P-2637

ISOLATION, PURIFICATION AND CHARACTERIZATION OF α-GALACTOSIDASE FROM *Aloe vera*

A PROJECT REPORT

Submitted by

SARANYA S	**71205214026**
THARANI J	**71205214036**
PRIYADARSINI M	**71205214701**

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE-641 006

ANNA UNIVERSITY :: CHENNAI 600 025

April 2009

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report entitled **"Isolation, Purification and Characterization of α-galactosidase from *Aloe vera* "** is the bonafide work of **Ms. S. Saranya , Ms. J. Tharani and. Ms M.Priyadarsini** who carried out the project work under my supervision.

SIGNATURE

DR.P.RAJASEKARAN

Head of the Department

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641006

SIGNATURE

MR.P.RAMALINGAM

Assistant Professor

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore-641006

CERTIFICATE OF EVALUATION

COLLEGE : **Kumaraguru College of Technology**

BRANCH : **Biotechnology**

SEMESTER : **Eighth Semester**

NAME OF THE STUDENTS	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
Saranya S (71205214026) Tharani J (71205214036) Priyadarsini M (71205214701)	Isolation, Purification and Characterization of α-galactosidase from *Aloe vera*	Mr.Ramalingam Assistant Professor

The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University, Chennai-600025, it was submitted for evaluation and viva voice held on

(INTERNAL EXAMINER) **(EXTERNAL EXAMINER)**

ACKNOWLEDGEMENT

With our deepest sense of gratitude we extend our heartfelt thanks to **Mr.Ramalingam,** Senior Lecturer, Department of Biotechnology, Kumaraguru College of Technology, for his relentless support, masterly guidance, creative ideas and patient efforts for successful completion of the project.

Our sincere thanks to **Dr. P. Rajasekaran**, Professor & Head, Department of Biotechnology, Kumaraguru College of Technology. His gracious and ungrudging guidance all through our project work is highly acknowledged with gratitude.

We are happy to thank **Mr. V. Stephen Rapheal** , our Class advisor and Lecturer, Department of Biotechnology, Kumaraguru College of Technology, for his unsolicited and timely help and encouragement without any hesitation.

We wish to extend our thanks to all **Teaching and Non-Teaching staffs** of the Department of Biotechnology for their kind and patient help throughout the project work.

We extend our heartfelt thanks to **Dr.S.Sadasivam**, Dean Academics, his concern and implication has been immensely helpful in the completion of the project.

We are happy to thank **Prof. R. Annamalai,** Vice-Principal, Kumaraguru College of Technology and **The Management**, Kumaraguru College of Technology for providing us all the facilities to carry out the project work.

We thank all our friends who physically and emotionally helped us to bring out the work successfully.

Finally, we wish to express our deep sense of gratitude to our beloved parents and family members for their constant encouragement and love, without whose inspiration this study would not have seen the dawn of day.

Saranya S

Tharani J

Priyadarsini M

ABSTRACT

ABSTRACT

α-Galactosidase activity was seen in both the inner and outer layer of *Aloe vera* and it was purified by ammonium sulphate fractional precipitation and ultra- filtration technique. The enzyme was optimally active at **55°C** and **45° C** for the outer layer and inner layer, respectively for the hydrolysis of p-nitrophenyl- α-galactopyranoside (PNPG). The optimum pH of the enzyme was **5.5** and **4.6** from the outer and inner layer, respectively. The metal ions Ag^{+} and Hg^{2+} strongly inhibited enzyme activity for the outer layer whereas the metal ions Fe^{2+} and Ag^{+} strongly inhibited enzyme activity for the inner layer. Fructose and galactose had the maximum inhibitory effect for the outer layer whereas galactose and arabinose had the maximum inhibitory effect for the inner layer. All other sugars had a mild inhibitory effect on the enzyme. Among the reagents, N-bromosuccinimide brought about maximum enzyme inhibition for both the layers. K_m and V_{max} for the enzyme from the outer layer was found to be 1.11 mM & 0.025U, respectively whereas K_m and V_{max} for the inner layer was found to be 0.833mM and 0.0055U. The TLC pattern revealed that the stachyose and raffinose in soymilk were completely hydrolyzed by the enzyme in 3 hours at 55°C.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
µg	Microgram
mg	Milligram
Ml	Millilitre
h	Hour
l	Litre
Min	Minutes
EDTA	Ethylene Diamine Tetraacetic Acid
PNPG	Para Nitro Phenyl Galacto-pyranoside
M	Molar
Nm	Nanometer

INTRODUCTION

1. 1. *Aloe vera*

Kingdom - Plantae

Division - Magnoliophyta

Class - Lilliopsida

Order - Asparagales

Family - Asphodelaceae

Genus - *Aloe*

Species - *vera*

Aloe vera is a stemless or very short - stemmed succulent plant. This is also known as medicinal Aloe. The stems, thick and fleshy, green to grey - green, with some varieties showing white flecks on upper and lower stem surfaces. The *Aloe vera* forms arbuscular mycorrhiza, a symbiosis that allows the plant better access to mineral nutrients in the soil. It belongs to the order Asparagales and family Asphodelaceae. It grows well in tropical climates. This succulence enables the species to survive in areas of low natural rainfall, making it ideal for rockeries and other low-water use gardens. This is frequently cited as being used in herbal medicine. This is also widely used in cosmetics. *Aloe vera* extracts are used in the treatment of diabetes and elevated blood lipids in humans. These positive effects are thought to be due to the presence of compounds such as polysaccharides, mannans, anthraquinones and lectins. This is proven to have good rejuvenating, healing, and soothing properties.

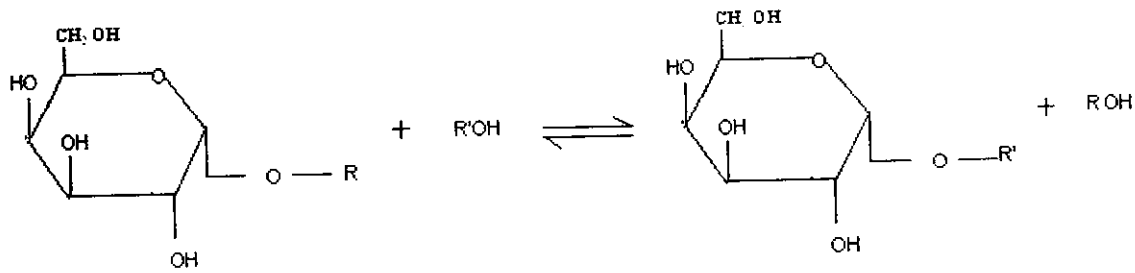
1.2. α-Galactosidase [α-D-galactoside galactohydrolase, EC 3.2.1.22]

Alpha-galactosidase catalyzes the hydrolysis of the alpha-1,6-

sugars), and polysaccharides (galactomannan and galactoglucomannans) (Dey and Pridham, 1972; Varbarnets et al., 2001). The hydrolytic action of α-galactosidase on various sugars is shown as below:

1. Melibiose yields D-galactose and D-glucose,
2. Raffinose yields one molecule of galactose and sucrose,
3. Stachyose yields two molecules galactose and sucrose,
4. Verbascose yields three molecules galactose and sucrose.

α-Galactosidase catalyses the following reactions:



the hydroxylic acceptor molecule, R'OH, is commonly water, although R and R' can be aliphatic or aromatic groups. This clearly indicates that the enzyme α-galactosidase may hydrolyze a number of simple α-galactosides to more complex polysaccharides.

1.2.1. Specificity:

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). α-Galactosidase exhibits two types of activity. They are as follows:

A. Hydrolase activity, and

B. Transgalactosylase activity

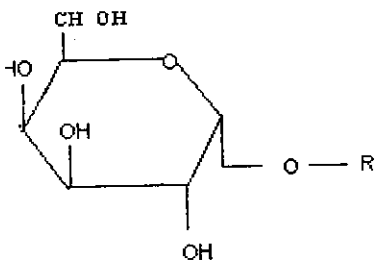
A. Hydrolase activity:

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

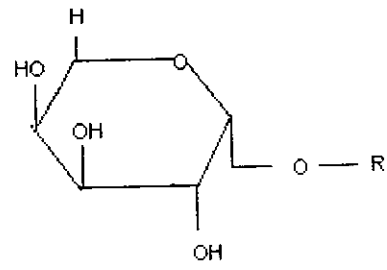
i. The ring structure of the substrate must be pyranoid, and

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

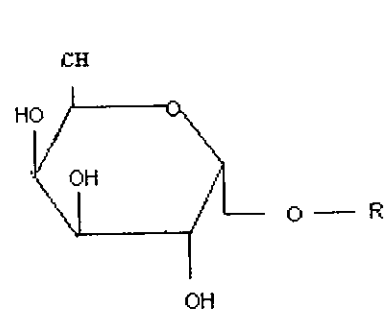
Like other carbohydrates, α-galactosidase can also tolerate changes at C-6 of glycosyl moiety of the substrate. Hence α-galactosidase from several sources has the capacity to hydrolyze ß-L-arabinosides (Figure 1.1). However, α-galactosidase from *Streptococcus bovis, Diplococcus pneumoniae* and *Calvatia cyathifomis* cannot act on arabinosides (Dey and Pridham, 1972). Dey and Pridham (1972) have reported that p-nitrophenyl-α-D-fucoside can be hydrolyzed by α-galactosidase, because p-nitrophenyl-α-D-fucoside has the similar configuration with that of D-galactose (Figure 1.1). α-Galactosidase from sweet almond and yeast are not able to hydrolyze the α-isomer of D-glycero-D-glyceroheptoside (Figure 1.1).

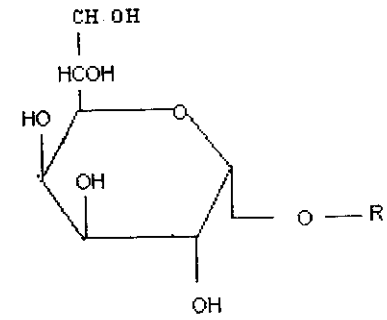


α-D-Galactoside



ß-L-Arabinoside





α-D-Fucoside D-Glycero-D-glyceroheptoside

Figure 1.1: α-D-Galactopyranoside and related glycosides.

B. Transgalactosylase activity:

Blanchard and Albon (1950) for the first time reported the transferase properties of α-galactosidase from yeast. They found that 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.

Transglycosylation occurs in two steps:

1. transfer of glycon preferably α-D-galactose , from donor to enzyme, and
2. transfer of glycon from the enzyme to the acceptor.

Usually, in step 2. the glycon moiety is transferred to hydroxyl groups other than water. Thus glycosylation leads to the formation of a triple complex, ES'S, where S' is a glycoside residue from donor and S is a second substrate molecule to which S' is transferred. In transglycosylation reactions, glycon moiety from donor is accepted by hydroxyl group of methionine present at the active site of α-galactosidase from *Trichoderma reesi*. The nature of galactosidic bond [α-(1,3) or α-(1,4) or α-(1,6)] in transgalctosylation products depend on the donor and the source from which

α-galactosidase is obtained. Because of competition in the transglycosylation reactions (self-condensation and condensation reactions), the end products are usually complex mixture.

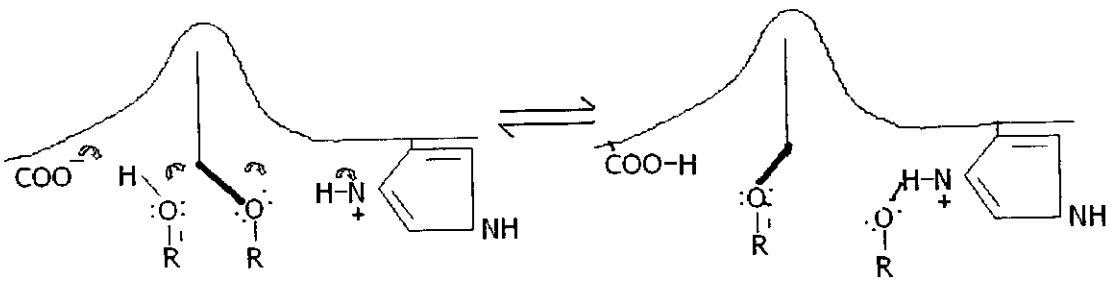
C. Polysaccharides:

Galactomannans are found as a major component of the endosperm in the seeds of many plants. Galactomannans consist of a backbone of ß-(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. The removal of terminal 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.

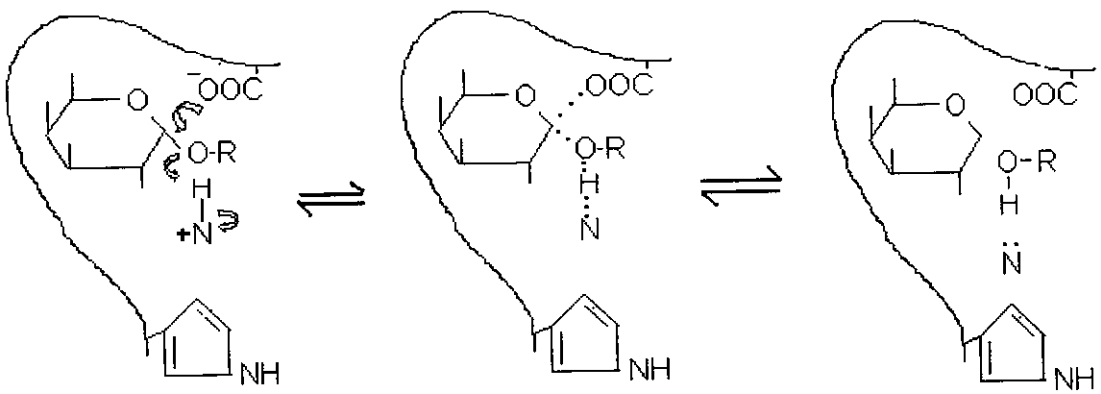
1.2.2. Mechanism of action:

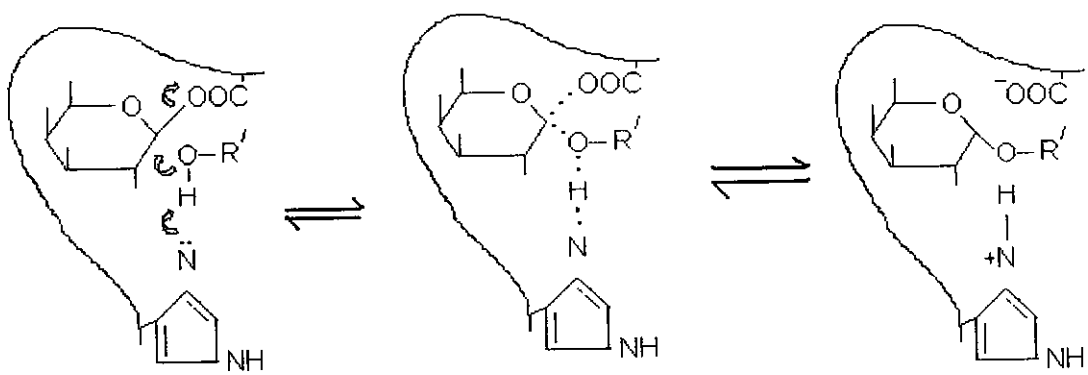
When compared with bond fission by other glycosylases on their substrates, α-galactosidase is also believed to cleave the galactose-oxygen bonds of substrates. Nuclear magnetic resonance and polarimeter studies have clearly indicated that the anomeric configuration of the liberated galactosyl residues has the same configuration as that of its substrates. Dey has proposed two alternative mechanisms for the action of α-galactosidase i.e. two-step mechanism and one step mechanism. Chemical modification studies suggest the presence of two carboxyl groups, a tryptophan and a tyrosine, at or near active site of enzyme. Based on the foregoing results, they proposed a new mechanism in which the ionizing group is a carboxyl group present in ionized form. This carboxylate anion not only help to stabilize the carbonium ion intermediate but also would direct the neucleophilic attack on the carbonium ion from one direction so that the product retains the same configuration as the substrate. The galactose molecule can assume the half chair conformation when carbonium ion is

formed, the 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.



A. One-step mechanism





B. Two-step mechanism

Figure 1.2. Mechanism of action of α-galactosidase.

1.3 Raffinose-family sugars:

The raffinose-family of oligosaccharides (RFO) are alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide, raffinose, the tetrasaccharide, stachyose and the pentasaccharide, verbascose. The RFO's are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to sucrose in abundance as soluble carbohydrates.

Humans and other monogastric animals (pigs and poultry) do not possess the alpha-galactosidase enzyme to break down these RFO and the oligosaccharides pass undigested through the stomach and upper intestine. In the lower intestine they are fermented by gas-producing bacteria that produce carbon dioxide, methane or hydrogen. Raffinose is broken down by the intestinal bacteria with the help of the enzyme α-galactosidase.

1.3.1. Stachyose

Stachyose[O-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-2)-ß-D-fructofuranoside] was first isolated from the rhizomes of *Stachys tubifera.* Stachyose is an oligosaccharide (trisaccharide) consisting of two D-galactose units and one sucrose sequentially linked Its molecular weight is 666.6 g/mol. Stachyose is naturally found in many vegetables (e.g. green beans, soybeans and other beans) and plants.

Stachyose is less sweet than sucrose, with about 28% on a weight basis. It is mainly used as a bulk sweetener or for its functional oligosaccharide property. Stachyose is not completely digestible by humans

1.3.2. Raffinose:

Raffinose is a complex carbohydrate. It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. Raffinose is hydrolysed to D-galactose and sucrose by α-galactosidase (α-GAL) (1). α-GAL also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol [1-O-(α-D-galactosyl)-myoinositol], if present. The enzyme does not cleave ß-linked galactose, as in lactose.

Raffinose is also known as melibiose and may be thought of as galactose + sucrose connected via an alpha-(1-6)-glycosidic linkage and so raffinose can be broken apart into galactose and sucrose via the enzyme alpha-galactosidase. Human intestines do not contain this enzyme.

1.3.3. Verbascose:

Verbascose[O-α-D-galactopyranosyl-(1-6)-α-D-galactopyranos yl-(1-6)-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-2)-ß-D-fructofuranoside] was first isolated from the roots of *Verbascum thapus* by Bourquelot and Bridel (1910). *In vitro* experiment using an enzyme preperation from the mature seeds of *Vicia faba* demonstrated the transfer of galactinol to stachyose yielding verbascose. When raffinose is galactose acceptor, stachyose if formed. The synthesis of verbascose is inhibited to greater extent by raffinose.

