 2008).

2.1.1 Hydrolase activity

α-Galactosidase from *Streptococcus bovis, Diplococcus pneumonia* and *Calvatia cyathifomis* cannot act on arabinosides (Dey and Pridham, 1972). Dey and Pridham (1969a) have reported that p-nitrophenyl-α-D-galactopyranosyl moieties of blood group-B substances by α-galactosidase to type-O has been reported for a number of enzymes , including coffee bean α-galactosidase (Harpez et al., 1975).

2.1.2 Transgalactosidase activity

The galactose from one melibiose was transferred to a second acceptor molecule leading to the formation of mannanotriose. This transglycosylation property of α-galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity , donor and acceptor concentration, pH, temperature and source of enzyme (Dey and Pridham, 1972).

Apart from hydrolase activity, α-galactosidase also exhibits transgalycosylase properties , but relatively at high substrate concentrations (Ohtakara et al., 1985; Van Laere et al., 1999). In recent years, transglycosylation catalysed by α –galactosidase is receiving a considerable attraction from investigators (Eneyskaya et al. 1998; Van Laere et al, 1999 ; Spangenberg et al, 1999; Andre et al. 2001).

The glycosylation leads to the formation of a triple complex,ES'S, where S' is transferred (Eneyskaya et al 1998). In transglycosylation reactions , glycon moiety from donor is accepted by hydroxyl group of methionine present at the active site of α-galactosidase from Trichoderma raesi (Eneyskaya et al. 1998). The nature of galactosidic the donor [α-(1,3) or α-(1,6)] in transglycosylation products depend on the donor and the source

from which α-galactosidase is obtained (Mitsutomi and Ohtakara, 1998; Van Laere et al., 1999). Because of competition in the transglycosylation reactions (seld-condensation reactions), the end products are usually complex mixture (Eneyskaya et al. 1998).

α-Galactosidase was able to form oligosaccharides with degree of polymerization (DP)> or =3 at higher concentration than DP=2, with a total yield of 20.5% (w/w) (Goulas et al., 2009). In the presence of PNPG as a donor , aga2, which is a novel galactosidase sene from Bifidobacterium breve, was able to catalyze glycosyl transfer to various acceptors including monosaccharides, disaccharides and sugar alcohols (Han Zhao et al., 2008). At high substrate concentrations , 64% of the enzyme showeh transglycosylation activity disaccharides are found to be good acceptors (Hinz et al, 2005). Transglycosylation activity showed that the enzyme has two galactose binding sites and hydrophobic site in its active center (Eneyskaya et al., 1997).

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 Materials :

Raffinose, melibiose, stachyose, p-nitrophenyl-α-D-galactopyranoside (PNPG) was obtained from Sigma Chemical Co. Galactose, fructose, glucose, sucrose, maltose, and phenylmethane sulphonylfluoride (PMSF) were procured from Hi-Media,, India. Standard protein molecular weight markers for SDS-PAGE were obtained from GENEI, India. All other reagents used were of analytical grade.

3.2 Methods :

3.2.1 Preparation of buffers :

The following buffers were used in our experiments and prepared according to Gomori (1955). All preparations were carried out using double distilled water. pH meter was standardized using 0.5 M potassium hydrogen phthalate.

(i).Acetate buffer :

Stock solution of A: 0.2 M solution of acetic acid (11.55 ml of acetic acid in 1000 ml), and stock solution B: 0.2 M solution of sodium acetate (16.4 g of sodium acetatein 1000 ml) was prepared. Acetate buffer (pH from 3.6 to 5.5) was prepared by mixing appropriate proportion of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

(ii) Sodium phosphate buffer :

Stock solution of A: 0.2 M monobasic sodium phosphate solution (27.89 in 1000ml), and

Solution B: 0.2 M dibasic sodium phosphate solution (95.36 g in 1000 ml) were prepared. Sodium phosphate buffer (pH 6.0 to 7.5 ml) was prepared by appropriate of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

(iii).Tris-HCl buffer :

Stock solution of A: 0.2 M Tris (hyroxymethyl aminomethane, 24.2 g in 1000 ml) solution, and solution B: 0.2 N HCl were prepared. Tris-HCl buffer (pH 8.0 to 9.0) was prepared by appropriate of A and B, diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

3.3.2 Preparation of dialysis tubing :

The dialysis tubing was cut into 10-20 cm length. The tubing's were boiled in 2% (w/v) sodium bicarbonate solution containing about 1 mM EDTA for about 10 minutes. The tubing's were cooled and rinsed with distilled water for three times. The tubing's were boiled again in distilled water for additional 10 minutes. After boiling, the tubing were allowed to cool and stored in seventy percent alcohol under refrigeration (2-5°C). Before use, the dialysis tubings were washed with distill

3.3 Characterization of α-galactosidase from *Penicillium chrysogenum* :

3.3.1 Morphological characteristics of *Penicillium chrysogenum* :

(i) Distribution and importance :

Penicillium chrysogenum is a mold that is widely distributed in nature, and is often

found living on foods and in indoor environments. It was previously known as *Penicillium notatum*. It has rarely been reported as a cause of human disease. It is the source of several β-lactam antibiotics, most significantly penicillin. Other secondary metabolites of *P.chrysogenum* include various different penicillins meleagrin chrysogine, xanthocillins etc.. The airborne spores of *P. chrysogenum* are important human allergens. Vacuolar and alkaline serine proteases have been implicated as the major allergenic proteins.