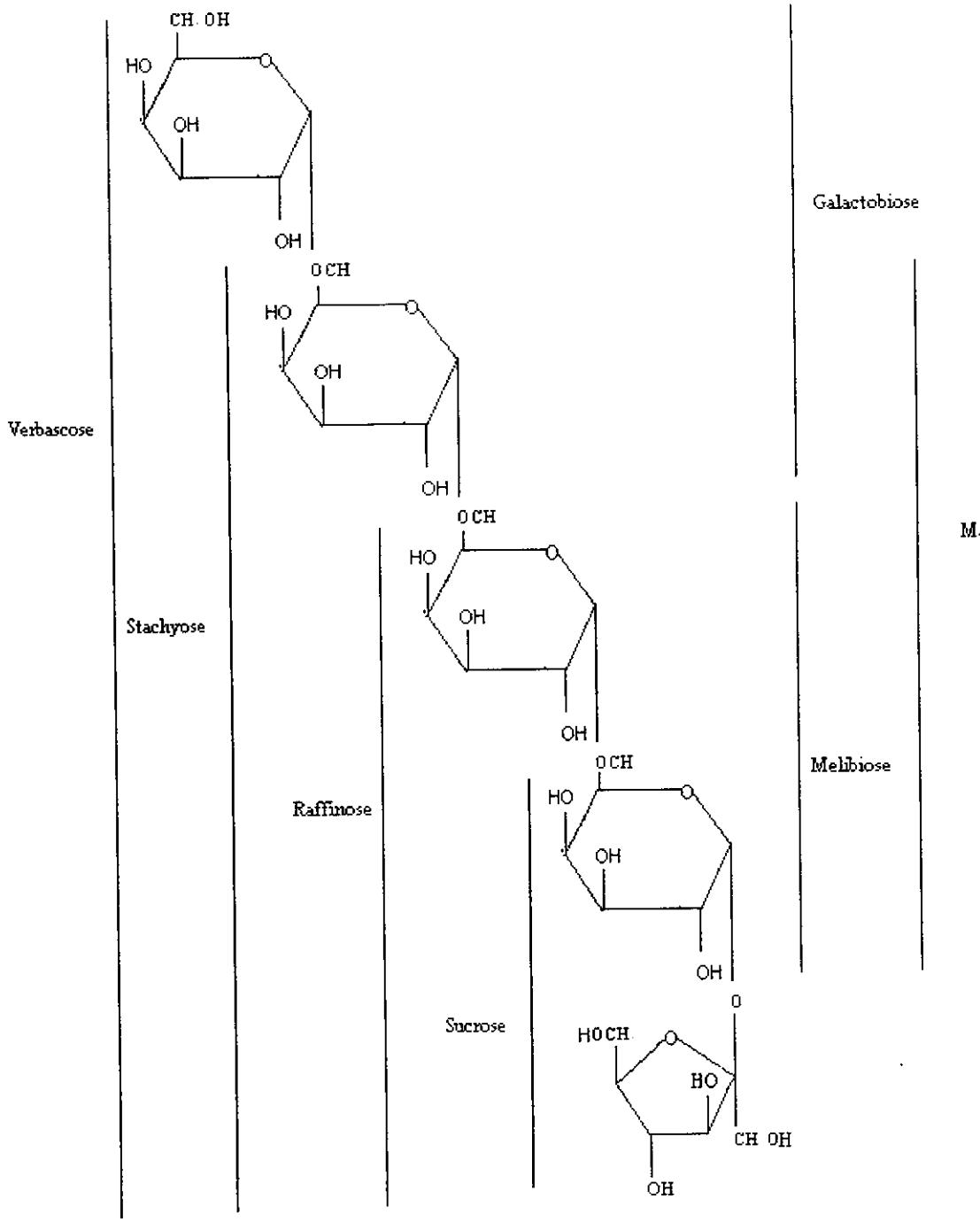


Figure 1.3. Raffinose-family sugars

1.4 Soy milk:

Soy milk (also called as soya milk, soybean milk, soy bean milk, soy drink, or soy beverage) is a milk-like product made from soybeans. Soy milk originated in Eastern Asia, China, a region where the soybean was native and used as food long before the existence of written records. Later on, the soybean and soybean foods were transplanted to Japan. Traditional soy milk, a stable emulsion of oil, water and protein, is simply an aqueous extract of whole soybeans. Soy milk contains about the same proportion of protein as cow's milk~ 3.5%; 2% fat, 2.9% carbohydrate and 0.5% ash.

Soy milk is nutritionally close to cow's milk, though most soy milk commercially available today contains artificially added vitamins such as vitamin B_{12} not naturally present. It naturally has about the same amount of protein as cow milk. Natural soy milk contains little digestable calcium as it is bound to the bean's pulp, which is insoluble in a human. To counter this, many manufacturers artificially enrich their products with calcium carbonate which can dissolve in the acid of the stomach. Notably it has little saturated fat, which many consider to be beneficial.

Soy milk is promoted as a healthy alternative to cow's milk for reasons including:

- contains fewer antibiotics, hormones, fat, cholesterol, excess protein, or links to cancer, diabetes, and other diseases
- Phytochemicals reduce the risk of cancer
- Soy protein reduces the levels of cholesterol and lessens the incidences of atherosclerosis
- Diabetes management through its ability to control blood sugar levels. However, diabetics should be aware that most brands of soymilk -

even those labelled "plain" or "organic" - are actually sweetened. Look for the word "unsweetened" on the label.

- Source of lecithin and vitamin E
- Lacks casein
- Soy milk is *pareve* and so may be consumed along with meat by Jews who keep kosher
- Safe for people with lactose intolerance or allergy to cow's milk
- Polyunsaturated and monounsaturated fats are good for your heart.
- Contains isoflavones, natural soy nutrients that are beneficial to health.

Using soybeans to make milk instead of raising cows is said to have ecological advantages, as the amount of soy that could be grown using the same amount of land would feed more people than if used to raise cows. Because the soybean plant is a legume, it also replenishes the nitrogen content of the soil in which it is grown.

1.5 Flatulence :

Leguminosae is the third largest family of flowering plants with approximately 650 genera and 18000 species. Legume seeds (also called beans, grainlegumes or pulses) are second to cereals as source of human and animal food. Nutritionally they are 2-3 times richer in protein than cereal grains. Dry beans are good source of proteins of reasonable quality and they also contain upto 60% carbohydrates.

The total carbohydrates of dry legumes range from 25% in winged beans to 68% in cowpeas. This carbohydrates include monosaccharides and oligosaccharides, starch and polysaccharides. Starch is most abundant legume carbohydrate and total sugars (monosaccharides and

the raffinose family sugars (raffinose, stachyose, verbascose and ajucose) are predominant in most legumes and account for a significant percentage (31-70%) of total sugars.

Certain legumes such as mung beans, black gram, Bengal gram (chick pea) contain higher amounts of raffinose-family oligosaccharides than others. The predominance of a particular oligosaccharide seems to depend on the type of legume.For example, verbascose is a major oligosaccharide in black gram, Bengal gram, red gram and mung beans whereas stachyose is a major oligosaccharide in California small white beans, navy beans, soybens, cowpeas and lupine seeds. Raffinose is present in moderate to low amounts in most legumes.

Flatulence (gas-production) is one of the constraints limiting consumption of legume seeds by humans and animals. Man cannot digest raffinose family sugars because the intestinal juice lacks the hydrolytic enzyme α-galactosidase and the raffinose family sugars themselves are unable to pass through the intestinal walls. In other words, when raffinose family sugars are injested by humans, two enzymes (invertase and α-galactosidase) are required for complete hydrolysis raffinose family sugars, because the gastrointestinal tract does not pocess α-galactosidase enzyme, the metabolic fate of raffinose family sugars is uncertain.

Many studies have shown that in man and animals these oligosaccharides are involved in flatulence production, which is characterised by the production of small amounts of methane, large amounts of carbon dioxide, hydrogen and lowered pH. These gases are responsible for the characteristic features of flatulence, namely nausea, cramps, diarrhoea, abdominal rumbling and so on. Clostridium and other organisms inhabiting the lower part of intestine were responsible for gas production (Richard et al., 1966).

Many attempts were made to eliminate flatus production by genetic selection. Murphy (1973) found that the addition of antibiotics (penicillin and streptomycin) along with leguminous seed preparations inhibited flatus production under *in vitro* and *in vivo*. However the addition of these compounds to beans may change their organoleptic properties and make them unacceptable.

Raffinose family sugars are soluble in water. Several investigators reported a reduction in the raffinose family sugars from beans by soaking, cooking, soaking followed by cooking, soaking followed by germination, various extraction methods, membrane filtration techniques and enzymatic processing. Among them fermentation improves organoleptic properties and nutrional quality of the legume based foods and also leads to subsequent reduction in flatulence- causing raffinose family sugars.

1.6 Biotechnological applications of α-galactosidase:

α-galactosidase is widely distributed in nature. α-galactosidase are group of exotype carbohydrates which release α-D-galactose from melibiose, raffinose, stachyose, verbascose, galactomannans, glycoproteins, ceramide trihexosides, etc. (Dey and Pridham, 1972; Spangenberg et al., 1999; Andre et al., 2001).

α-Galactosidase has the following biotechnological applications:

1. Beet sugar industry
2. Pulp and paper industry
3. Food processing industry
4. Medical application and
5. Hydraulic fracturing of oil and gas wells.

1.6.1 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 increases gradually during storage and usually comes to 0.15%. In the beet sugar industry raffinose is an obstacle substance for normal crystallization of beet sugar. When the contents of raffinose in the beet molasses gets increased gradually to the range of 6-10%, the crystallization of beet sugar is abandoned and the molasses is discarded because of the preventive action of raffinose. If the raffinose in beet sugar can be removed or decomposed by some means, it is possible that the crystallization of beet sugar from molasses is further improved and the yield of beet sugar can be increased.

Crude α-galactosidase from microbial sources can be used to hydrolyze raffinose (Ganter et al., 1988; Puchart et al., 2000) into galactose and sucrose; this improves the crystallization efficiency and yield of sucrose. For the aforesaid purpose microorganisms exhibiting an appreciable α-galactosidase activity but with only a slight or no invertase activity is preferred for decomposition of raffinose.

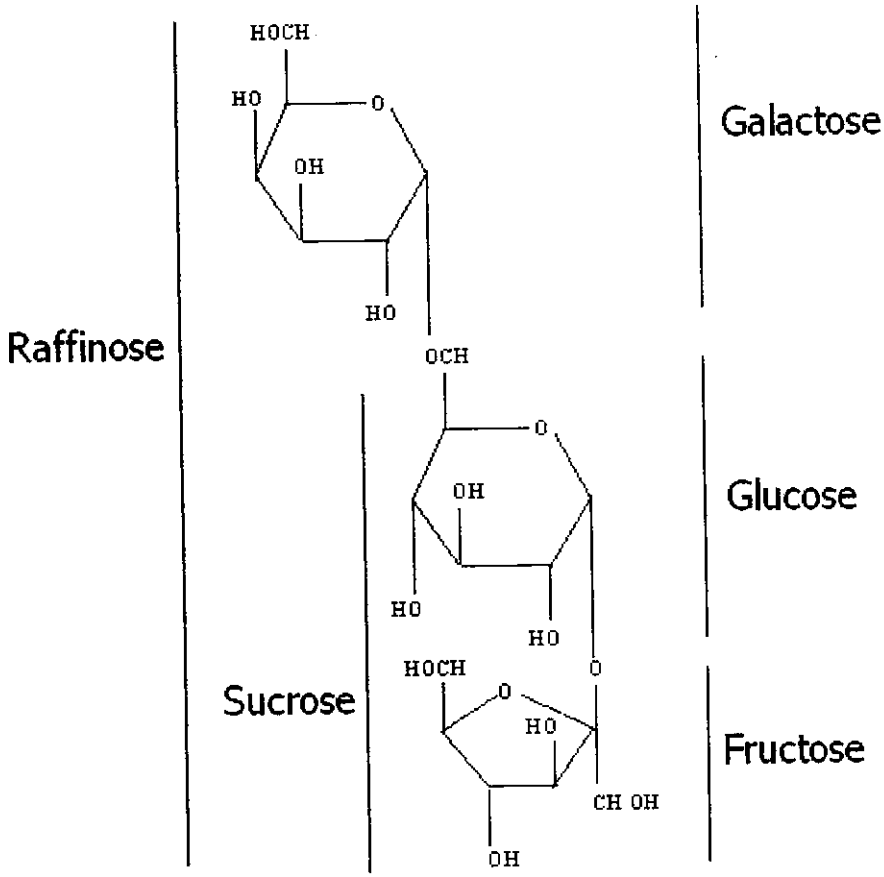


Figure 1.4 Structure of Raffinose

1.6.2 Use of α-Galactosidase in pulp and paper industry

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature. The major constituents of hemicelluloses are the hetero-1,4-ß-D-xylans and the hetero-1,4-ß-D-mannans (galactoglucomannans and glucomannans). In the case of galactomannans (softwood), enzymatic hydrolysis occurs with the concerted action of the following hydrolytic enzymes: endo-ß-1,4-mannanases, ß-mannosidase, α-galactosidase, ß-glucosidase. In the softwood pulp bleaching, microorganisms which can produce enzymes ß-mannanase and α-galactosidase without cellulose are preferred to avoid the degradation of cellulose (Zeilinger et al., 1993).

1.6.3 Applications of α-galactosidase in food processing industry

α-Galactosidase is potentially important in the hydrolysis of raffinose-family oligosaccharides (raffinose, stachyose, verbascose and ajugose) in pulses. Pulses provide major protein requirement and consists of raffinose-family oligosaccharides. Raffinose and stachyose present in soymilk are responsible for intestinal discomfort and flatulence (Steggerda et al., 1966). Soymilk can be used as a substitute for cow's milk, especially for infants suffering from lactose intolerance. Furthermore soymilk can be used as a protein beverage (Mulimani et al., 2000). A few microbial commercial preparations of α-galactosidase have been used to hydrolyze raffinose and stachyose present in soymilk (Thananunkul et al., 1976; Cruz and Park, 1982; Schuler et al., 1993; Garro et al., 1993; Kotwal et al., 1998).

1.6.4 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.

In humans, red blood cells have some 100 known blood group determinants (epitopes) that comprises 15 genetically distinct blood group system and the rhesus (Rh) blood group have major clinical importance. The A, B and O differ in sugar residues at their non-reducing ends. The precipitation-inhibition experiment of Kabat and Leskowitz (1955) first time indicated the role of an α-D-galactosyl group in blood group, type-B specificity.

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 type-B. However hydrolysis of terminal α-D-galactopyranosyl moieties of

blood group-B substances has been reported for a limited number of α-galactosidases, including those from coffee bens, soybeans, Steptomyces sp. and Trichomonas foetus (Zhu et al., 1995). Harmening (1994) for the first time reported that only rare individuals produce clinically significant antibodies to the 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 & Zaprometova, 1982). α-Galactosidase may be used in the near future for such medical purposes as enzyme therapy.

1.6.5 Hydraulic fracturing of oil and gas wells

In hydraulic fracturing application, 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 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 then be pumped out, after which oil or gas production can proceed. Well stimulation is achieved by flooding the wellbore with viscous, water based fracturing fluid (Clark, 1949; King, 1989). The fluid is water soluble solution containing guar gum, a natural product derived from leguminous plant, *Cyamopsis tetragonolobus.* Chemical oxidizers (persulfates) have been used to 'break' the fracturing fluid as they have mixtures of hemicellulases. However, temperature can exceed 120°C in the deeper reaches of 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 ß-1,4-mannanases) are highly desirable (McCutchen et al., 1996).

1.7 Applications of *Aloe vera* in the field of medicine:

Aloe has been well known for centuries for its healing properties, and both oral intake and topical dressings have been documented to facilitate healing of any kind of skin wound, burn, or scald - even speeding recovery time after surgery. It is also used for blisters, insect bites, rashes, sores, herpes, urticaria, athlete's foot, fungus, vaginal infections, conjunctivitis, sties, allergic reactions, and dry skin. The raw plant is best, but commercial preparations can also be used, especially for taking orally, as this plant tastes horrible. Other topical uses include acne, sunburn, frostbite (it appears to prevent decreased blood flow), shingles, screening out x-ray radiation, psoriasis, preventing scarring, rosacea, warts, wrinkles from aging, and eczema.

Internally, aloe is showing real promise in the fight against AIDS, and the virus has become undetectable in some patients who used it on a regular basis, due to its immune system stimulant properties. It also seems to help prevent opportunistic infections in cases of HIV and AIDS. It appears to be of help in cancer patients (including lung cancer) by activating the white blood cells and promoting growth of non-cancerous cells. Taken orally, aloe also appears to work on heartburn, arthritis and rheumatism pain and asthma, and studies have shown that it has an effect on lowering blood sugar levels in diabetics. Other situations in which it appears to work when taken internally include congestion, intestinal worms, indigestion, stomach ulcers, colitis, hemorrhoids, liver problems such as cirrhosis and hepatitis, kidney infections, urinary tract infections, prostate problems, and as a general detoxifier.

Commercially, aloe can be found in pills, sprays, ointments, lotions, liquids, drinks, jellies, and creams. The following gives the list of various products and their aloe content:

Sunburn treatments - 20% or more aloe content

Creams & Ointments - 20% or more aloe content

Juices - 95% or more aloe content

Beverages - 50% or more aloe content

Drinks - 10% or more aloe content

Capsules - 5-10% or more aloe content

In capsule form, it is used as a natural laxative. The gel, when squeezed from a freshly picked leaf, can be used to aid in the healing of burns, scars, and skin rashes. In tablet form, this herb has been known to aid kidney infections, help relieve arthritis, and ulcers. Used in cosmetics, this herb has been known to help in anti-wrinkle creams and make up. It also is beneficial for the hair and scalp.

OBJECTIVES

Cinnamoyl, pcoumaroyl, feruloyl, caffeoyl aloesin, and related compounds isolated from *Aloe* species were examined for anti inflammatory and antioxidative activities. It was suggested that the bioactivities may link to acyl ester groups in aloesin, together with those of aloesin related compounds. However, investigations using the contact hypersensitivity response indicated a preventive effect of aloesin on the UVB induced immune suppression (Yagi *et al.*, 2003).

An active glycoprotein fraction containing 58 %protein isolated from *Aloe vera* gel showed a radical scavenging activity against superoxide anion generated by the xanthine xanthine oxidase system as well as inhibition of cyclooxygenase2 and reduction of thromboxane A 2 synthase level in vitro (Yagi *et al.*, 2003).

Two dihydrocoumarins isolated from *Aloe* showed antioxidant activity against superoxide and hydroxyl radicals (Zhang *et al.*, 2006).

2.2.4. Antimicrobial activity

Agarry *et al.* (2005) compared the antimicrobial properties of ethanolic extract of *Aloe* leaf and gel. It was found that *Aloe gel* was effective against *Staphylococcus aureus* and *Trichophyton mentagrophytes.*

Invitro susceptibility of *Shigella flexneri*, *Streptococcus pyogenes* to *Aloe* gel was studied by Valerie *et al.* (2003).

Antifungal activity of *Aloe* gel was studied in phytopathogenic fungi like *Rhizoctonia solani,Fusarium oxysporum, Colletotrichum coccodes* by Jasso de Rodriguez *et al.*(2005).

2.3. α-Galactosidase

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-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 and *o*NPG had lower K_m and higher K_{cat} as compare to natur al substrates, melibiose, raffinose, and stachyose (Note et al., 2006).

Germinating seeds of Cassia sericea Sw. contain two molecular forms of α-galactosidase which were partially purified and characterized. K_m values for the substrate p-nitrophenyl-α-D-galactoside (PNPG) were 0.91 mM and 1.05 mM for the two forms, and substrate inhibition was observed at high concentrations of PNPG (Geeta Ramachandra et al., 1989)

Heat stability of the enzyme decreased as purity increased. This trend was partially reversed by addition of 2-mercaptoethanol, NADH, cysteine, 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 imbibition proteins' (SIPs) which are present in a wide range of plant families (Carmi, Nir et al., 2003).

The Cucurbitaceae translocate a significant portion of their photosynthate as raffinose and stachyose, which are galactosyl derivatives of sucrose. These are initially hydrolyzed by α-galactosidase to yield free

galactose and, accordingly, Gal metabolism is an important pathway in Cucurbitaceae sink tissue (Dai N et al., 2006)

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

2.3.1. Hydrolase activity

α-Glactosidase from *Streptococcus bovis*, *Diplococcus pneumoniae* and *Calvatia cyathifomis* cannot act on arabinosides (Dey and Pridham, 1972). Dey and Pridham (1969a) have reported that p-nitrophenyl-α-D-fucoside can be hydrolyzed by α-galactosidase, because p-nitrophenyl-α-D-fucoside has the similar configuration with that of D-galactose.

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 number of enzymes, including coffee bean α-galactosidase (Harpez et al., 1975).

2.3.2. Transgalactosylase 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 transglycosylase properties, but relatively at high substrate concentrations

(Ohtakara et al., 1984; 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 a glycoside residue from donor and S is a second substrate molecule to which 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 reesi* (Eneyskaya et al. 1998).The nature of galactosidic bond [α-(1,3) or α-(1,4) or α-(1,6)] in transgalctosylation products depend on the donor and the source from which α-galactosidase is obtained (Mitsutomi and Ohtakara, 1988; Van Laera et al., 1999). Because of competition in the transglycosylation reactions (self-condensation and condensation reactions), the end products are usually complex mixture (Eneyskaya et al. 1998).

α-Galactosidase was able to form oligosaccharides with degree of polymerisation (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 gene 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 showed 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 EV et al , 1997)

Objectives:

- To purify the α-galactosidase from *Aloe vera* by classical purification techniques
- To study the effect of pH , temperature, metal ions and some reagents on α-galactosidase
- To evaluate the effect of α-galactosidase on flatulence-causing oligosaccharides of soy milk

LITERATURE REVIEW

2.1. Medicinal plants

The recent years have witnessed a great intrest among researchers in herbal medicine and scientific validation of herbal drugs by isolation of active components from potential medicinal plants. Approximately 119 pure natural compounds isolated from higher plants are used in medicine throughout the world (Farnsworth *et al.*, 1985).

There are about 2000 plant species, which have been found to posses the medicinal value in all the five traditional systems of medicine viz Ayurveda, Siddha, Unani, Tibetian and Homeopathy (Baruah *et al.*, 1984). Most of these medicinal plants have been identified and their uses are well documented and described by different author (Nadkarni, 1997; Jain, 1991; Kirtikar and Basu, 1991; Ambasta, 1992) but the efficacies of many of these plants are yet to be verified

2.2. *Aloe vera*

2.2.1. Constituents of *Aloe vera*

The main constituents of *Aloe vera* include amino acids, anthroquinones, hormones, vitamin, and carbohydrates (Dr Peter Atherton, 1997; Lawrence G Plaskett, 1998; Robert H Davis, 1997; Bill C Coates, 1996; Diane Gage, 1996).

The constituents of the outer layer of the *Aloe vera* leaves include the anthranoids aloin A and B, hydroxyaloin, 1,8-dihydroanthrachinon, 2-alkylchromon, flavanone and minerals (Hansel *et al.*, 1992).*Aloe vera* gel contains water (95%), and polysaccharides (mainly mannose, pectin, hemicellulose, glucomannan, acemannan). It also contains amino acids,

lipids, sterols (lupeol, campesterol, ß-sitosterol), tannins and enzymes (Bruneton., 1995).

2.2.2. Medicinal applications

Aloe vera gel is used externally for numerous skin problems, especially for burns (Bruneton., 1995; Shelton., 1991; Hirt *et al.*, 1995). *Aloe ferox*, a species with very a similar composition to *A. barbadensis*, is mixed with hot water and used as a purgative. In South and West Africa *Aloe ferox* is used for eye complaints and sexual diseases (Hutchings *et al.*, 1996; Watt *et al.*, 1962) .In vivo studies indicate that, with *Aloe vera* gel, wounds heal more rapidly (Davis *et al.*, 1994; Shelton RM., 1991; Davis *et al.*, 1994).

The laxative effect of *A. barbadensis* and *A. ferox* can be attributed to their content of anthranoids (Ishii *et al.*, 1990).The dried sap is used for the short-term treatment of constipation (Haller JS, 1990).*Aloe vera* gel is used externally for burns (Visuthikosol *et al.*, 1995).In two studies, each with two patients with burns induced by X-rays, freshly collected *Aloe vera* gel had a positive effect (Collin., 1935; Loveman., 1937).

Aloe vera is used to control pain, edema in gum injuries, gum abscesses, cracks, gum problems associated with AIDS, leukemia, Oral disorders like Candidiasis, Desquamative Gingivitis, Vesiculobullous etc. (Timothy *et al.*)

Aloe vera gel enhanced the anti-acne properties of Ocimum oil and it was more effective than clindamycin used in the treatment of Acne (Orafidiya *et al.*, 2004).

2.2.3. Antioxidant activity

Growth stage plays a vital role in the composition and antioxidant activity of *Aloe vera*. It was found that three year old extract of *Aloe vera* exhibited highest radical scavenging activity (Hu *et al.*, 2003).

2.3.3. Mechanism of action

Dey (1969) has proposed two alternative mechanisms for the action of α-galactosidase i.e. two-step mechanism and one step mechanism.

Mathew and Balasubramaniam (1987a) have proposed to alternative mechanism of action of α-galactosidase from coconut. Shabalin et al. (2002) reported that steriochemical 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.

2.3.4. Raffinose-family sugars

The raffinose - family sugars have been identified as one of the important contributors of flatus (gas production) in human and experimental animals (Cristafaro et al., 1973).

2.3.4.1. Raffinose

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)

2.3.4.2. Stachyose

Stachyose was first isolated from the rhizomes of *Stachys tubifera* (Dey, 1985).

2.3.4.3. Verbascose

Verbascose was first isolated from the roots of *verbascum thapus* by Bourquelt and Bridel (1910).

2.3.5. Flatulence

Dry beans are good source of proteins of reasonable quality and they also contain upto 60% carbohydrates (Reddy et al., 1984).

Starch is most abundant legume carbohydrate and total sugars (monosaccharides and oligosaccharides) represent only a small percentage of total carbohydrates of the raffinose family sugars (raffinose, stachyose, verbascose and ajucose) are predominant in most legumes and account for a significant percentage (31-70%) of total sugars(Hiomowitz et al., 1972; Akpapunan and Markakis., 1979; Fleming , 1981).

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).

The raffinose family sugars themselves are unable to pass through the intestinal walls (Rackis, 1975).

Many studies have shown that in man and animals these oligosaccharides are involved in flatulence production, which is characterised by the production of small amounts of methane, large amounts of carbon dioxide, hydrogen and lowered pH (Rackis, 1975).

Raffinose family sugars are soluble in water. Several investigators reported a reduction in the raffinose family sugars from 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).

NDO (Non Digestable Oligosaccharides) can be fermented by gas-producing microorganisms present in the cecum and large intestine, which in turn can induce flatulence and other gastrointestinal disorders in sensitive individuals. The use of microorganisms expressing α-galactosidase

is a promising solution to the elimination of NDO before they reach the large intestine (LeBlanc et al., 2004).

L. fermentum a-galactosidase is able to partially alleviate α-galactosidase deficiency in rats. This offers interesting perspectives in various applications in which lactic acid bacteria could be used as a vector for delivery of digestive enzymes in man and animals (Florence Ledue-Clier et al., 2008).

MATERIALS & METHODS

3.1 Materials:

The *Aloe vera* was collected from Palladam. The chemicals such as raffinose, stachyose, p-nitrophenyl α-galactopyranoside (α-PNPG) were purchased from Sigma, USA. The galactose, fructose, glucose, sucrose, maltose and PMSF were purchased from Hi-Media, Mumbai. The protein molecular weight marker used for SDS-PAGE was purchased from Bangalore Genei. All other reagents used were of analytical grade.

3.2 Methods:

3.2.1 Preperation of buffers:

The following buffers were used :

- Acetate buffer: Stock solution of A, 0.2 M acetic acid (7.55 ml of Acetic acid in 100 ml) and stock solution of B, 0.2 M sodium acetate (16.4 g of sodium acetate in 100 ml) was prepared. Acetate buffer pH (4 to 5.5) was prepared by mixing appropriate proportions of A and B (Table 3.1), diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.
- Sodium Phosphate buffer: Stock solution of A , 0.2 M monobasic sodium phosphate (27.89 g in 1000 ml) and stock solution of B, 0.2 M dibasic sodium phosphate (95.365 g in 1000 ml) was prepared. Sodium phosphate buffer pH (6 to 8.5) was prepared by mixing appropriate proportions of A and B (Table 3.3), diluted to 100 ml and pH of the solution was adjusted and checked with pH meter.

Table 3.1 : Acetate buffer

A (in ml)	B (in ml)	pH
46.3	3.7	3.6
41.0	9.0	4.0
25.5	24.5	4.6
14.8	35.2	5.0
4.8	45.2	5.6

Table 3.2 : Phosphate buffer:

A (in ml)	B (in ml)	pH
87.7	12.3	6.0
73.5	26.5	6.4
39.0	61.0	7.0
16.0	84.0	7.5
5.3	94.7	8.0

3.3. Preparation of enzyme extract:

3.3.1. Outer layer:

The outer layer of *Aloe vera* was mashed with mixie by using 25 mM phosphate buffer, pH 6.0. Ultra-sonication was done for 10 cycles (1 cycle -10 seconds sonication followed by 2 minutes rest) at 0-4°C. Sonicated sample was centrifuged at 10000 rpm for 10 minutes. The supernatant thus obtained was checked for enzyme activity.

3.3.2. Inner layer:

The inner layer of *Aloe vera* was mashed to homogeneity in 10 mM phosphate buffer, pH 6.0 using mortar and pestle. The homogenized sample was then centrifuged at 7000 rpm for 10 minutes. The supernatant was assayed for enzyme activity.

3.4 Characterization:

3.4.1. Screening of enzyme extracts for invertase and ß-galactosidase :

3.4.1.1 Invertase assay:

Invertase activity in the crude enzyme extract from *Aloe vera.* was carried out. The reaction mixture contained 0.5 ml of enzyme extract, 0.3 ml of 1% sucrose and 1.5 ml acetate buffer (0.2 M , pH 5.5). The tubes were incubated at 50°C water bath for 15 min. 1 ml of DNS was added and incubated in boiling water bath for 5 min. 1ml of 40% Rochelle salt (sodium potassium tartarate) solution was added and the color developed was measured spectrophotometrically at 540nm.

3.4.1.2. ß -galactosidase assay:

ß-Galactosidase activity was assayed by spectrometric measurement of the release of p-nitrophenol from p-nitrophenyl-ß-D-galactopyranoside (ß-PNPG) at 405 nm.The reaction mixture consists of 100 µl of 10 mM PNPG in water, 800 µl of acetate buffer (0.2M, pH 4.6) and 100 µl of appropriately diluted enzyme. After incubation at 37°C for 15 minutes,

the reaction was stopped by the addition of 3 ml of 0.2 M sodium carbonate and the color developed was measured spectrophotometrically at 405 nm.

3.4.1.3. α-Galactosidase assay:

α-Galactosidase activity was assayed for both outer and inner layer of *Aloe vera* by spectrometric measurement of the release of p-nitrophenol from p-nitrophenyl-α-D-galactopyranoside (a-PNPG) at 405nm by modified method of Dey and Pridham (1969). For outer layer the reaction mixture consists of 100 µl of 10mM PNPG in water, 800 µl of acetate buffer (0.2 M, pH 5.5) and 100 µl of appropriately diluted enzyme. For inner layer the reaction mixture consists of 100 µl of 10Mm PNPG in water, 800 µl of acetate buffer (0.2M, pH 4.6) and 100 µl of appropriately diluted enzyme. After incubation at 55°C and 45°C for outer and inner layer respectively for 15 minutes, 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 of enzyme releasing 1 µmol of p-nitrophenol per minute under assay conditions.

Preparation of α-PNPG:

30 mg of p-nitrophenyl-α-D-galactopyranoside was dissolved in distilled water and the volume was made upto 10 ml. This was used as substrate for α-galactosidase assay.

3.4.2. Effect of different factors on α-galactosidase activity:

3.4.2.1. Optimum pH:

The effect of pH on the enzyme was measured in the pH range from 4.0 to 7.0. The following buffers were used: 200 mM acetate buffer (pH 4 to 5.5) and 1M phosphate buffer (pH 6 to 7).

3.4.2.2. Optimum temperature:

The influence of the temperature on α-galactosidase activity was determined by incubating the assay mixture for 15 min at temperature from 10-70°C.

3.4.2.3. pH stability:

Outer layer:

pH stability was determined by incubating the enzyme extract at pH 5.5 and 6.0 for 2h. Every 30 min time interval sample was collected and the residual activity was determined under normal assay conditions.

Inner layer:

pH stability was determined by incubating the enzyme extract at pH 5.0 and 5.5 for 1h. Every 30 min time interval sample was collected and the residual activity was determined under normal assay conditions.

3.4.2.4. Temperature stability:

Outer layer:

The enzyme extracts were incubated at 60°C and 65°C up to 120 minutes in a thermostat water bath. At particular time intervals a sample was withdrawn and assayed for remaining α-galactosidase activity.

Inner layer:

The enzyme extracts were incubated at 50°C and 55°C up to 90 minutes in a thermostat water bath and the samples collecgted were assayed for remaining α-galactosidase activity.

3.4.3. Effect of metal ions, sugars and some reagents.

3.4.3.1. Outer layer:

A reaction mixture consisting of 0.1 ml of enzyme solution, 0.7 ml of 0.2 M acetate buffer (pH 5.0) and 0.1 ml of metal ions (K^+ , Ca^{2+}, Hg^{2+}, and Fe^{3+} as chloride salts, Ag^+ as nitrate salt, Cu^{2+}, Mg^{2+},and Fe^{2+}, as sulphate salts) or sugars (glucose, galactose, fructose, lactose, sucrose, raffinose, ribose, maltose, arabinose and melibiose). Some reagents

(EDTA, 1,10-phenanthroline, N-bromosuccinmide, iodoacetate, iodoacetamide, and PMSF, at 5 mM) were incubated for 15 minutes at 50°C and α-galactosidase activity was determined after adding 0.1 ml of 10 mM α-PNPG. The activity was expressed as a percentage of the activity level in the absence of the compound.

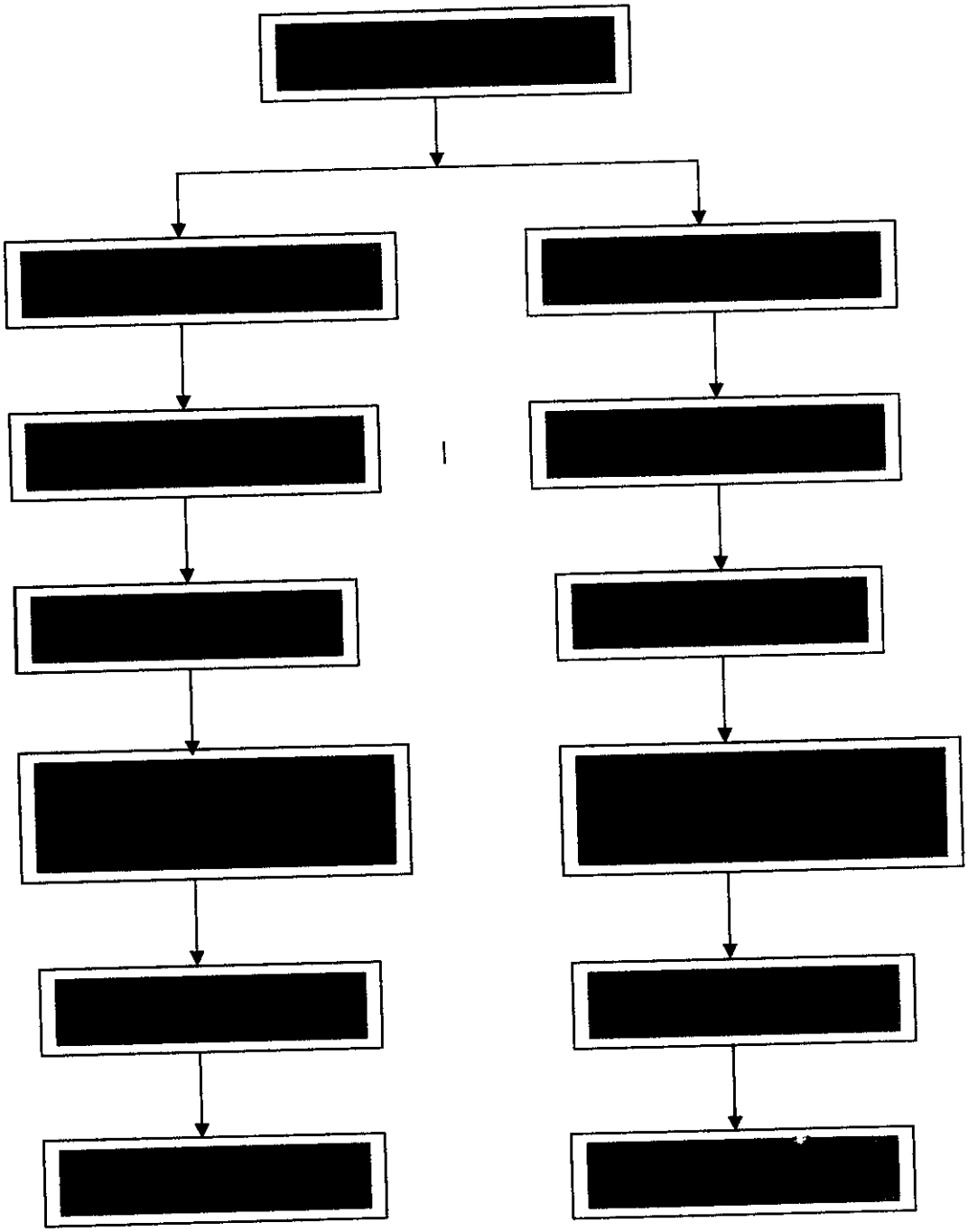
3.4.3.2. Inner layer:

A reaction mixture consisting of 0.1 ml of enzyme solution, 0.7 ml of 0.2 M acetate buffer (pH 4.6) and 0.1 ml of metal ions (K^+ , Ca^{2+}, Hg^{2+}, and Fe^{3+} as chloride salts, Ag^+ as nitrate salt, Cu^{2+}, Mg^{2+},and Fe^{2+}, as sulphate salts) or sugars (glucose, galactose, fructose, lactose, sucrose, raffinose, ribose, maltose, arabinose and melibiose). Some reagents (EDTA, 1,10-phenanthroline, N-bromosuccinimide, iodoacetate, iodoacetamide, and PMSF, 5 mM) were incubated for 15 min at 45°C and α-galactosidase activity was determined after adding 0.1 ml of 10 mM α-PNPG. The activity was expressed as a percentage of the activity level in the absence of the compound.