3.3.2 Production of α-galactosidase from *P.chrysogenum:*

The Czapek-dox medium used in the production studies composed of :

KH_2PO_4	1.0 g
Kcl	0.5 g
Na_2SO_4	10 g
$MgSO_4$	0.5 g
$ZnSO_4$	0.05 g
$(NH_4)_2MoO_4$	0.05 g
$FeSO_4$	0.01 g
$CuSO_4$	0.005 g
$MnSO_4$	0.005g
Distilled water	1 L
pH	5.5 (before autoclaving)

For production studies, galactose at 2% (w/v) was added to the above medium before autoclaving. Batch, submerged fermentations were carried out on an orbital shaker (160 rpm) in Erlenmeyer flasks (250 ml, each containing 125 ml of czapek-dox medium) was inoculated with 1ml of *P. chrysogenum* culture. After 2 weeks of growth, filtrate was collected by filtration through Whatman filter paper no.1. The resultant mixture was centrifuged at 10,000 x g for 20 min and the clear supernatant was used as enzyme source.

3.3.3 Assay of α-galactosidase activity :

α-Galactosidase activity was assayed by spectrophotometric measurement of the release of p-nitrophenol from p-nitrophenol-α-D-galactopyranoside (PNPG) at 405 nm by the modified method of Dey and Pridham (1969a). The reaction mixture consisted of 100 µl of 10 mM PNPG in water, 800 µl of acetate buffer (0.2 M, pH 4.5), and 100 µl of appropriately diluted enzyme. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 3 ml of 0.2 M sodium carbonate. One unit of enzyme activity is defined as the amount releasing 1 µmol of p-nitrophenol per min under assay conditions.

3.3.4 Effect of different factors on α-galactosidase :

3.3.4.1 Determination of pH and temperature optima :

The effect of pH on the enzyme activity was measured at **60**°C in the pH range from 3.5 to 9.0. The following buffers were used: 100 mM acetate buffer (pH 3.6 to 5.5), 100 mM phosphate buffer (pH 6.0 to 7.5). For the determination of the temperature optimum, enzyme activity was assayed in the range of 30-100°C using acetate buffer (100 mM, pH 5.0).

3.3.4.2 Effect of metal ions, sugars and some reagents :

A reaction mixture consisting of 0.1 ml of enzyme solution, 0.7 ml of 0.2 M acetate buffer (pH 4.5), and 0.1 ml metal ions or sugars or some reagents (final concentration indicated) were added for 15 min at 37°C and α-galactosidase activity was determined after adding 0.1 ml of 10 mM PNPG. The activity was expressed as a percentage of the activity level in the absence of the compound.

3.3.4.3 Kinetic studies (K_m and V_{max}) :

α-Galactosidase assay was performed by varying the concentrations of PNPG from 10 µl to 50 µl. The absorbance of the p-nitrophenol released was measured at 405 nm and readings were tabulated.

3.4 Purification:

3.4.1 Ammonium sulphate fractional precipitation :

Ammonium sulphate (80%) was added to the culture filtrate (180 ml) with stirring and the mixture was kept aside at 4°C for 12 hours. At the end of the 12 hours, the mixture was centrifuged at 10000 rpm for 10 min and the supernatant was collected. The pellet was dissolved in a minimum amount of phosohate buffer (50 mM, pH 4.5).

3.4.2 Ultra-Filtration :

The fractions having high α-galactosidase activity were pooled and dialysed overnight to remove ammonium sulphate ions. The dialysed samples were subjected to ultra-filtration (membrane cut-off is 70 kDa). The dialysed enzyme solution was filtered through the ultra-filtration membrane.

3.4.3 Polyacrylamide gel elctrophoresis :

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

(a) Monomer solution:

The solution contained 29.2 g of acrylamide and 0.8 g of N,N'-methylenebisacrylamide in a final volume of 100 ml made-in distilled water . The solution was filtered through Whatman filter paper no.1 and stored at 4°C

(b) 1.875 M Tris-buffer, pH 8.8 :

22.7 g of Tris was dissolved in distilled water. The pH was adjusted to 8.8 with 1 N HCl and the final volume made up to 100 ml with distilled water. The solution was stored in refrigerator.

(c) 1.25 M Tris-HCl buffer, pH 6.8 :

15.125 g of Tris was dissolved in distilled water. The pH was adjusted to 6.8 with 1 N HCl and the final volume made up to 100 ml with distilled water. The solution was stored in refrigerator.

(d) Sodium dodecylsulfate (SDS), 10% (w/v)

10 g of SDS was dissolved in distilled water and final volume made up to 100 ml with distilled water.

(e) Ammonium persulfate, 1% (w/v) :

100 mg of ammonium persulfate was dissolved in distilled water and final volume made up to 10 ml with distilled water. Freshly prepared ammonium sulfate was used.