3.4.4. Kinetic studies (K_m and V_{max}):

α-Galactosidase assay was performed for both outer and inner layer by varying the concentrations of PNPG from 10 µl to 100 µl. The absorbance of the p-nitrophenol released was measured at 405nm and readings were tabulated.

Purification chart:



3.5. Purification

3.5.1. Ammonium sulphate fractional precipitation:

Ammonium sulphate (30%) was added to the culture filtrate (295 ml of outer layer and 810 ml of inner layer) with stirring and the mixture was kept aside at 4°C for 12 h. At the end of the 12 h, the mixture was centrifuged at 10000 rpm for 10 min and the supernatant was collected. The pellet was dissolved in a minimum amount of phosphate buffer (10 mM, pH 6.0). The above procedure was repeated with supernatent for 60% ammonium sulphate fractionation.

3.5.2. Ultra-filtration:

The fractions having high α-galactosidase activity were pooled and dialyzed overnight to remove ammonium sulfate ions. The dialyzed samples were subjected to ultra-filtration (membrane cut-off is 50 kDa, PALL Life Sciences, India). The dialyzed enzyme solution was filtered through the ultrafiltration membrane.

3.5.3. Polyacrylamide gel electrophoresis

- Monomer solution:

The solution contained 29.2 g of acrylamide and 0.8 g of N-N- methlyene bis acrylamide in final volume of 100 ml made in distilled water. The solution was filitered through Whatman number 1 filter paper.

- 1.875 M Tris buffer pH 8.8

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

- 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 volume of the solution was made up to 100 ml. The solution was stored at refrigerator.

- Sodium dodecyl sulphate (SDS) 10% w/v

10 g of SDS was dissolved in distilled water and final volume was made up to 100 ml and stored at room temperature.

- Ammonium persulfate (APS), (1% w/v)

Ammonium persulfate 100 mg was dissolved in distilled water and the volume was made up to 10 ml. Freshly prepared APS was used every time.

- Electrophoresis buffer (10X)

Tris base 6 g, glycine 14.4 g and SDS 10g was dissolved in distilled water and the volume was made up to 1000 ml and stored at room temperature.

- Separating gel mixture (10%)

Stock acrylamide solution	3.71ml
Tris-HCl (pH 8.8)	2 ml
Distilled Water	4.5 ml
APS	100µl
TEMED	20 µl

The mixture was poured into cassette kept in the gel casting apparatus and was allowed to polymerize.

- Stacking gel mixture (4%)

Stock acrylamide solution	340 µl
Tris-HCl (pH 6.8)	250 µl
Distilled Water	1.87 ml
APS	50µl
TEMED	10 µl

The mixture was poured into the cassette above the separating 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 the gel was inserted into the gasket of electrophoretic apparatus. The bromophenol blue containing sample buffer was mixed with [illegible] sample and was loaded in the wells. Electrophoresis was

performed at room temperature and at constant current of 100 volts. When the bromophenol blue dye stopped 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from glass plate and was stained.

3.5.3.1. Coomassie brilliant blue staining:

The gel was soaked in staining solution. After allowing it to stain for 1 hour the gel was immersed in the destaining solution. Destaining was stopped when the gel background was colorless.

- Staining solution

1g Coomassie brilliant blue in a solution containing 40 ml methanol, 10 ml glacial acetic acid and 50 ml distilled water.

- Destaining solution

Solution containing 40ml methanol, 10ml glacial acetic acid and 50 ml distilled water.

3.5.4. BSA calibration curve.

Bovine serum albumin (BSA) 1mg/ml was used as standard protein. Different concentrations of BSA solutions were pipetted out in the test tube and the total volume was made upto 1ml with distilled water. 2.1ml of alkaline copper reagent was added to each tube, mixed thoroughly and allowed to stand at room temperature for10min. Then to each tube 0.2ml of 1N Folins-Ciocalteau's reagent was added with immediate mixing. The extinction was read after 20min incubation against appropriate reagent blank at 660 nm in a ELICO spectrophotometer. A standard graph was constructed.

Alkaline copper reagent.

- Reagent A was prepared by dissolving 2 g sodium carbonate in 0.1N sodium hydroxide.
- Reagent B was prepared by dissolving 1g copper sulfate in 100ml of distilled water.
- Reagent C was prepared by dissolving1g sodium potassium tartarate in100 ml of distilled water.
- 98 ml of reagent A was mixed with 1ml of reagent B and 1ml of reagent C just before use.

Folin-Ciocalteau's reagent.

- 2N Folin-Ciocalteau reagent commercially available was diluted with distilled water in the ratio 1:1(v/v) just before use.

Table 3.3: BSA calibration table:

Reagents	B	S_1	S_2	S_3	S_4	S_5
Volume of working std. (ml)	0.0	0.2	0.4	0.6	0.8	1.0
Concentration of protein (µg)	0	20	40	60	80	100
Volume of distilled water (ml)	1.0	0.8	0.6	0.4	0.2	0.0
Volume of alkaline copper reagent (ml)	2.1	2.1	2.1	2.1	2.1	2.1
Incubate at room temperature for 10 min						
Volume of Folin's reagent(ml)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate at room temperature for 20 min						
Absorbance at 660 nm.	0.0	0.087	0.180	0.211	0.284	0.398

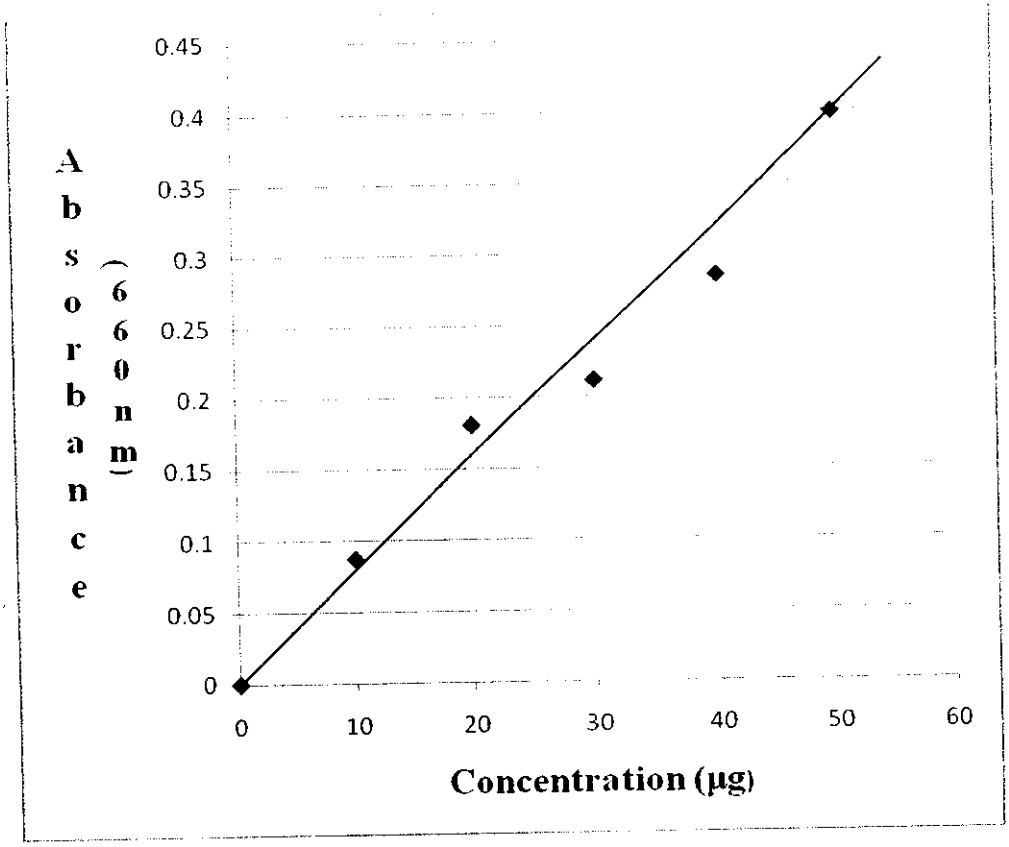


Figure.3.1. BSA Calibration curve

3.6. Applications of α-galactosidase :

3.6.1 Preparation of soymilk.

Soymilk was prepared by boiling 100g of whole dry seeds of soyabean in 250mL of distilled water for 60min to soften it and was allowed to attain room temperature. The boiled seeds were ground to fine paste in mortar and pestle and about 200mL of distilled water was added to the above paste, stirred thoroughly. The soymilk was obtained by filtering the above mixture. The filtrate was centrifuged at 10000 rpm for 10 minutes and the supernatant was used for enzymatic (a-galactosidase) treatment.

3.6.2 Treatment of soymilk by crude α-galactosidase:

100ml of soymilk and 40ml of a-galactosidase from the outer layer was taken and incubated at 55°C for 3 hours. 65 ml of the mixture was withdrawn for the first one and half hour and 55 ml of the mixture was

withdrawn for the rest of the 3 hours. The samples collected was then concentrated by allowing it to evaporate at 80-100°C.

3.7. Thin layer chromatography

3.7.1. Preparation of samples

Soy milk was concentrated to 5 ml to form a concentrated syrup. This was used as such as the sample before treatment. The samples after enzymatic treatment was also loaded on the TLC plate. Both these samples were loaded at 10 µl concentration. Sucrose and raffinose solutions were also loaded at 30 µl concentration (2 mg / ml).

3.7.2. Procedure

Thin layer chromatography was performed to analyze the end products of raffinose and stachyose of soymilk by α-galactosidase . TLC plate coated with silica gel was used to detect hydrolyzates of α-galactosidase. The plates developed in solvent system consisted of n-propanol : ethyl acetate : water (6:1:3, v/v). At the end of the run, plates were air dried, sprayed with 1% a-naphthol in absolute alcohol containing 10% (v/v) o-phosphoric acid to detect the fructose- containing sugars (Albon and Gross, 1950).

RESULTS & DISCUSSION

4.1 Purification:

4.1.1 Ammonium sulphate fractional precipitation:

Ammonium sulphate fractional precipitation was carried out and purification of 1.115 fold was obtained for the outer layer and it was 1.738 fold for the inner layer.

4.1.2 Ultra-filtration

Ultra-filtration was carried out and purification of 1.825 fold for the outer layer and it was 1.133 fold for the inner layer.

Table 4.1.1. Purification chart of α-galactosidase from the outer layer of *Aloe vera*

Enzyme Fraction	Volume (ml)	Protein conc. (mg)	Activity (Units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude filtrate	295	858.8	35.057	0.139	1	100
Ammonium sulphate fractionation	250	68.69	4.75	0.2416	1.738	13.549
Ultra filtration	8	58.32	0.0736	0.2537	1.825	0.209

Table 4.1.2. Purification chart of α-galactosidase from the inner layer of *Aloe vera*

Enzyme Fraction	Volume (ml)	Protein conc. (mg)	Activity (Units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude filtrate	810	83.41	9.414	0.139	1	100
Ammonium sulphate fractionation	55	61.68	0.528	0.155	1.115	5·608
Ultra filtration	20	34.48	0.1086	0.1575	1.133	1·153

4.1.3 PAGE:

Native-PAGE was carried out for the purified enzymes and the result is shown in figure 4.1.1. From the figure it is clear that the molecular weight of the enzyme from inner layerwas found to be 22±2 kDa. On the other hand, the molecular weight of the enzyme from outer layer was found to be 24±2 kDa. Molecular weight of alpha-galactosidase from *Aspergillus oryzae* was reported to be 99kDa (Ramalingam & Mulimani, 2004).

SDS-PAGE was also carried out for the purified enzyme and the results are depicted in figure 4.1.2. The results clearly shows that the enzyme from both inner and outer layers were made of a single polypeptide chain. Molecular weight of the alpha-galactosidase from inner layer and outer layer coincides with the results of Native-PAGE. Molecular weight of alpha-

galactosidase from *Aspergillus oryzae* was reported to be 99kDa (Ramalingam & Mulimani, 2004).

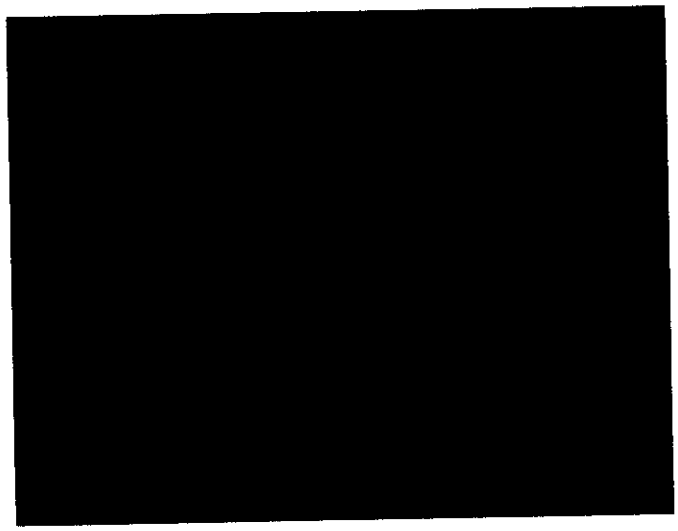


Fig 4.1.1. Native-PAGE for purified α-Galactosidase from *Aloe vera*

Fig 4.1.2. SDS-PAGE for purified α-Galactosidase from *Aloe vera*

4.2 Characterization:

α-Galactosidase activity was detected in both out and inner layers of *Aloe vera.* Invertase was not found in *Aloe vera.*

As expected, α-galactosidase activity was found to vary with pH. The effect of pH on enzyme activity is shown in figure 4.2. The enzyme exhibited optimal α-galactosidase activity in a pH range between 3.6 to 7.0, the optimum pH being 5.0 for the outer layer and 4.6 for the inner layer. α-galactosidase from *Giberella fujikuroi* (Thippeswamy and Mulimani, 2002) showed an optimum pH of 5.8. α-galactosidase from *Lactobacillus fermenti* (Mudgett et al., 1984) showed an optimum pH 5.0. α-Galactosidase from *L.fermentum* (Garro et al., 1993) showed an optimum pH of 5-6.5. α-galactosidase from *Ganoderma lucidum* (Sripuran et al., 2003) showed an optimum pH of 6. α-galactosidase from *Aspergillus flavipes* (Ozsoy et al., 2003) showed an optimum pH 4.5-5. α-galactosidase from *Lactobacillus reuteri* (Tzortzis et al., 2003) showed an optimum pH of 5.0. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) showed an optimum pH of 5.0. α-Galactosidase from Lactic acid bacteria (Mital et al., 1979) showed an optimum pH of pH 5.5. α-Galactosidase from mature leaves of *Cucurbita pepo* (Richard et al.,1983) showed an optimum pH of 7.5. α-Galactosidase from *Bifidobacterium breve* 203 (Sakai et al.,1987) showed an optimum pH of 5.5. α-Galactosidase from alkalophilic bacteria (Akiba et al.,1976) showed an optimum pH of 6.5 to 7.5. α-Galactosidase from yeast *Torulaspora delbrueckii IFO1255*, (Oda et al.,1996) showed an optimum pH of 4.5 to 5.5. α-Galactosidase from *Bifidobacterium adolescentis* (Van den Broek et al.,1999) showed an optimum pH of 6.5. α-Galactosidase from *Bifidobacterium breve* (Xiao et al.,2000) showed an optimum pH of 5.5 to 6.5. α-Galactosidase from *Corynebacterium murisepticum* (Nadkarni et al.,1992) showed an optimum pH of 7.5. α-Galactosidase from *Bacillus Stearothermophilus* NUB 3621 (Fridjonsson et al.,1999) showed an optimum

pH of 6.0 to 7.5. α-Galactosidase from *Thermus brockianus* ITI 360 (Fridjonsson et al.,1999) showed an optimum pH of 5.5 to 6.5. α-Galactosidase from *Penicillium purpurogenum* (Shibuya et al.,1998) showed an optimum pH of 4.0 to 6.0.