(f) Electrophoresis buffer (10 X) :

Tris base 30 g, glycine 144 g, and SDS 10 g were dissolved in distilled water and final volume made up to 100 ml with distilled water. The solution was stored at room temperature.

(g) Running gel (12%) :

Acrylamide (30%)	40.0 ml
Tris-HCl buffer (1.875 M, pH 8.8)	20.0 ml
Distilled water	34.0 ml
Ammonium persulfate	5.0 ml

The mixture was degassed, 1 ml of 10% SDS and 12 µl of TEMED were added. The solution was poured into cassette kept in the gel casting apparatus. The solution was over layered with distilled water using a syringe without distributing the surface and was allowed to polymerize.

(h) Stacking solution :

Acrylamide	12.0 ml
Tris-HCl buffer (1.25 M, pH 6.8)	3.0 ml
Distilled water	13.2 ml
Ammonium persulfate	1.5 ml

After degassing the mixture 0.3 ml of 10% SDS and 12 µl of TEMED were added. The over layered water on running gel was poured into the cassette over the running gel. A comb was introduced into the spacer gel and was allowed to polymerize in presence of light. After polymerization the comb was removed from the cassette and gel was inserted into the gasket of the electrophoretic apparatus. Electrophoresis was performed at room temperature and constant current of 30 mA. When the bromophenol blue dye reached 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from the glass plates and soaked in staining solution (0.25 g Coomassie Brilliant Blue R-250- in 45 ml methanol, 10 ml glacial actic acid and 45 ml distilled water). After immersed in destaining solution, which was the staining solution minus the stain. Destaining was stoped when the gel background was colorless. The purified enzyme was checked for glycoprotein nature using the Schiff reagent according to the method of Geranrd (1990), as described in this chapter, section 2.2.5.7.

3.5 Application of α-galactosidase :

Soymilk is a milk-like product obtained by aqueous extraction of soybeans. Apart from beany flavor, the presence of anti-nutritional factors like raffinose-family oilgosaccharides in soymilk limits its wider acceptance for human consumption. *Lactobacillus brevis* could be used as starter culture for the preparation of yogurt-like products. These microorganisms unable to utilize sucrose (the most abundant metabolizable sugar present in soymilk), but during fermentation they effectively utilize flatulence-causing raffinose and stachyose of soymilk. Ramalingam and Mulimani (2004) reported that α-galactosidase from *Aspergillus oryzae* hydrolyzed raffinose and stachyose in soymilk. Ramalingam et al. ((2007) reported that α-galactosidase from *Pleurotus florida* completely degraded the flatulence-causing raffinose and stachyose in soymilk

3.6 Thin-layer chromatography :

Thin layer chromatography (TLC) was performed to analyze the end products of raffinose and stachyose of soymilk by the partially purified / purified enzyme (26 U/mg) in acetate buffer (0.1 M, pH 5.0), 20 ml of soymilk was added and incubated at 60°C for 4 h. TLC plate coated with silica-G was used to detect hydrolyzates of α-galactosidase. The plates were developed in solvent system consisted of n-propanol : ethylacetate : water (6:1:3, v/v). At the end of the run, plates were air dried, sprayed with 1% α-naphthol in absolute ethanol containing 10% (v/v) o-phosphoric acid to detect the fructose-containing sugars (Albon and Gross, 1950).

RESULT & DISCUSSION

4.1 Purification of α-galactosidase from *P.chrysogenum* :

4.1.1 Ammonium sulphate fraction precipitation :

Ammonium sulphate fractional precipitation was carried out and purification fold was obtained.

4.1.2 Ultra-filtration :

Ultra-filtration was carried out and purification of 3.33 fold was obtained.

Table 4.1: Purification of α-galactosidase from *P.chrysogenum*

Purification step	Total volume (ml)	Total enzyme (U)	Total protein (mg)	Specific acivity (U/mg)	Yield (%)	Fold purifi--cation
Crude extract	740	17.76	2368	0.024	100	1
Ultra-filtration Retentate	180	14.4	792	0.08	81	3.33
Ultra-filtration Filtrate	560	2.24	896	0.004	12.6	1.66

4.2 Electrophoresis of α-galactosidase (from *P.chrysogenumi*) on native-PAGE :

After simple three purification steps, PAGE of the final enzyme preparation showed a single band. The molecular weight of the purified by native-PAGE was estimated to be between 200 to 205 kDa. *M.vinacea* secretes multiple forms of extracellular α-galactosidase (α-gal I & II) (Shibuya et al., 1997). Luonteri et al. (1998) have reported the secretion of multiple forms of α-galactosidases (AGLI – AGLIII) by *Penicillium simplicissimum*. De Vries et al. (1999) have reported multiple forms of α-galactosidases (AglA, AglB and AglC) from *A. niger* N400. Ademark et al. (2001) have reported occurrence of multiple α-galactosidases (α-gal I-IV) from A. niger ATCC 46890.