α-Galactosidase from *Aloe vera* had an optimum temperature at 55°C for the outer layer and 45°C for the inner layer (Fig 4.3). α-Galactosidase from *G. fujikuroi* (Thippeswamy and Mulimani, 2002) showed an optimum temperature of 56°C. α-Galactosidase from *Lactobacillus fermenti* (Mudgett et al., 1984) showed an optimum temperature of 45°C. α-Galactosidase from *L.fermentum* (Garro et al., 1993) showed an optimum temperature of 45°C. α-Galactosidase from *Ganoderma lucidum* (Sripuran et al., 2003) showed an optimum temperature of 70°C. α-galactosidase from *Aspergillus flavipes* (Ozsoy et al., 2003) showed an optimum temperature of 45°C. α-Galactosidase from *Lactobacillus reuteri* (Tzortzis et al., 2003) showed an optimum temperature of 50°C. a-Galactosidase from lactic acid bacteria (Mital et al., 1979) showed an optimum temperature of 55°C. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) showed an optimum temperature of 60°C. α-Galactosidase from alkalophilic bacteria (Akiba et al., 1976) showed an optimum temperature of 35°C. α-Galactosidase from yeast *Torulaspora delbrueckii IFO1255* (Oda et al., 1996) showed an optimum temperature of 55°C. α-Galactosidase from *Bifidobacterium adolescentis* (Van den Broek et al., 1999) showed an optimum temperature of 45°C. α-Galactosidase from *Bifidobacterium breve* (Xiao et al., 2000) showed an optimum temperature of 37°C. α-Galactosidase from *Bacillus Stearothermophilus* NUB 3621 (Fridjonsson et al., 1999) showed an optimum temperature of 75°C. α-Galactosidase from *Thermus brockianus* ITI 360 (Fridjonsson et al., 1999) showed an optimum temperature of 93°C. α-Galactosidase from *Penicillium purpurogenum* (Shibuya et al., 1998) showed an optimum temperature of 55°C.

α-Galactosidase lost only 45% & 53% of the activity in 60 & 90 minutes at pH 5.5 while it lost 19% & 26% of its activity in 60 & 90 minutes at pH 6.0 for the outer layer. α-Galactosidase from the inner layer lost only 33% & 10% of the activity in 30 minutes at pH 5.0 and 5.6, respectively. But it lost all its activity at both the pH in 60 minutes. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) showed enzyme stability in the pH range 4.5-6.5. The α-galactosidase activity was found to decrease at temperatures 65°C and 70°C hence it is not a thermostable enzyme. α-Galactosidase from *Bifidobacterium breve* (Xiao et al.,2000) showed thermostabilty at 50°C. α-Galactosidase from *Bacillus Stearothermophilus* NUB 3621 (Fridjonsson et al., 1999) showed thermostability at 70°C, 75°C, 80°C. α-Galactosidase from *Thermus brockianus* ITI 360 (Fridjonsson et al., 1999) was a thermostable enzyme. α-Galactosidase from *Penicillium purpurogenum* (Shibuya et al., 1998) showed thermostability at 40°C.

Table 4.6 shows the effect of metal ions on α-galactosidase activity. Among the cations tested, heavy metal ions such as Ag^{+} and Hg^{2+} strongly inhibited the enzyme activity to about 73.03% and 73.33%, respectively for the outer layer whereas the heavy metal ions Fe^{2+} and Ag^{+} strongly inhibited the enzyme activity to about 48.28% and 25.22%, respectively for the inner layer. The inhibition of α-galactosidase activity by Hg^{2+} shows the presence of tryptophan residues at or near the active site of the enzyme. α-Galactosidase from *L. fermentum* (Garro et al., 1993) was inhibited by Hg^{2+} and Zn^{2+}. α-Galactosidase from *G. lucidum* (Sripaun et al., 2003) was inhibited by Ag^{2+} and Hg^{2+}. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) was inhibited by Hg^{2+}, $Mn^{2+}$and Cu^{2+} . α-Galactosidase from mature leaves of *Cucurbita pepo* (Richard et al., 1983) was inhibited by Ag^{2+}, Hg^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+}. α-Galactosidase from alkalophilic bacteria (Akiba et al., 1976) was inhibited by Ag^{2+} and

Hg^{2+}. α-Galactosidase from yeast *Torulaspora delbrueckii IFO1255*, (Oda et al., 1996) was inhibited by Ag^{2+}, Hg^{2+} and Cu^{2+}.

Table 4.7 shows the effect of sugars on α-galactosidase activity. Among the sugars tested, fructose and galactose inhibited α-galactosidase activity by 77.49% and 64.50%, respectively for the outer layer whereas galactose and arabinose inhibited α-galactosidase activity by 50.00% and 53.69%, respectively for the inner layer. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) was inhibited by D-glucose, D-galactose and D-mannose. α-Galactosidase from *G.lucidum* (Sripuan et al., 2003) was inhibited by galactose.

Table 4.7 shows the effect of reagents on α-galactosidase activity. Among the reagents tested n-bromosuccinimide inhibited the enzyme activity to about 88.79% for the outer layer and about 89.41% for the inner layer, respectively. Inhibition of enzyme activity by n-bromosuccinimide indicated the presence of tryptophan at or near active site of an enzyme. The reagents such as EDTA and 1, 10- phenanthroline did not inhibit the enzyme activity suggesting that the metal ion is not required for enzyme activity. α-Galactosidase from *L. fermentum* (Garro et al., 1993) was not inhibited by EDTA. α-Galactosidase from *G. lucidum* (Sripuran et al., 2003) was not inhibited by EDTA.

.The kinetic studies of α-galactosidase from *Aloe vera* with different substrates showed that the K_m for p-nitrophenyl α-D-galactopyranoside, was 1.11 mM and V_{max} was 0.025, respectively for the outer layer and K_m was 0.833 mM and V_{max} was 0.0055, respectively for the inner layer . α-Galactosidase from *L. fermenti* (Mudgett et al., 1984) showed a K_m value (a-PNPG) of $5.7^* 10^{-4}$ M. α-Galactosidase from *G. lucidum* (Sripuran et al., 2003) showed K_m for PNPG, ONPG and MNPG were 0.4 mM, 0.67 mM, and 16.7 mM, respectively. α-Galactosidase from *L. reuteri* (Tzortzis et al., 2003) showed K_m for PNPG 0.55 mM. α-Galactosidase from

lactic acid bacteria (Mital et al., 1979) showed K_m of 0.957 mM and 4.12 mM for PNPG and raffinose, respectively. α-Galactosidase from *Humicola* sp. (Kotwal et al., 1999) showed K_m for PNPG, ONPG, melibiose, raffinose and stachyose were 0.31 mM, 0.54 mM, 0.70 mM , 3.3 mM and 7.6 mM, respectively. α-Galactosidase from mature leaves of *Cucurbita pepo* (Richard et al., 1983) showed K_m for PNPG was 1.4 mM. α-Galactosidase from yeast *Torulaspora delbrueckii*, (Oda et al.,1996) showed K_m for PNPG, ONPG, MNPG, melibiose, stachyose and raffinose were 2.8 mM, 1.3 mM, 2.8 mM, 4.2 mM, 230 mM and 170 mM, respectively. α-Galactosidase from *Bifidobacterium breve* (Xiao et al., 2000) showed K_m for melibiose and raffinose were 2.14 mM and 0.66 mM, respectively. α-Galactosidase from *Bacillus Stearothermophilus* NUB 3621 (Fridjonsson et al., 1999) showed K_m for PNPG, melibiose and raffinose were 0.38 mM, 12.0 mM and 16.4 mM, respectively.

4.3. Applications of α-galactosidase

Fig 4.3. Hydrolysis of stachyose and raffinose in soymilk by α-

Stachyose and raffinose from soymilk was completely hydrolysed by α-galactosidase from *Aloe vera*

Table 4.2: Effect of pH on activity of α-galactosidase from the outer layer of *Aloe vera*

pH	Activity (U)	Relative activity (%)
3.6	0.0844	16.10
4.0	0.0854	15.06
4.6	0.0865	13.96
5.0	0.1006	100.0
5.6	0.0831	17.37
6.0	0.0806	19.89
6.5	0.0759	4.51
7.0	0.0752	25.26

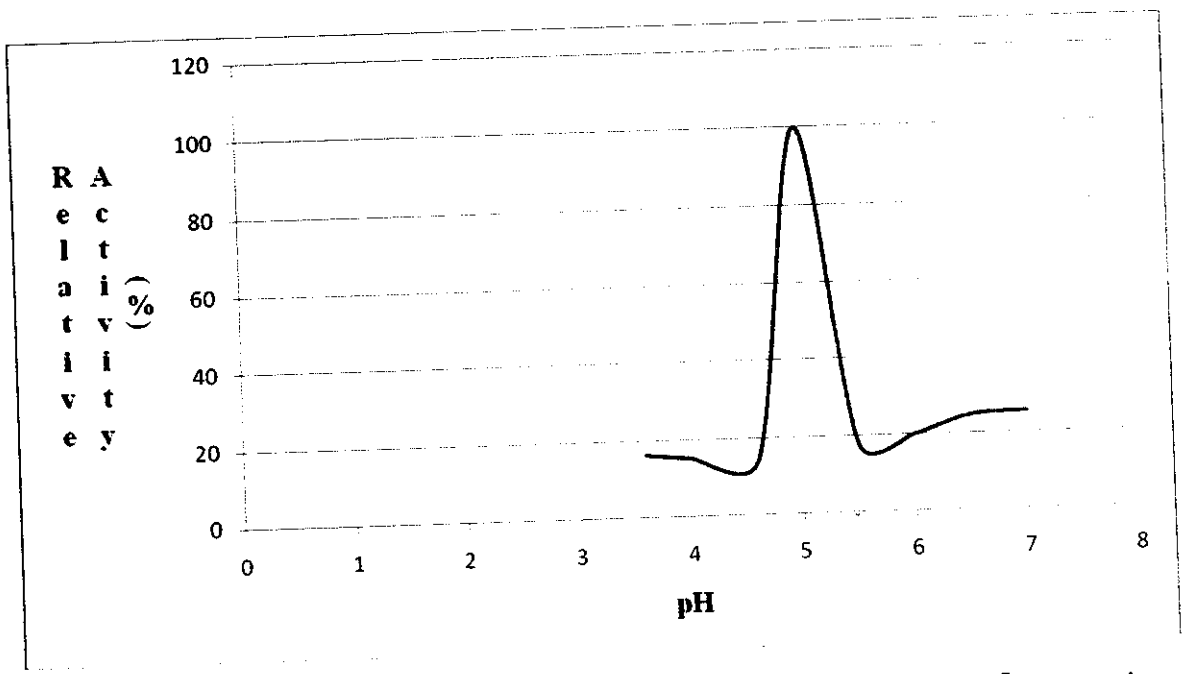


Figure 4.2.1. Effect of pH on the activity of α-galactosidase from outer

Table 4.2.2: Effect of pH on activity of α-galactosidase from the inner layer of *Aloe vera*

pH	Activity (U)	Relative activity (%)
3.6	0.0062	46.15
4.0	0.0092	20.29
4.6	0.0116	100.0
5.0	0.0097	16.41
5.6	0.0081	30.03
6.0	0.0073	36.48
6.5	0.0059	49.16
7.0	0.0031	73.40

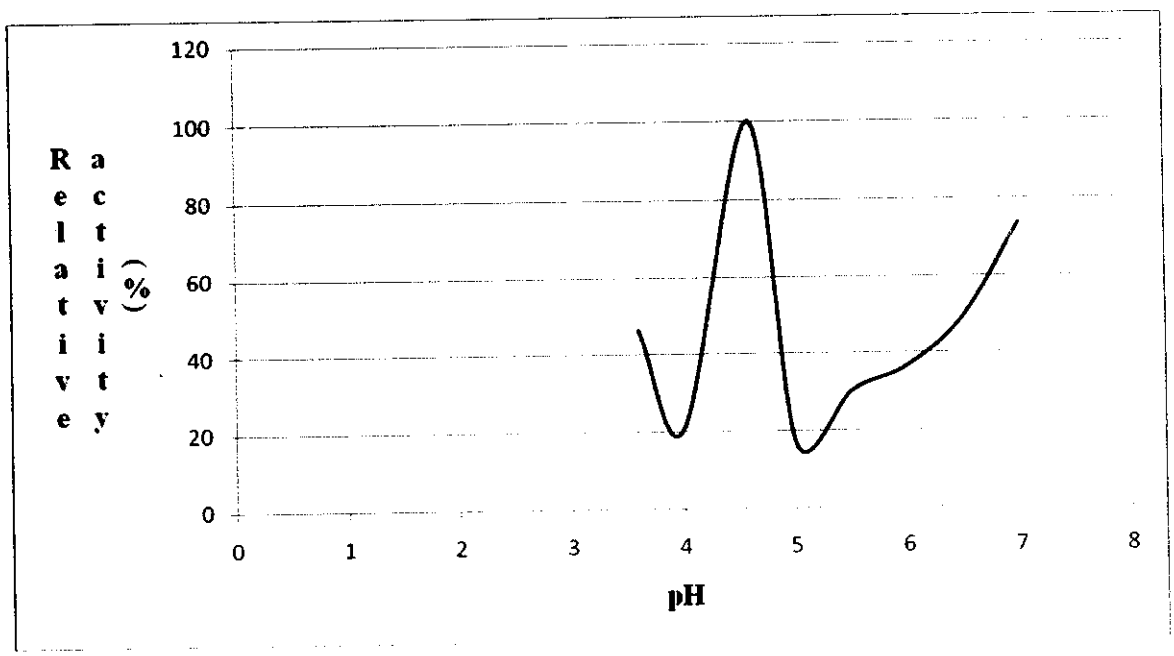


Figure 4.2.2.: Effect of pH on the activity of α-galactosidase from inner

Table 4.3. Effect of temperature on activity of α-galactosidase from outer layer of *Aloe vera*

Table 4.3.1 Outer layer

Temperature (°C)	Activity (U)	Relative Activity (%)
30	0.0166	74.10
35	0.0313	51.14
40	0.0333	47.97
45	0.0413	35.53
50	0.0495	22.74
55	0.0641	100.0
60	0.0596	06.90
65	0.0551	13.92

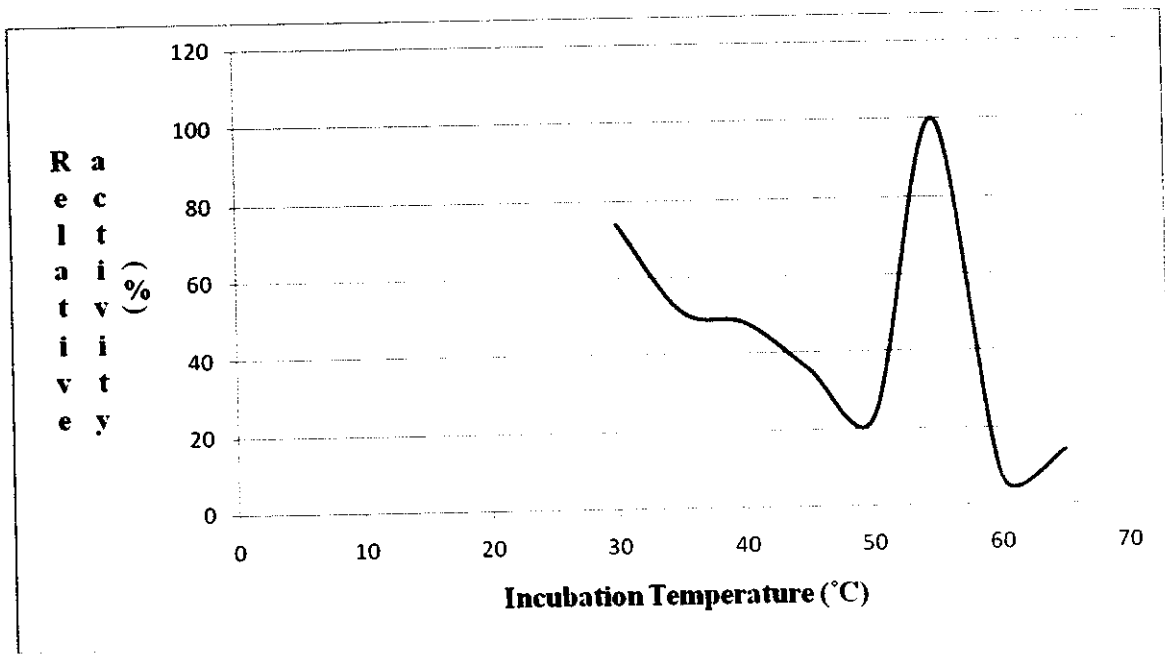


Figure 4.3.1. Effect of temperature on the activity of α-galactosidase from outer layer of *Aloe vera*

Table 4.3.2. Inner layer

Temperature (°C)	Activity (U)	Relative Activity (%)
30	0.0003	98.18
35	0.0027	83.18
40	0.0039	74.99
45	0.0159	100.0
50	0.0038	76.36

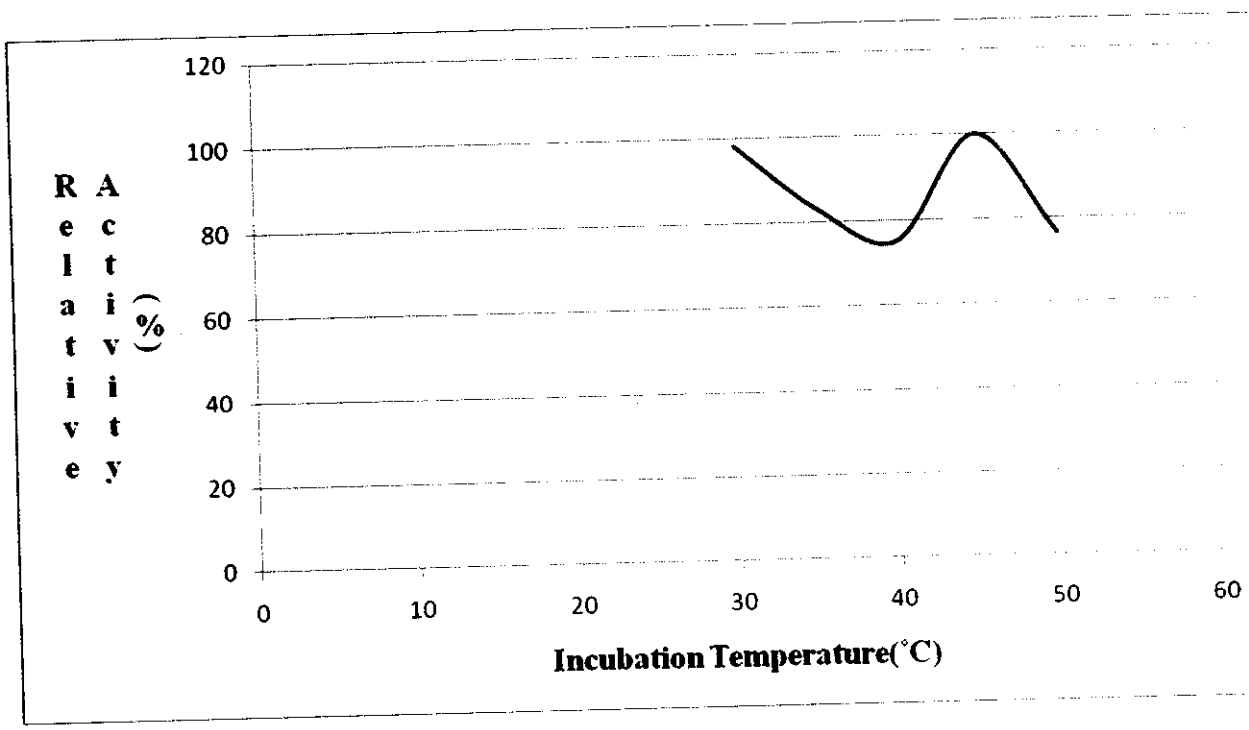


Figure 4.3.2. Effect of temperature on the activity of α-galactosidase

Table 4.4 : Effect pH on stability of the α-galactosidase from the outer layer of *Aloe vera*