4.3 Characterization of α-galactosidase from *P.chrysogenum* :

(i) Effect of pH on activity :

The pH dependence of α-galactosidase activity was determined by incubating in buffer varying pH using PNPG. The pH-activity profile for the hydrolysis is shown Figure 4.2 . From Figure 4.2 it is observed that enzyme was found to be active between pH 4.0 to 6.0. The enzyme exhibited the maximum activity at 4.5. Mulimani and Ramalingam (1995) reported that cell-free extract from *P.chrysogenum* showed optimal activity at pH 5.0 with p-nitrophenyl-α-galactopyranoside (PNPG). Kaji and Yoshihara (1972) reported that α-galactosidase from *Corticium rolfsii* exhibited maximum activity at pH 5.0. α-Galactosidase from *P. cinnabarinus* exhibited the optimum pH at pH 5.0. (Ohtakara et al., 1984) . α-Galactosidase from *M.pilosus* showed the optimum pH of 5.0 for the activity

(Wong et al., 1986). Nadkarni et al. (1992) have reported that α-galactosidase from *Corynebacterium murisepticum* exhibited an optimum pH at 7.5. Dey et al. (1993) have reported that α-galactosidase from *P.ochrochloron* exhibited optimum pH at 4.5. α-Galactosidase from thermophilic fungus *Humicola sp.* was optimally active at pH 5.0 (Kotwal et al., 1995).

(ii) Effect of pH on the stability of the enzyme activity :

The effect of pH on the stability of the enzyme is shown in figure 4.3. The enzyme was incubated in buffers of various pH values for 1h at 60°C and the residual activity was assayed. The enzyme was less stable. α-Galactosidase from *P. cinnabarinus* was stable between 3 and 9, and retained about 95% of its activity (Ohtakara et al., 1984). α-Galactosidase from *M.pilosus* was stable in buffer at pH 3 to 8. Kotwal et al. (1995) have reported that α-galactosidase from *Humicola sp.* lost its 12% of its original activity at pH 3.0 when enzyme was incubated at 4°C for 16 h.

(iii) Effect of temperature on activity :

The temperature–dependence of α-galactosidase activity was also determined by incubating the enzyme in acetate buffer (pH 5.0) using PNPG. The effect of temperature on enzyme activity profile for hydrolysis of PNPG is shown in the table 4.4. From the Figure 4.4, it is observed that enzyme was found to be active between temperature 40 - 70°C. α-Galactosidase from fungal sources were optimally active at 60 °C. α-Galactosidase from *A. saitoi* was optimally active at 55 °C (Sugimoto and Van Buren, 1970). α-Galactosidase from *P. cinnabarinus* showed optimum temperature at 75°C (Ohtakara et al., 1984). The purified

α-galactosidase from *M. pilosus* showed its optimum temperature at 55°C (Wong et al., 1986). α-Galactosidase from *P.ochrochloron* exhibited maximal activity at 55 °C (Dey et al., 1993). α-Galactosidase from *Humicola* sp. had an optimal temperature at 60°C (Kotwal et al., 1995).

α-Galactosidase from *Torulaspora delbrueckii* showed optimal activity at 55°C (Oda and Tonomura, 1996). α-Galactosidase from *Trichosporon cutaneum* exhibited maximal activity at 45°C (Oda and Tonomura, 1997). α-Galactosidase from *Thermomyces lanuginosus* is most active at 65-70°C (Puchart et al., 2000).

(iv) Thermal stability :

The effect of temperature on the stability of the enzyme is shown in figure 4.5. The enzyme was incubated at 70°C and 80°C in buffer of pH 4.5. From the Figure 4.5, it is observed that the enzyme is less stable. The enzyme lost its total activity at 80°C. α-Galactosidase from *P. cinnabarinus* exhibited no loss in activity below 75°C and completely lost its activity at 90°C and pH 5.0 for 15 min (Ohtakara et al., 1984). α-Galactosidase from *A.nidulans* was very sensitive to temperature (unstable above 30°C) (Rios et al., 1993). Puchart et al. (2000) have reported that α-galactosidase from thermophilic fungus *T. lanuginosus* was stable at 50°C and pH range 3.0-7.5. Kotwal et al. (1995) have reported that α-galactosidase from *Humicola sp.* was stable at 60°C for 1 h and was rapidly inactivated above 60°C.

(V) Effect of metal ions, sugars and some reagents on enzyme activity :

Table 4.6, shows that the enzyme was strongly inhibited by metal ions such as Ag^{2+}, Cu^{2+} and Hg^{2+}. The enzyme activity was completely inhibited by 1 mM Hg^{2+}, indicating that the tryptophan residue appears to play a role in the catalytic processes of the α-galactosidase. Significant inhibitory effects of enzyme activity were observed in the presence of K^{+} and Zn^{2+}. EDTA did not inhibit the α-galactosidase from *P.chrysogenum*, indicating that the enzyme is not a metalloenzyme. Mg^{2+} exerted a slight positive effect upon α-galactosidase activity from *P.chrysogenum*. α-Galactosidase from *P. cinnabarinus* was strongly inhibited by Ag^{2+}, and Hg^{2+}, whereas EDTA didn't affect the enzyme activity (Ohtakara et al., 1984). α-Galactosidase from *M.pilosus* was also strongly inhibited by Ag^{2+}, Cu^{2+} and Hg^{2+} (Wong et al., 1986). The addition of Hg^{2+} to the reaction mixture produced a strong inhibitory effect on α-galactosidase from *Bifidobacterium longum* (Garro et al., 1993). α-Galactosidase from *Humicola* sp. was strongly inhibited by Hg^{2+} (Kotwal et al., 1995).

The effects of sugars on α-galactosidase activity are summarized in Table 4.7. Among the sugars tested lactose and melibiose were powerful inhibitors. Fructose exerted a slight positive effect upon enzyme activity. Lactose and melibiose strongly inhibited α-galactosidase activity from *P.cinnabarinus* (Ohtakara et al., 1984). Glucose, maltose and stachyose also markedly inhibit the enzyme activity, while xylose, arabinose, lactose, sucrose, and raffinose showed weak inhibition on α-galactosidase from *P.cinnabarinus* (Ohtakara et al., 1984). Kotwal et al. (1999) have reported that galactose inhibited the purified α-galactosidase from *Humicola* sp.

Table 4.2: Effect of pH on activity of α-galactosidase from *P.chrysogenum*

pH	Activity(U)	Relative Activity(%)
3.5	-0.006	-11.53
4.0	0.039	75
4.5	0.052	100
5.0	0.043	82.69
5.5	0.012	23.07
6.0	0.002	3.84
6.5	-0.002	-3.84
7.0	-0.007	-13.46
7.5	-0.006	-11.53

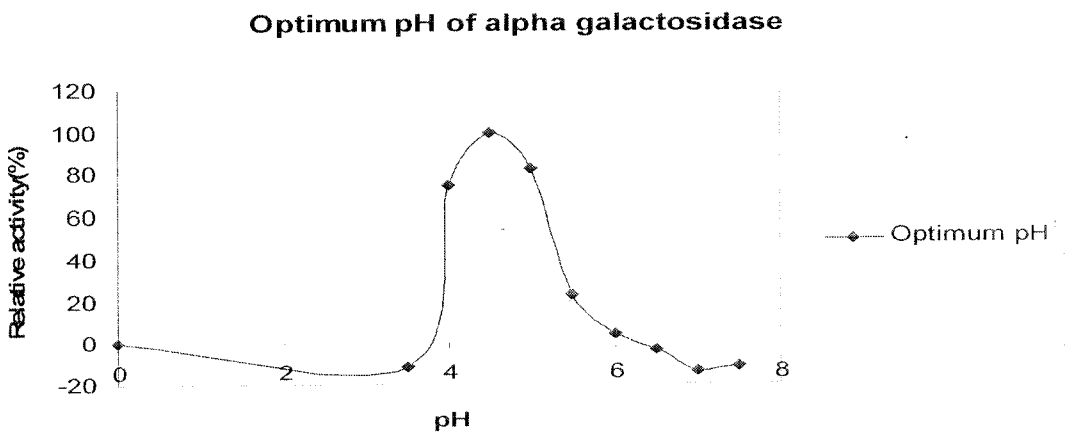


Figure 4.1: Effect of pH on activity of α-galactosidase fron *P.chrysogenum*

Table 4.3: Effect of pH on stability α-galactosidase from *P.chrysogenum*

Time (minutes)	Residual activity at pH-3.5(%)	Residual activity at pH-4.0(%)	Residual activity at pH-4.5(%)	Residual activity at pH-5.0(%)	Residual activity at pH-5.5(%)
0	100	100	100	100	100
30	0.0049	0.0081	0.010	0.010	0.009
60	0.0042	0.0079	0.010	0.010	0.012
90	0.0011	0.0008	0.009	0.007	0.006

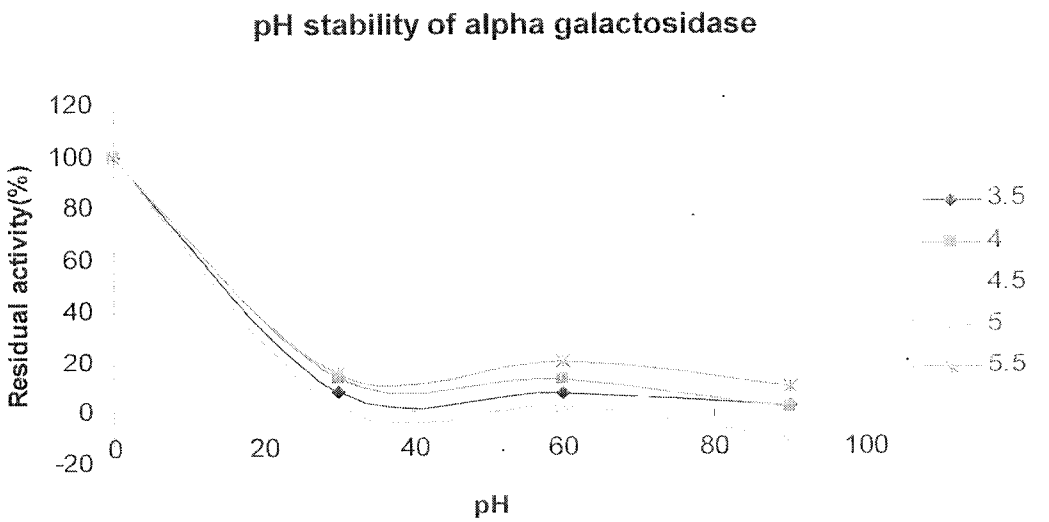


Figure 4.2: Effect of pH on stability α-galactosidase from *P.chrysogenum*

Table 4.4: Effect of temperature on activity of α-galactosidase from *P.chrysogenum*