Time (minutes)	Residual activity at pH 5.5 (%)	Residual activity at pH 6.0 (%)
0	100.00	100.00
30	99.25	99.10
60	54.82	80.56
90	46.84	74.34
120	34.38	38.39

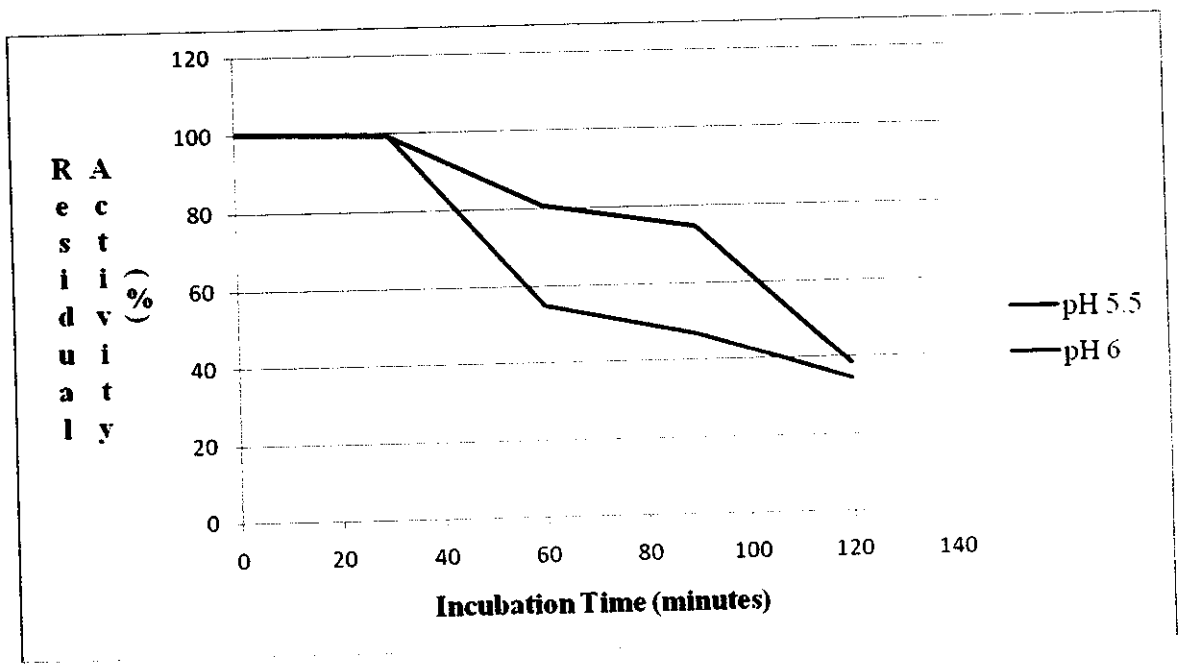


Figure 4.4.1: Effect of pH on stability of the α-galactosidase from outer

Table 4.4.2: Effect of pH on stability of α-galactosidase from the inner layer of *Aloe vera*

Time (minutes)	Residual activity at pH 5.0 (%)	Residual activity at pH 5.5 (%)
0	100.00	100.00
30	67.33	90.23
60	0.00	0.00

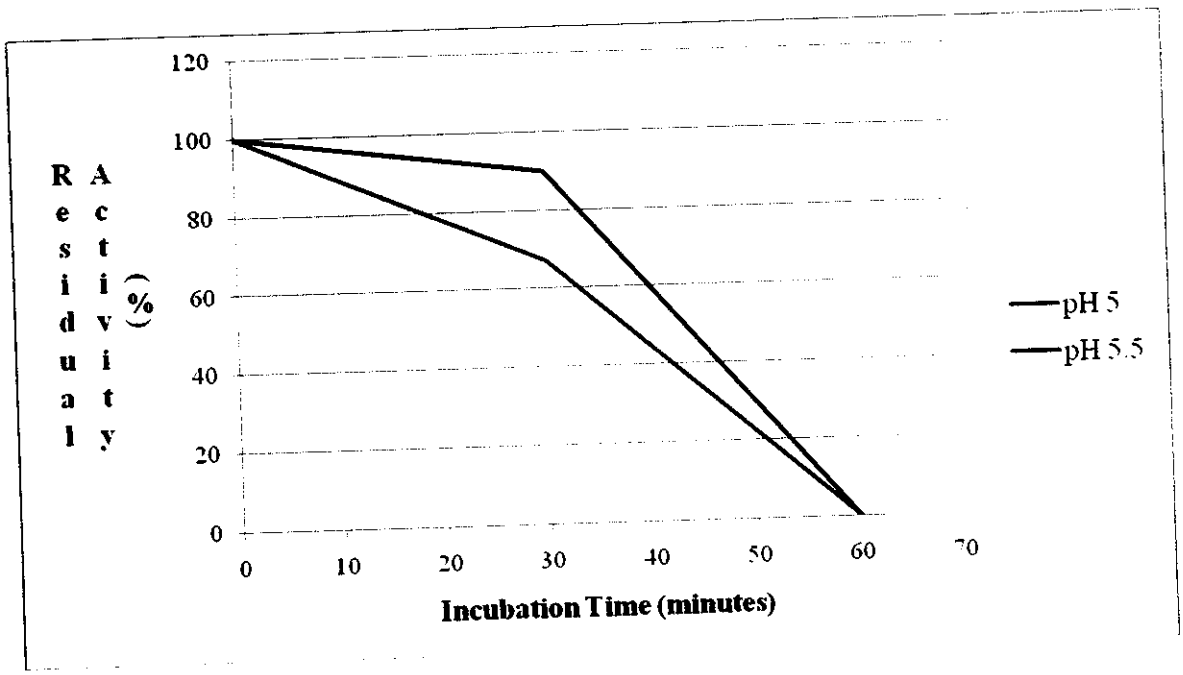


Figure 4.4.2: Effect of pH on stability of α-galactosidase from inner layer of *Aloe vera*

Table 4.5: Effect of temperature on the stability of α-galactosidase from *Aloe vera*

Table 4.5.1. Outer layer

Time (Minutes)	Residual Activity at 60°C (%)	Residual Activity at 65°C (%)
0	100.00	100.00
30	88.97	24.57
60	77.41	24.47
90	0.00	0.00

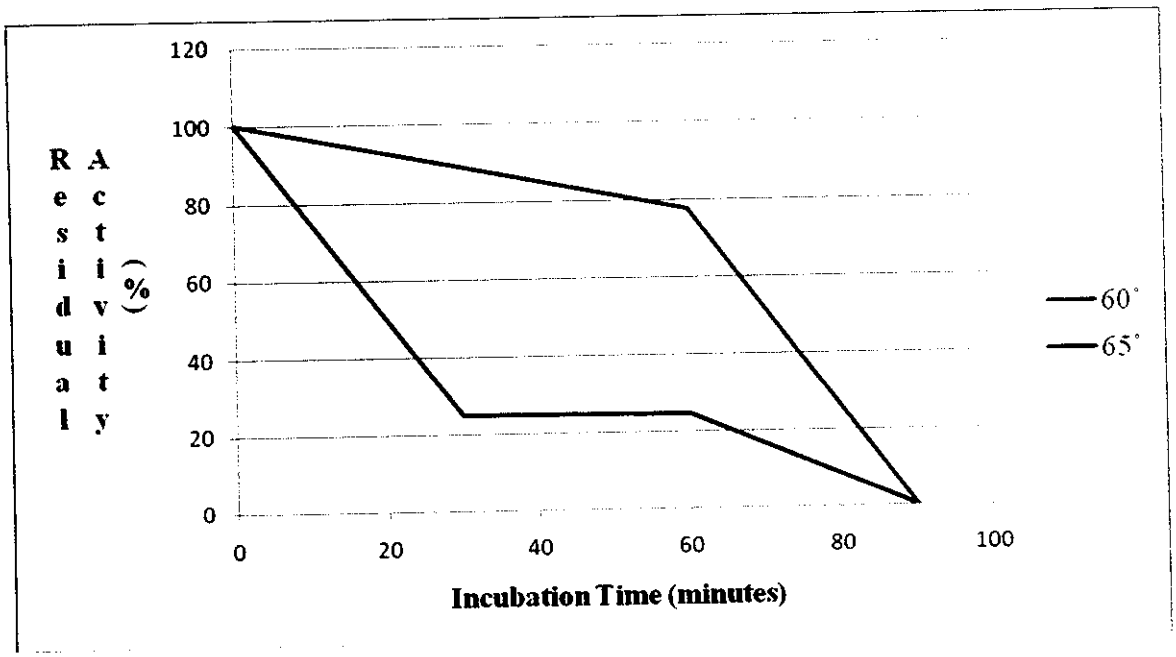


Figure 4.5.1: Effect of temperature on the stability of α-galactosidase from the outer layer of *Aloe vera*

Table 4.5.2. Inner layer

Time (Minutes)	Residual Activity at 50°C (%)	Residual Activity at 55°C (%)
0	100.00	100.00
30	60.01	68.00
60	60.01	62.00
90	12.00	62.00
120	0.00	37.98

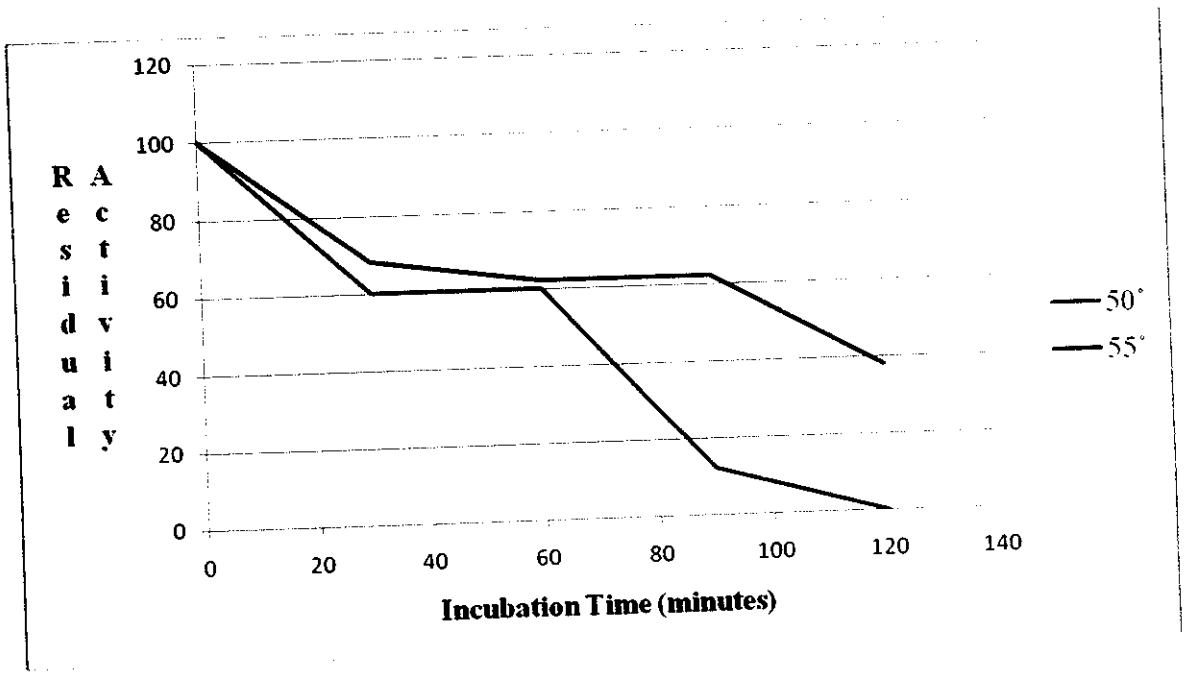


Figure 4.5.2: Effect of temperature on the stability of α-galactosidase from the inner layer of *Aloe vera*

Table 4.6: Effect of metal ions on α-galactosidase from the outer layer of *Aloe vera*

Metal ions	Concentration (mM)	Activity (U)	Residual activity (%)
Control	Nil	0.02310	100.0
$FeSO_4$	5	0.00717	31.04
KCl	5	0.00725	31.39
$CaCl_2$	5	0.00739	31.99
$FeCl_3$	5	0.00804	34.81
$CuSO_4$	5	0.00790	31.20
$HgCl_2$	5	0.00616	26.67
$MgSO_4$	5	0.00681	29.48
$AgNO_3$	5	0.00623	26.97

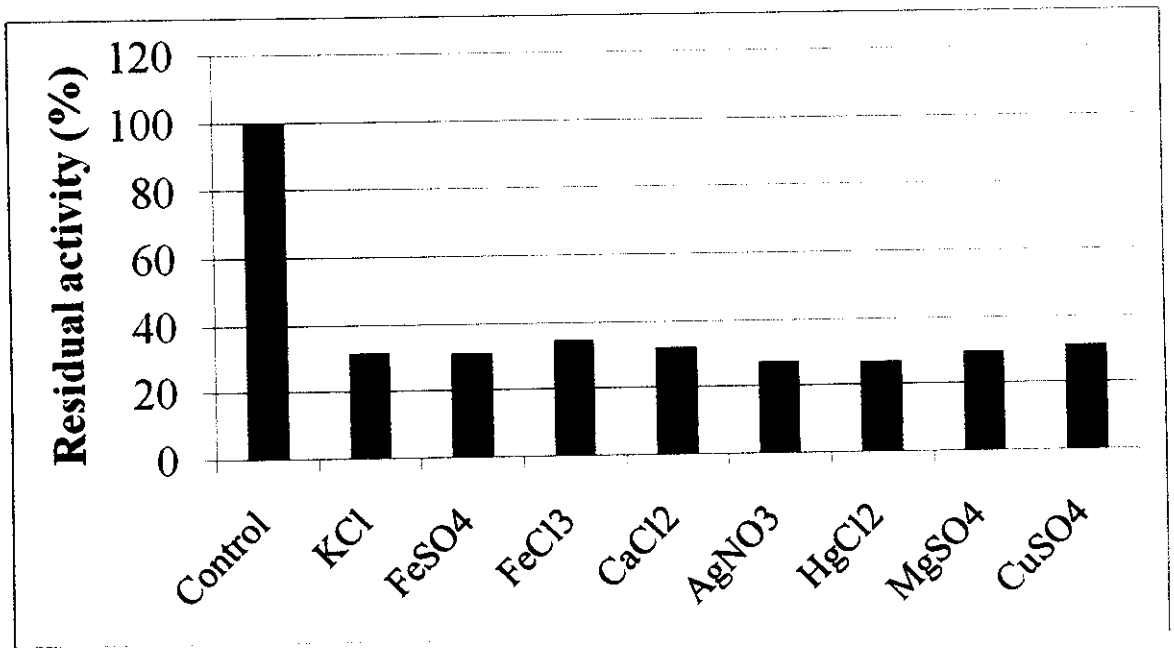


Figure 4.6.1: Effect of metal ions on α-galactosidase from the outer

Table 4.6.2. Inner layer

Metal ions	Concentration (mM)	Activity (U)	Residual activity (%)
Control	Nil	0.02310	100.0
$FeSO_4$	5	0.00717	31.04
KCl	5	0.00725	31.39
$CaCl_2$	5	0.00739	31.99
$FeCl_3$	5	0.00804	34.81
$CuSO_4$	5	0.00790	31.20
$HgCl_2$	5	0.00616	26.67
$MgSO_4$	5	0.00681	29.48
$AgNO_3$	5	0.00623	26.97

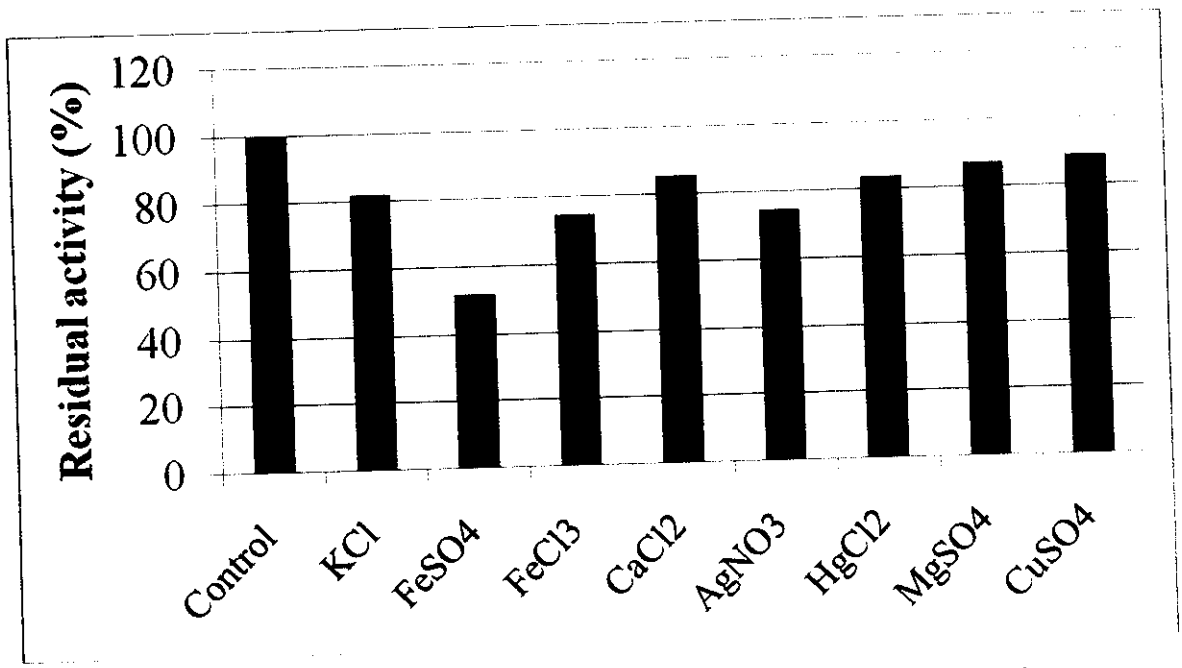


Figure 4.6.2: Effect of metal ions on α-galactosidase from the inner layer of *Aloe vera*

Table 4.7: Effect of sugars on α-galactosidase from outer *Aloe vera*

Table 4.7.1. Outer layer

Sugars	Activity (U)	Relative activity (%)
Control	0.0231	100.0
Lactose	0.0161	69.70
Sucrose	0.0128	55.41
Glucose	0.0082	35.50
Galactose	0.0078	33.77
Raffinose	0.0125	54.11
Ribose	0.0119	51.52
Maltose	0.0138	59.74
Melibiose	0.0103	44.59
Fructose	0.0052	22.51
Arabinose	0.0121	52.38

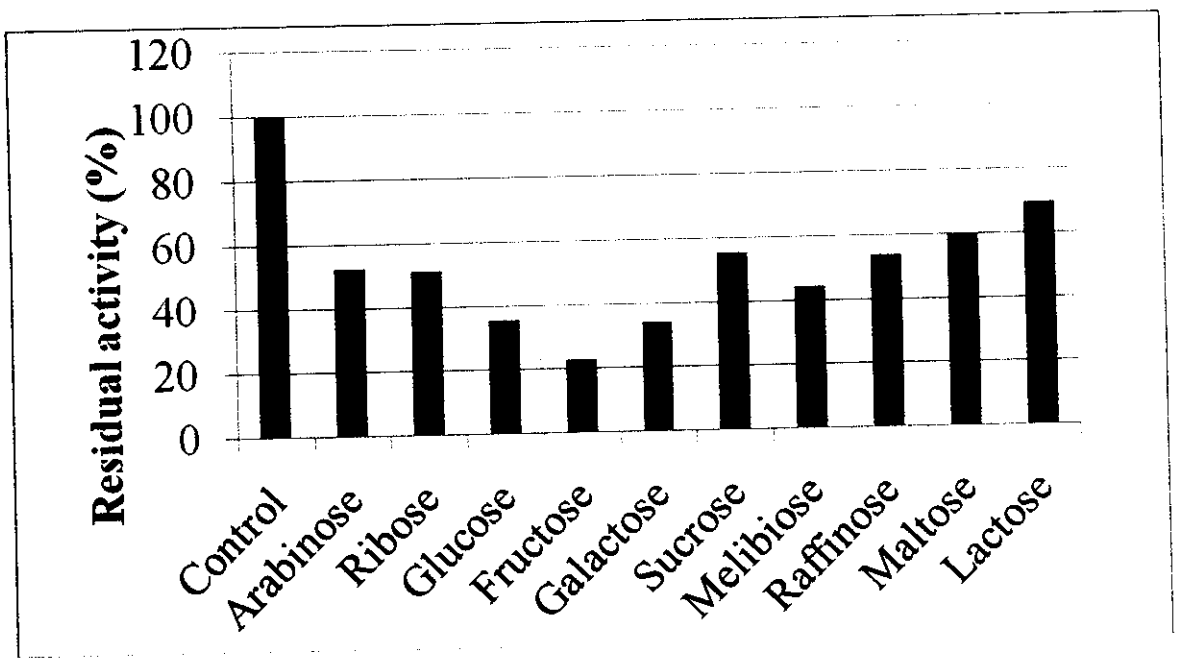


Fig 4.7.1: Effect of sugars on α-galactosidase from outer layer of

Table 4.7.2. Inner layer

Sugars	Activity (U)	Relative activity (%)
Control	0.00406	100.0
Lactose	0.00210	51.72
Sucrose	0.00225	55.42
Glucose	0.00203	50.00
Galactose	0.00203	50.00
Raffinose	0.00239	58.87
Ribose	0.00210	51.72
Maltose	0.00210	51.72
Melibiose	0.00326	80.30
Fructose	0.00232	57.14
Arabinose	0.00188	46.31

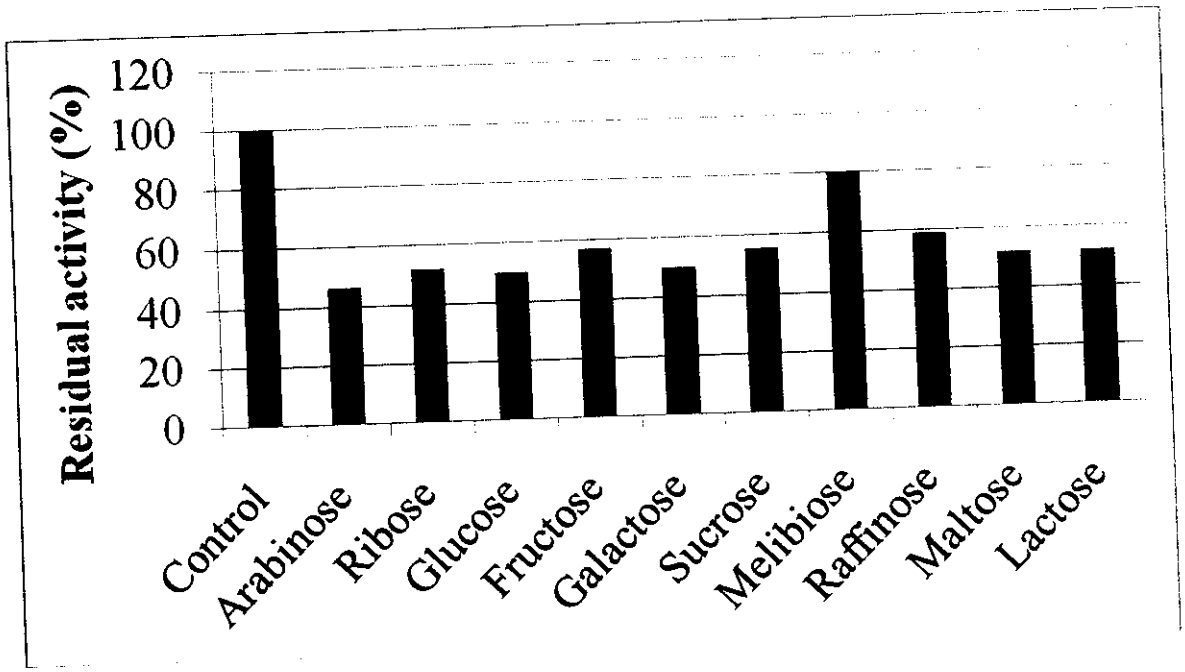


Figure. 4.7.2. Effect of sugars on α-galactosidase from inner layer of

Table 4.8. Effect of reagents on α-galactosidase from *Aloe vera*

Table 4.8.1. Outer layer

Reagents	Activity (U)	Residual activity (%)
Control	0.0231	100.0
1,10-phenanthroline	0.0113	49.22
EDTA	0.0117	50.78
N-Bromosuccinimide	0.0049	21.21
PMSF	0.0155	67.10
Iodoacetamide	0.0146	63.20
Iodoacetate	0.0147	63.64

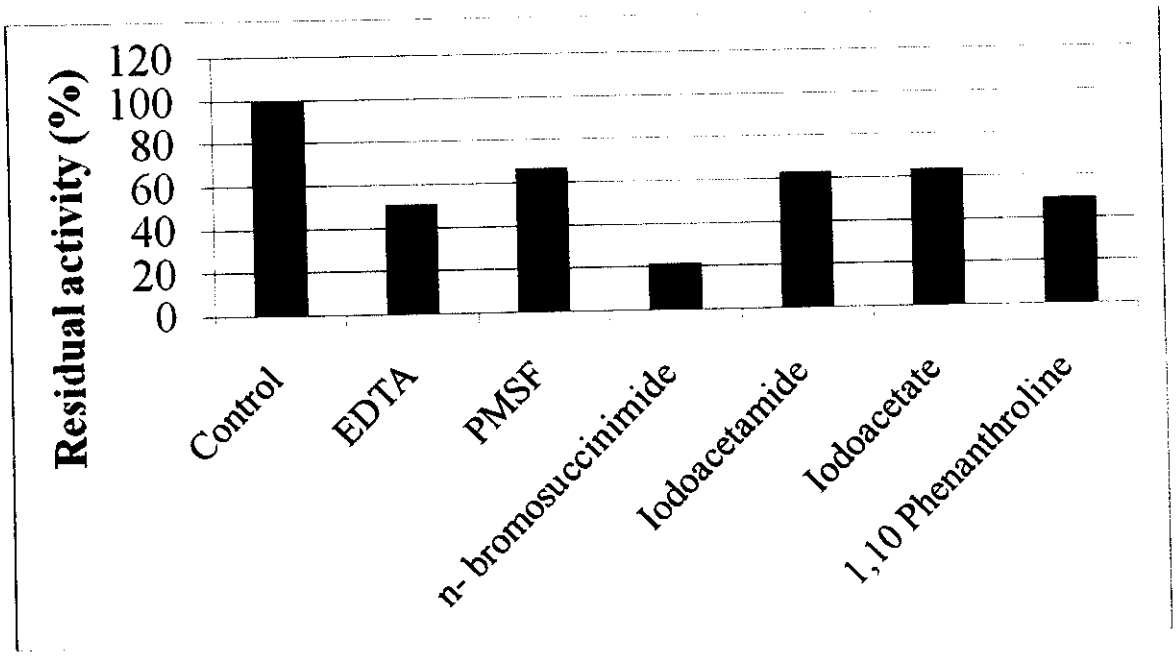


Figure 4.8.1: Effect of reagents on α-galactosidase from outer layer of

Table 4.8.2. Inner layer

Reagents	Activity (U)	Residual activity (%)
Control	0.00406	100.0
1,10-phenanthroline	0.00326	80.30
EDTA	0.00355	87.44
N-Bromosuccinimide	0.00043	10.59
PMSF	0.00333	82.02
Iodoacetamide	0.00370	91.13
Iodoacetate	0.00333	82.02

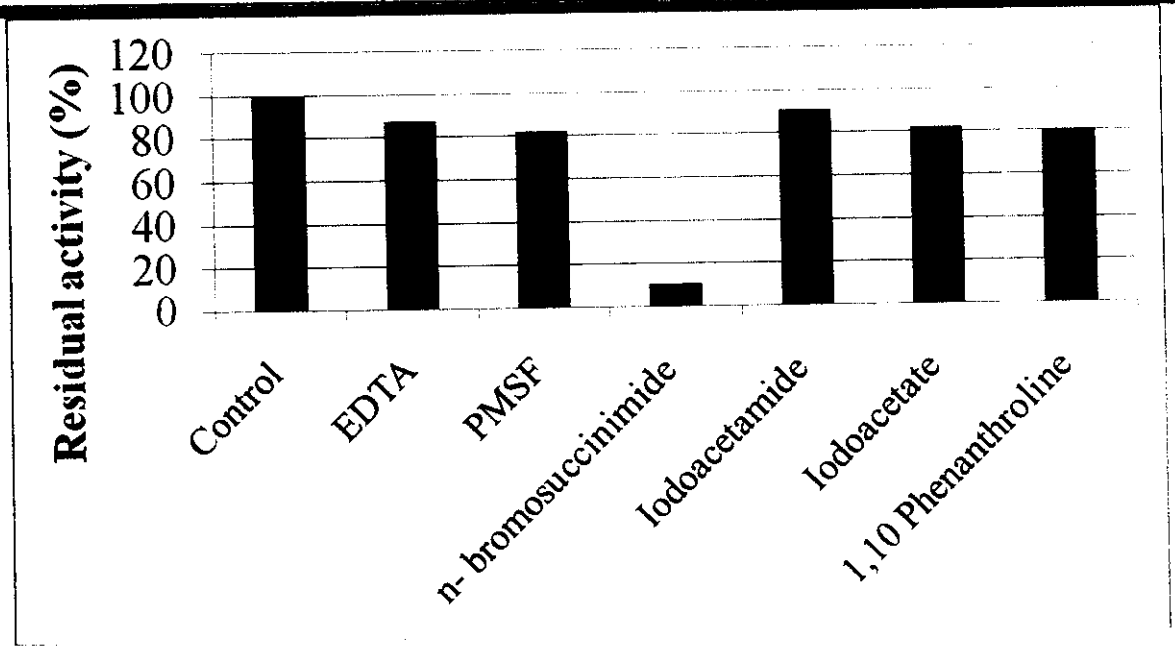


Fig. 4.8.2: Effect of reagents on α-galactosidase from inner layer of *Aloe vera*

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

Table 4.9.1.Outer layer

Substrate Concentration , S (mM)	Activity (U)	1/S	1/V
0.33	0.00580	3.03	172.41
0.66	0.00942	1.51	106.16
0.99	0.01290	1.06	77.52
1.32	0.01507	0.75	66.36
1.65	0.01522	0.60	65.70
1.99	0.01667	0.50	59.99
2.33	0.01696	0.42	58.96
2.66	0.01725	0.37	57.97
2.99	0.01841	0.33	54.32
3.33	0.01884	0.30	53.08

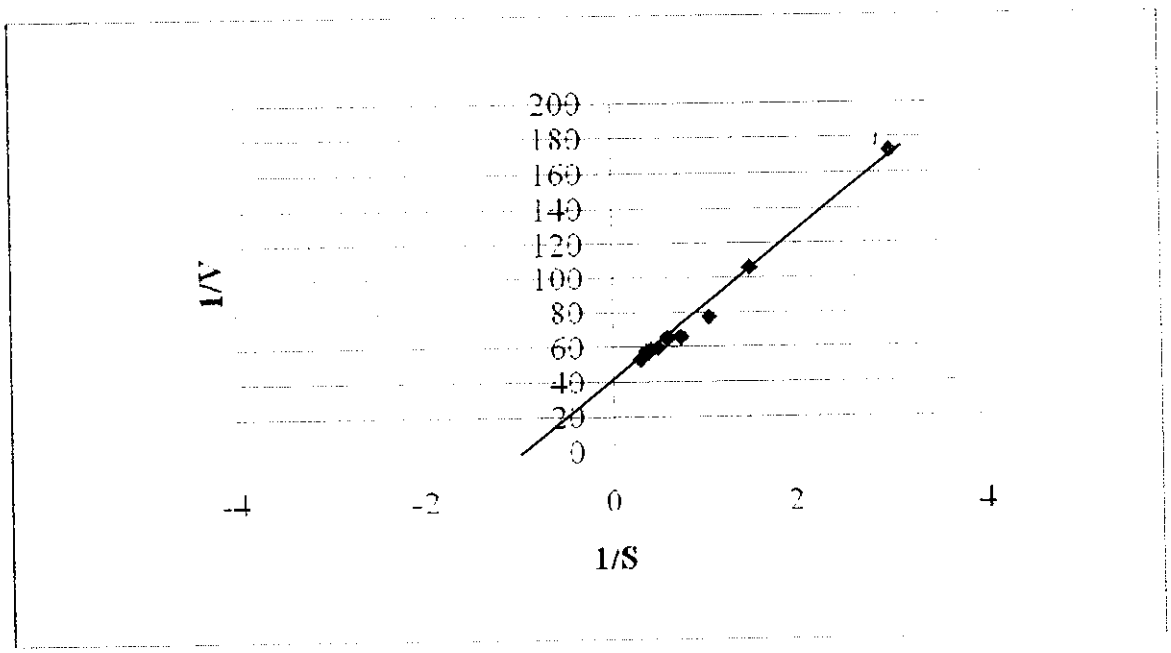


Figure 4.9.1: Effect of varying substrate concentration (PNPG)

Table 4.9.2.Inner layer

Substrate Concentration , S (mM)	Activity (U)	1/S	1/V
0.33	0.00232	3.03	431.03
0.66	0.00246	1.51	406.50
0.99	0.00261	1.06	383.14
1.32	0.00275	0.75	363.64
1.65	0.00348	0.60	287.36
1.99	0.00362	0.50	276.24
2.33	0.00377	0.42	265.25
2.66	0.00391	0.37	255.75
2.99	0.00420	0.33	238.10
3.33	0.00449	0.30	222.72

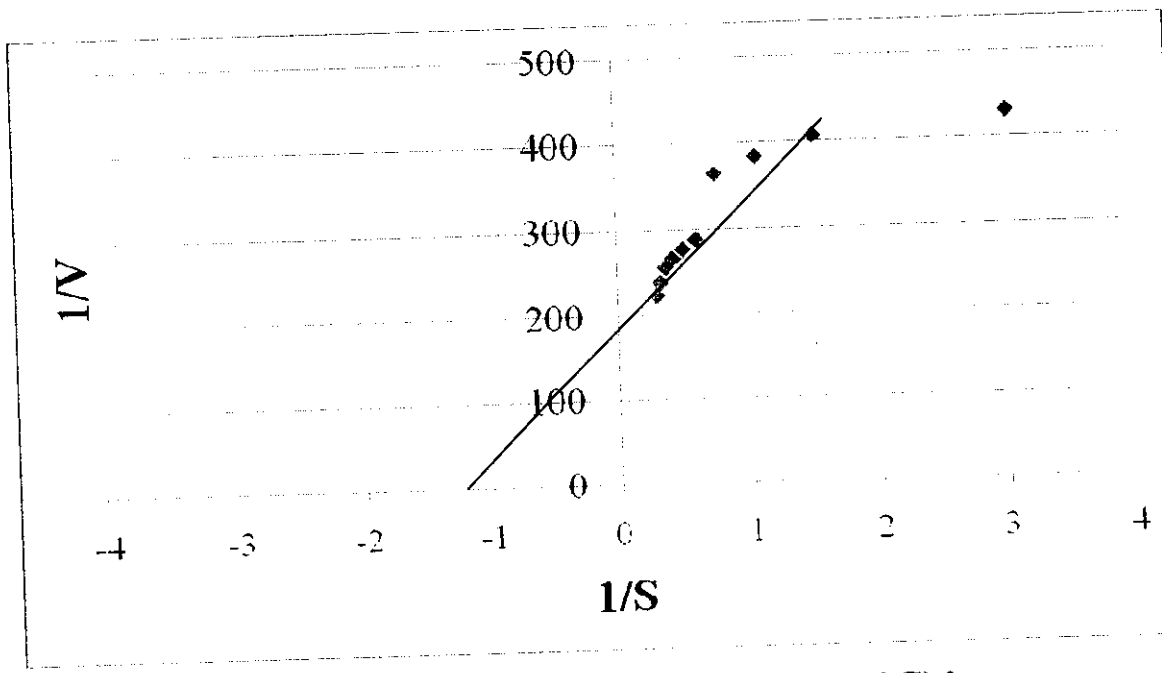


Figure 4.9.2: Effect of varying substrate concentration (PNPG) inner layer of *Aloe vera*

SUMMARY & CONCLUSION

The α-galactosidase from the outer and inner layers of *Aloe vera* 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-flavour makes soymilk unfit to human consumption. Thus, α-galactosidase from *Aloe vera* can be used in various food processing industries to improve the nutritive value of food products.