Temperature(°c)	Activity(U)	Relative Activity(%)
30	-0.006	-18.75
40	0.014	43.75
50	0.023	71.87
60	0.032	100
70	0.029	90.62
80	-0.007	-21.87
90	-0.01	-31.25
100	-.013	-40.62

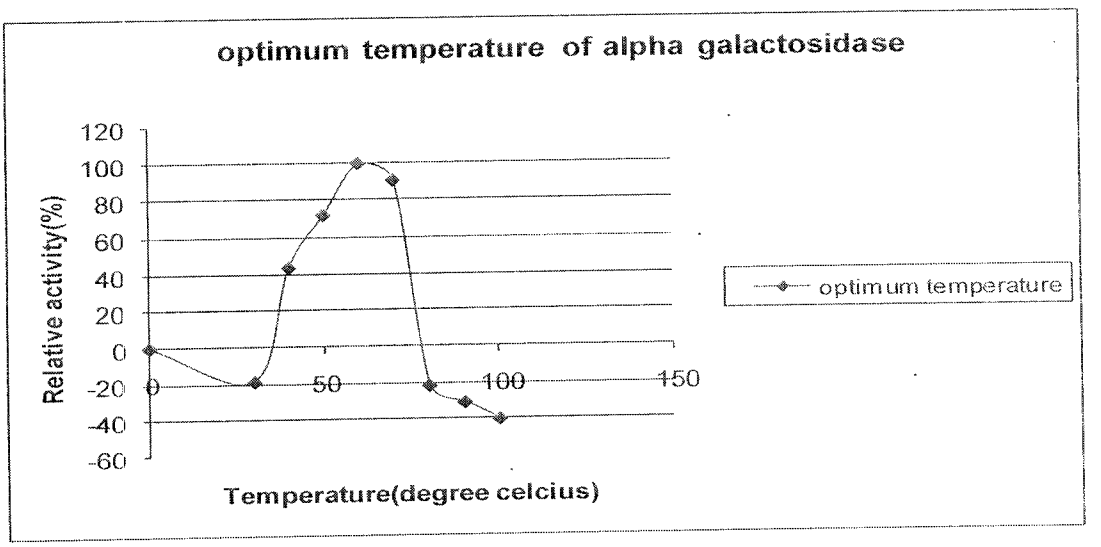


Figure 4.3: Effect of temperature on activity of α-galactosidase from *P.chrysogenum*

Table 4.5: Effect of temperature on the stability of α-galactosidase from *P.chrysogenum*

Time(minutes)	Residual activity at 70°c(%)	Relative activity at 80°c(%)
0	100	100
30	18.33	23.33
60	16.66	1.66

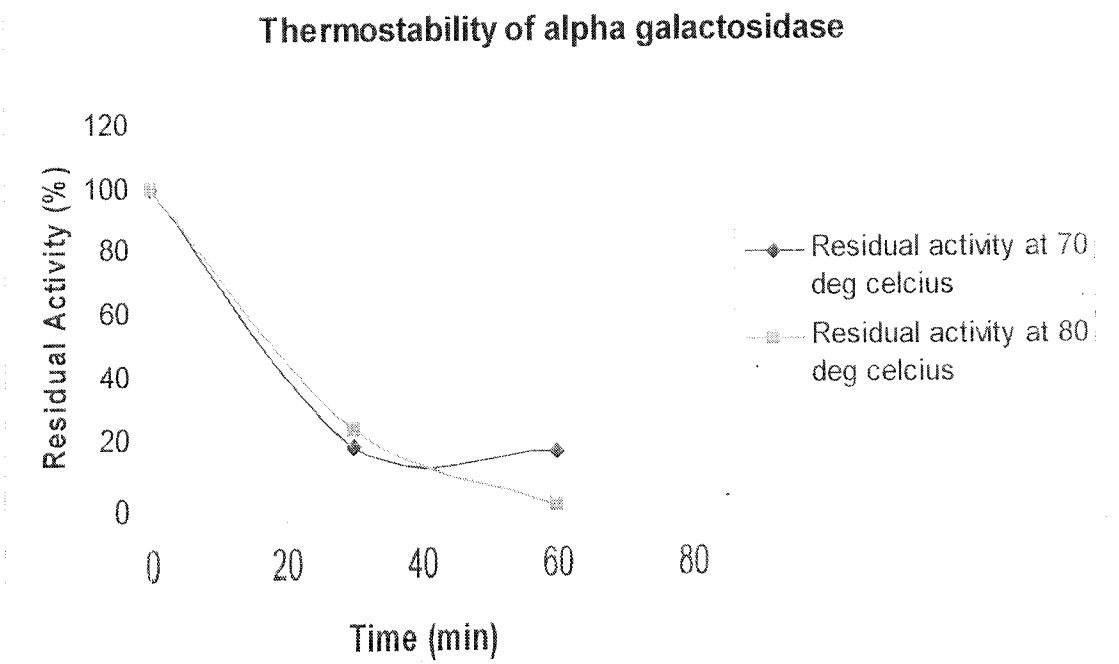


Figure 4.4: Effect of temperature on the stability of α-galactosidase from *P.chrysogenum*

Table 4.6: Effect of metal ions on α-galactosidase from *P.chrysogenum*

Metal ions	Concentration(mM)	Residual activity(%)
Control	5	–
$AgNO_3$	5	–
$HgCl_2$	5	–
$CuSO_4$	5	75
$ZnSO_4$	5	105
$MnSO_4$	5	89
$CaCl_2$	5	113
$FeSO_4$	5	100

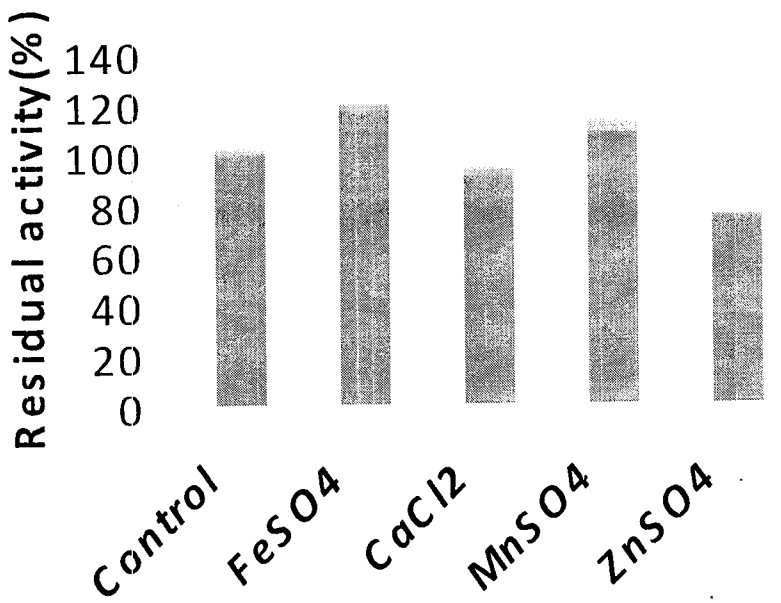


Figure 4.5 : Effect of metal ions on α-galactosidase from *P.chrysogenum*

Table 4.7: Effect of sugars on α-galactosidase from *P.chrysogenum*

Sugars	Concentration(mM)	Relative activity(%)
Control	–	100
Xylose	10	104
Galactose	10	92
Melibiose	10	74
Sucrose	10	94
Fructose	10	99
Raffinose	10	96
Maltose	10	94
Glucose	10	95
Lactose	10	86
Arabinose	10	100

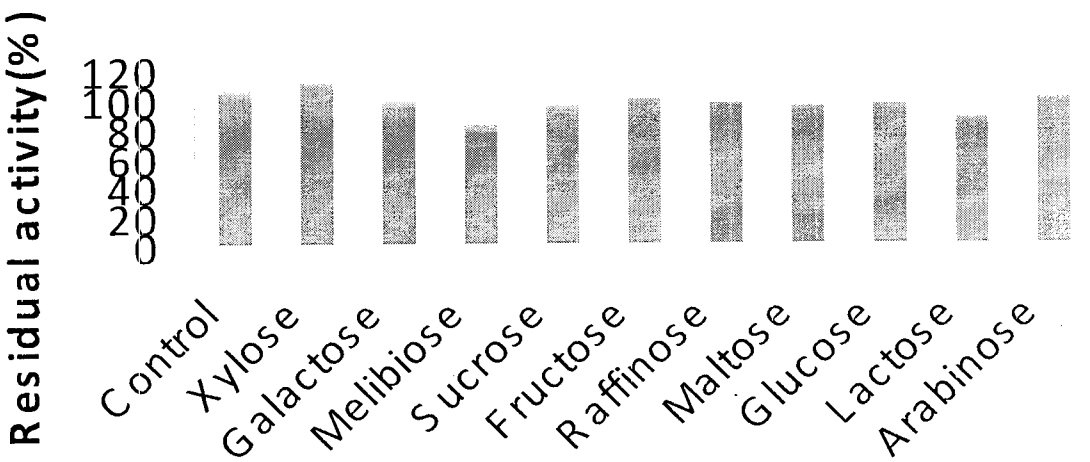


Figure 4.6: Effect of sugars on α-galactosidase from *P.chrysogenum*

Table 4.8: Effect of reagents on α-galactosidase from *P.chrysogenum*

Reagents	Concentration(mM)	Relative activity(%)
Control	-	100
Iodoacetic acid	5	103
N-Bromosuccinamide	5	_
Iodoacetamide	5	118
EDTA	5	118
1,10-Phenanthroline	5	95