REFERENCES

1. Agarry O.O., Olaleye M.T., and Bello-Michael C.O. (2005). Comparative antimicrobial activities of *Aloe vera* gel and leaf. African Journal of Biotechnology 4 ; 1413-1414.
2. Akiba, H., Ohishi, N., & Yagi, K. (1976), Properties of α- galactosidase in alkalophilic bacteria, Current microbial., 55; 129
3. Akira Ohtakara & Gold, P. (1984), Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus, Agri.biol.chem* 48; 1319.
4. Andre, Pierre & Sandrene, KL (2001), Influence of carbohydrates on α-galactosidase from *Pleurotus florida*, Appln Microbiol & Biotechnol, 92; 132
5. Grace Sathyanesan Anisha, Rajeev Kumar Sukumaran and Parukuttyamma Prema (2008), α-Galactosidase Production by *Streptomyces griseoloalbus*, Food Technol. Biotechnol. 46 ; 171–177
6. Baruah, Parukutty and Sharma, G.C (1984),Studies on medicinal uses of the plants by Boro tribals of Assam .II. J.Econ.Taxon.Bot. 53 ; 599-604.
7. Bennett, C. & Richard, K. (1997), use of Fungi in Biodegradation. In: Environmental Microbiology, *Env Sci Technol.*, 35; 59A-62A
8. Blanchard & Albon (1950), Transferase properties of α- galactosidase from yeast, Appl. Microbiol & Biotechnol, 56; 73-79
9. Bourquelot & Bridel (1910), Isolation of verbascose from *Verboscum thapus*, J. of Indl. Microbiol. & Biotech, 26; 29
10. Bruneton J.,(1995), Pharmacognosy, phytochemistry, medicinal plants. Lavoisier, Paris.
11. Carol Mc.Cutchen & Bulpin, K. (1996), characterization of extremely thermostable enzymatic breakers (α-1,6-galactosidase and ß-mannanase) from the hyperthermic bacterium *Thermotoga neapolitana* 5068 for hydrolysis of guar gum., *Biotech & Bioengg*, 52; 332.

12.Carmi, Nir, Zhang, Genfa, Petreikov, Marina, Gao, Zhifang, Eyal, Yoram, Granot, David, Schaffer, & Arthur A(2003), Cloning and functional expression of alkaline [alpha]-galactosidase from melon fruit: similarity to plant SIP proteins uncovers a novel family of plant glycosyl hydrolases. Plant journal 33 ; 97-106

13.Clark (1949), Bioremediation & biodegradation, Env. Sci. Technol., 31; 75-79

14.Clarke & Miller (2000), comparison of enzyme aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase, *J. of Indl. Microbiol. & Biotech,* 25; 102

15.Collin C.(1935), Roentgen dermatitis treated with fresh whole leaf of Aloe vera. Am J Roe 33 ; 396-7 in 23.

16.Cruz & Park (1982), Hydrolysis of soymilk, Appl. Biol. Chem. 17; 152-155

17.Dai N, Petreikoy M, Portnoy M, Katzir N, Pharr DM, Schaffer AA (2006), Cloning and Expression Analysis of a UDP-galactose/glucose/ Pyrophosphorylase from Melon Fruit Provides Evidence for the Major Metabolic Pathway of galactose Metabolism in Raffinose Oligosaccharide Metabolizing Plants. Plant Physiol.

18.Davis RH., DiDonato JJ., Johnson RW (1994), Anti-inflammatory and wound healing of growth substance in *Aloe vera.* J Am Pediatr Med Assoc 84 ;77-81.

19.Dr Peter Atherton, (1997).The Essential *Aloe Vera*, the Actions and the Evidence, 2nd Edition.

20.Grace Sathyanesan Anisha, Rajeev Kumar Sukumaran and Parukuttyamma Prema (2008), α- galactosidase Production by *Streptomyces griseoloalbus,* Food technol. Biotechnol. 46 ; 171-177

21.Dey & Pridham (1972), Characteristic properties of α- galactosidase

22. Eneyskya, Cruz, F. & Daniel O Hart (1998), production of α-galactosidase by thermophilic fungus *Humicola* sp. In solid state fermantation and its application in soyamilk hydrolysis *Carbohydr.res.*, 305; 83.

23. Falco, Dubois & Fischer, P. (2000), degradation of textile dyes by white-rot fungi *Braz J.chem.eng*, 17; 4.

24. Florence Ledue-Clier, Jean Guy LeBlanc1Martine Bensaada, Graciela Savoy de Giori, Theodora Guerekobaya, Fernando Sesma, Vincent Juillard, Sylvie Rabot, and Jean-Christophe Piard (2008), Ability of *Lactobacillus fermentum* to overcome host a-galactosidase deficiency, as evidenced by reduction of hydrogen excretion in rats consuming soya α-galacto-oligosaccharide . BMC Microbiol. 8 ; 22

25. Fransworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D and Guo, Z.G. (1985). Medicinal plants in therapy. *Bull.* WHO. 63 ; 965-981.

26. Fridjonson, Paller, K.S., Moiki & Ferris (1999), Thermostability studies of α- galactosidase in *Thermus brockiansus*, Mol.biol,biophy, 13; 178-19

27. Garro, P. & Dey, O (1993), characterization of α-galactosidase from *Lactobacillus fermentum. J.Appln. Bacteriol*, 75; 485.

28. Ganteral, Tan,D.X., Manchester, L.C & Calvo J.R. (1988), Hydrolysis of raffinose by α- galactosidase in *Pleurotus eous*, Biochem, 120; 175

29. Geeta Ramachandra, B.Bhaskar, & T.K.Virupaksha (1989), a-galactosidase of Germinating seeds of Cassia Sericea sw Food.Biochem.14 ; 45-59

30. Goulas T, Goulas A, Tzortzis G, & Gibson GR(2009), A novel alpha-galactosidase from Bifidobacterium bifidum with transgalactosylating properties: gene molecular cloning and heterologous expression. Appl. Microbiol. Biotechnol.

31.G.Tzortzis & Gitezelmann, R. (2003), synthesis of α-galactosidase from *Lactobacillus reuteri* of canine origin, *Appl. Microbiol. Biotechnol*, 63; 286.

32.G.Tzortzis et al. (2004), *in vitro* evaluation of the fermentation prperties of galactooligosaccharides synthesised by α-galactosidase from *Lactobacillus reuteri*, *Appl. Microbiol. Biotechnol*, 64; 106.

33.Haller JS (1990), A drug for all seasons, medical and pharmacological history of aloe. Bull N Y Acad Med. 66 ; 647-59.

34.Hansel R., Keller K., Rimpler H., Schneider G., eds (1992) Hagers Handbuch der Pharmazeutischen Praxis, 5. Auflage, Bd 4, Springer - Verlag, Berlin, Heidelberg, New York, 213-23.

35. Han Zhao, Lili Lu, Min Xiao, Qinpeng Wang , Yu Lu, Chunhui Liu , Peng Wang , Hidehiko Kumagai & Kenji Yamamoto, Cloning and characterization of a novel α-galactosidase from Bifidobacterium breve 203 capable of synthesizing Gal-a-1,4 linkage. FEMS Microbiology Letters, 285 ; 278-283

36.Hajime Shibuya & Kobayashi, K. (1998), cloning and high level expression of α-galactosidase cDNA from *Penicillium purpurogenum*, *Applied & Envt. Microbial*, 64; 4489.

37.Hutchings A., Scott AH., Lewis G. (1996), Zulu Medicinal Plants. University of Natal Press, Scottsville, South Africa, 33.

38.Hu Y., Xu J., Hu Q (2003),Evaluation of antioxidant potential of aloe vera(*Aloe barbadensis miller*) extracts. J.Agric.Food Chem. 51 ; 7788-7791.

39.Jasso de Rodriguez D., Hernandez Castillo D., Rodriguez Garcial R. And Angulo Sanchez.(2005), Antifungal activity of *Aloe vera* gel and juice against plant pathogenic fungi.Industrial crops and Products.21 ; 81-87

40.Jean Guy LeBlanc, Aurelio Silvestroni, Cristelle Connes, Vincent Juillard, Graciela Savoy de Giori, Jean-Christophe Piard and Fernando

Sesma(2004), Reduction of non-digestible oligosaccharides in soymilk: application of engineered lactic acid bacteria that produce α-galactosidase. Genet. Mol. 3; 432-440

41.Kenji Sakai & Hyomowitz (1987), hydrolysis of a-D-galactosyl oligosaccharide in soymilk by α-D-galactosidase of *Bifidobacterium breve* 203.*Agri.Biol.Chem*, 51; 315.

42.King (1989), Biodegradation of Industrial waste, Env. Sci. Technol., 15; 91-93

43.Kobat & Lestkowitz (1995), Role of α- D galactosyl group in blood group, Biochem 12; 1021-1029

44.Kotwal, Dey & Pridham (1999), purification and characterization of extracellular α-galactosidase from thermophilic fungus *Humicola* sp. *J. of Indl. Microbiol. & Biotech*, 23; 661.

45.Kotwal, Dey & Pridham (1999), purification and characterization of a constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp. *J. Biochem, Molbiol, biophy*, 3; 9.

46.Kotwal, Dey & Pridham (2000), chemical modification of a-galactosidase from the thermophilic fungus *Humicola* sp. *Biochem, Mol.biol, biophy*, 4;665.

47.Kotwal, Yang, Y enRoque, P. (1998), Application & Purification of α-galactosidase in *Pleurotus eous*, Appl. Biol. Chem. 152; 172

48.Kritikar, K.R., Basu, B.D. (1911), Indian Medicinal plants Vols.I-IV. Bishen Sing Mahendra Pal Sing, Dehradun, India

49.LeBlanc.JG & Kay, L. (2004), reduction of non-digestible oligosaccharides in soymilk and application of engineered lactic acid bacteria that produce α-galactosidase, *Appln Microbiol. & Biotechnol*, 65; 119.

50.Mangala.A.Nadkarni et al. (1992), characterization of α-galactosidase from *Corynebacterium murisepticum* and mechanism of its induction, *J.Gen. Appl. Microbiol*, 38; 23.

51.Mc cutchen, Robak, J. & Gryglewski. (1996), Thermal stability of α-galactosidase, Mol. biol, biophy, 131; 138

52.Mital, M & Kabet, K. (1979), the enzymatic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from *Gibberella fujikuroi. J. of food protection*, 42; 895.

53.Mitsunori Ishiguro & Cristafaro (2001), Applications of oyster mushrooms in environmental biotechnology, *Appl. & Envtal microbiol.*, 67; 1601.

54.M.M. Gote, M.I. Khan, D.V. Gokhale, K.B. Bastawde and J.M. Khire (2006), Purification, characterization and substrate specificity of thermostable α-galactosidase from *Bacillus stearothermophilus* (NCIM-5146), Process.biochem, 41; 1311-1317

55.Mudgett, Gary, L. Joshua,H (1984), Characterisation of a-galactosidase from *Lactobacillus fermentii*, Biochem, 11; 34

56.Mulimani & Thippeswamy (2002), the enzymatic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from *Gibberella fujikuroi, Process biochem.*, 38; 635.

57.Murphy (1973), Inhibition of flatus production by addition of antibiotics with leguminous seed preparations, Agri. Biol. Chem, 56; 247

58.Nadkarni, Sai, M, & Sena, S (1992), Characterization of α-galactosidase from *Cornybacterium murisepticum*, Cell Biochem Funct, 10; 457

59.Nurten Ozsoy (2003), production and characterization of α-galactosidase from *Aspergillus flavipes, Cell Biochem, Funct.*, 21; 387.

60.Oda.Y (1996), the enzymatic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from *Gibberella fujikuroi.J. Appl. Bacteriology*, 80; 203.

61.Olafur Fridjonsson & Li (1999), production and characterization of α-galactosidase from *Aspergillus flavipes*, *FEMS Microbiol. Letters*, 176; 147.

62.Olafur Fridjonsson & Li (1999), cloning of the gene encoding a novel thermostable α-galactosidase from *Thermus brockianus, Appl. & envtal microbial*, 65; 3955.

63.Orafidiya L.O., Agbani E.O., Oyedele O.O., Babalola O.O., Onayemi O., & Aiyedun F.F. (2004), The effect of *Aloe vera* on the anti-acne properties of the essential oil of *Ocimum gratissimum* Linn leaf- a preliminary clinical investigation. International Journal of Aromatherapy. 14 ; 15-21

64.Pierre Marraccini, W. John Rogers, Victoria Caillet, Alain Deshayes, Dominique Granato, Françoise Lausanne, Sylvianne Lechat, David Pridmore and Vincent Pétiard (2005), Biochemical and molecular characterization of *α*-d-galactosidase from coffee beans.Plant physiology and Biochem 43 ; 909-920

65.Puchart, Suhaila, M., Hassan, A.A. & Jayamalar, P. (2000), Hydrolysis of raffinose by α- galactosidase in *Pleurotus florida*, Biotech & Bioengg, 510; 522

66.Rackis & Pierre Richard (1975), Production of a-galactosidase from *Thermus brockianus , Physiological effects of food carbohyd.*, 15; 207.

67. Ramalingam & Mulimani (2004), Purification and properties of alpha-galactosidase from *Aspergillus oryzae*, J. Micribiol. Biotechnol, 11, 534.

68.Richard, Morris, H. & Jani, D.C (1983), Production of a-galactosidase from *Thermus brockians*, Physiological effects of food carbohyd., 19; 51

69. Rolfe J,Bryant Damann R, & Rao (2009), Purification and characterization of α-galactosidase from Peanuts, Food.Biochem,25 ; 139-156

70. Sakai, Ohkawa, N, Shiloh, M.U & Moidal (1987), Hydrolysis of soymilk by α- galactosidase of *Bifidobacterium breve*, Appl. Biol. Chem, 17; 112

71. Schuler & Kane (1984), kinetic properties of α-D-galactosidase from *Lactobacillus fermenti*, *Phytochem*,56, 25

72. Schuler, Vaca, C.E., Wilhelm, J. & Harms, M. (1993), Hydrolysis of soymilk by α- galactosidase in *Pleurotus eous*, Appl. Biol. Chem. 15; 73-75

73. Sebastiao Tavares de Rezende, Valeria Monteze Guimaraes, Marilia de Castro Rodrigues, & Carlos Roberto Felix(2005), Purification and characterization of an α-galactosidase from *Aspergillus fumigates,* Braz. arch. biol. Technol, 48; 2

74. Shabalin , K.A., Kulminskaya, A.A., Savel`ev, A.N., Shishlyannikov., and Neustroev, K.N. (2002), Hydrolysis of galactooligosaccharides by α-galactosidase from *Trichoderma reesei* , Enzyme Microbiol. Technol ; 30, 231.

75. Shibuya et al. (1998) cloning and high level expression of α-galactosidase cDNA from *Penicillium purpurogenum*, *Current microbial.*, 35; 129

76. Spangenberg, Kijneau, K. & Dereck, M. (1999), Characterization, Applications of α- galactosidase from *Pleurotus eous*, Appln Microbiol & Biotechnol, 92; 148

77. Sripuan & Rao (2003) purification and characterization of thermostable α-galactosidase from *Ganoderma lucidum.*, *J.Food Sci.*, 41; 173.

78. Staggerda, Alexander, D. & Hart, M. (1966), Flatulation due to fermentation of sugars, Appln . Microbiol & Biotechnol, 24; 52

79. Teruhiko akiba et al. (1976) properties of α-galactosidase of alkalophilic bacteria, *Agr. Biol. Chem*, 40; 1851.

80.Thananaunkal, Keller, N. & Foster, J (1976), Hydrolysis of soymilk by enzymes from Oyster mushrooms, Appl. Biol. Chem, 40; 1851

81.Thida Sripuran et al. (2003), *BioSci, Biotechnol, Biochem.*, 67, 1485.

82.Timothy Durance & Brent Skura(1984), Purification and characterization of a CIostfidium perfringens α-galactosidase Food.Science 50; 518-522

83.Ulezlo & Zaprometora (1982), Deficiency of α- galactosidase A-Fabry disease, Appln . Microbiol & Biotechnol, 17; 92

84.Valerie A.Ferro, Fiona Bradbury, Pamela Cameron, Eisin Shakir, Sabita R. Rahman and William H. Stimson (2003), Invitro susceptibility of *shigella flexneri*, *streptococcus pyogenes* to inner gel of Aloe barbadensis Miller. Antimicrobial Agents And Chemotherapy 47 ; 1137-1139

85.Van den Broek & Lowry (1999), synthesis of a-galactosidase oligosaccharides by a cloned α-galactosidase from *Bifidobacterium adolescentis, Biotech. Letters*, 21; 441.

86.Van Laera, Billy, N & Joseph, J (1999), α- galactosidase activity in high substrate concentration, Braz .J. Chem. engg, 20; 26-31

87.Varbarnels, Mohly & Friere (2001), Purification, Characterisation of α-galactosidase from *Lactobacillus fermentii*, Phy chem, 57; 17

88.Visuthikosol V., Chowchuen B., Sukwanarat YJ.(1995),Effect of Aloe vera gel on healing of burn wounds: A clinical and histological study. J Med Assoc Thai 78 ; 403-409

89.Xiao, T. & Harumi Kyaski (2000), high yield production and characterization α-galactosidase from *Bifidobacterium breve* grown on raffinose, *Biotech. Letters*, 22, 747.

90.Xiu-feng Zhang, Hong-mei Wang, Yuan-li Song, Li-hua Nie, Lan-fen Wang, Bin Liu, Ping-ping Shen and Yang Liu (2006), Isolation, structure elucidation, antioxidative and immunomodulatory properties of two novel dihydrocoumarins from *Aloe vera* .Bio organic and medicinal chemistry Letters 16 : 949-953

91.Yagi A., Takeo S.(2003), Antiinflammatory constituents, aloesin and aloemannan in *Aloe* species and effects of tanshinon VI in Salvia miltiorrhiza on heart. Yakugaku Zasshi. 123 ; 517321

92.Zeilinger, Sun, Y. Stark, A & Madar, Z. (1993), Biodegradation & Bioremediation, I.biosci, 51;53

93.Zhu, Rhmm, E.B., Stampfer, B. & Willet, W.C (1995), Medical application of α- galactosidase, Mol. Biol, 70; 75