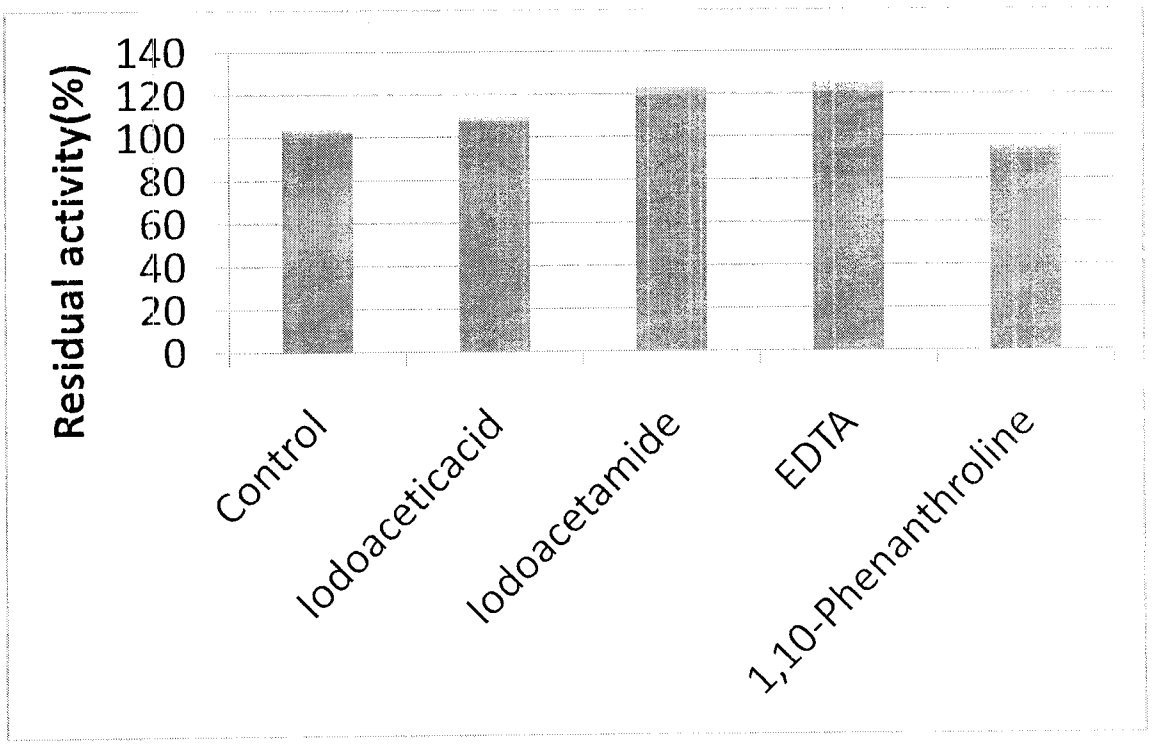


Figure 4.7: Effect of reagents on α-galactosidase from *P.chrysogenum*

Table 4.9: Determination of k_m and v_{max} for α-galactosidase using PNPG as substrate

Substrate concentration,s (mM)	1/s	1/v
0.33	3.03	3.787
0.66	1.51	2.578
0.99	1.06	1.388
1.32	0.75	1.116
1.65	0.60	0.965

4.4 Applications of α-Galactosidase :

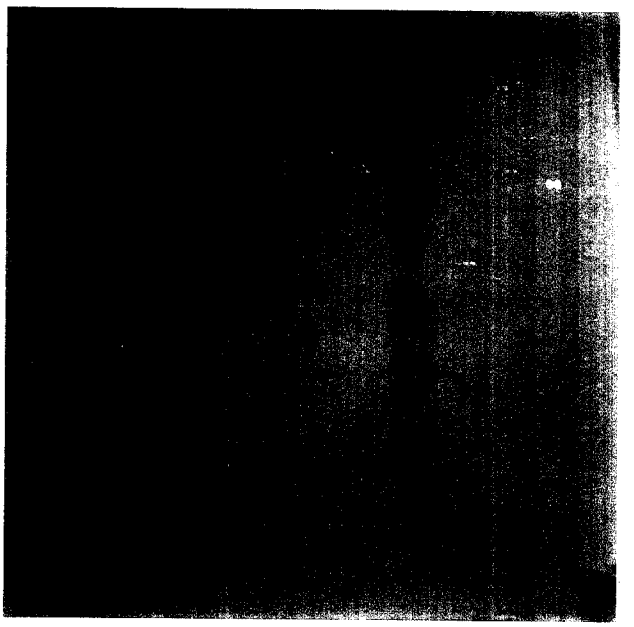


Figure 4.8. Hydrolysis of stachyose and raffinose in soymilk by α-galactosidase from *Penicillium chrysogenum.* Stachyose and raffinose from soymilk was completely hydrolyzed in 4 h by α-galactosidase from *P.chrysogenum.*

SUMMARY & CONCLUSIONS

sThe α-galactosidase from P.chrysogenum was purified to homogeneity by ammonium sulphate fractionational precipitation,dialysis and ultra-filtration. The optimum pH,temperature,pH stability and thermal stability of the purified enzyme was carried out the effect of metal ions,sugars and some reagents were also studied on the purified enzyme.the enzyme has the ability to degrade raffinose-family sugars in soymilk. The presence of raffinose-family sugars and the beany flavor makes soymilk unfit to human consumption. Thus, α-galactosidase from Penicillium chrysogenum can be used in various food processing industries to improve the nutritive value of food products.

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