PURIFICATION, CHARACTERIZATION, AND HYDROLYTIC ACTIVITY OF ALPHA-GALACTOSIDASE FROM LACTOBACILLUS HELVETICUS ATCC 10797

By

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Your file Votre référence
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DEDICATION

To My Father, Hassan, My Mother, Mariam, who supported me with love and prayers

To My Husband, Salem And My Son Abdullah

To My Family And Friends

ABSTRACT

In this study, α-Galctosidase (α-D-galactoside-galactohydrolase, EC 3.2.1.22) has been isolated, purified, and characterized from intracellular fraction of *Lactobacillus helveticus* ATCC 10797. It was purified by a combination of ammonium sulfate precipitation and fast performance liquid chromatography system using ion exchange and gel-filtration columns. This enzyme was purified to only 9.44 fold over the crude extract with a recovery of 1.8%. The km of 3.83 mM and Vmax of 416.44 μmol/min/mg protein were calculated from PNPG. The molecular mass was estimated to be 188 kDa by gel-filtration, but 90 kDa by SDS-PAGE, indicating two similar molecular weight subunits. The optimum temperature for enzyme activity was 37 °C, but with a stability below 30 °C. The optimum pH was at 6 with a stability of pH 4-8 range.

This enzyme was activated by 10 mM monovalent ions such as K^+, NH_4^+, Li^+ and CS^+, while the activity was inhibited by divalent ions such as Cu^{+2}, Zn^{+2}, Fe^{+2}. About 40% of the enzyme activity was inhibited with 100 mM EDTA. α-Galactosidase was inhibited by 1mM glucose and galactose, 10 mM sucrose, high concentrations of melibiose, or raffinose and stachyose, but the least inhibitory effect was shown with fructose.

When the sugars were incubated with α-galactosidase, melibiose was hydrolyzed to glucose and galactose, raffinose to galactose and sucrose, while stachyose to galactose and sucrose with raffinose as intermediate product.

RÈSUMÈ

Dans cette étude, l'enzyme α-Galctosidase (α-D-galactoside-galactohydrolase, EC 3.2.1.22) a été isolée, purifiée, et caractérisée à partir de la fraction intracellulaire de *Lactobacillus helveticus* ATCC 10797. Elle a été purifié par une combinaison de précipitation de sulfate d'ammonium et de système rapide de chromatographie liquide en utilisant des colonnes échangeur d'ions et de gel-filtration. Cette enzyme a été purifiée seulement à 9.44 fois par rapport à l'extrait brut avec un recouvrement de 1.8 %. Le Km de 3.83 mM et un Vmax de 416.44 μmol/min/mg de protéine ont été calculé à partir du PNPG. La masse moléculaire a été estimée à 188 kDa par gel-filtration mais de 90 kDa par le gel SDS-PAGE, indiquant deux sous-unités de poids moléculaire semblable. La température optimale pour l'activité enzymatique était de 37°C, mais avec une stabilité en-dessous de 30 °C. Le pH optimum était de pH 6 avec une stabilité entre pH 4-8. Cette enzyme a été activée par 10 mM des ions monovalents K^+, NH_4^+, Li^+ et CS^+, alors que l'activité était inhibée par les ions bivalents Cu^{+2}, Zn^{+2}, Fe^{+2}. Environ 40 % de l'activité enzymatique a été inhibée avec de l'EDTA 100 mM.

La α-galactosidase a été inhibée par 1mM de glucose et de galactose, 10 mM de sucrose ainsi que des concentrations élevées de melibiose, de raffinose ou de stachyose, mais le fructose démontrait un moindre effet inhibiteur. Quand les sucres ont été incubés avec la α-galactosidase, le melibiose a été hydrolysé en glucose et en galactose, le raffinose en galactose et sucrose, alors que le stachyose était transformé en galactose et en sucrose avec du raffinose en tant que produit intermédiaire.

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In the name of Allah, the most gracious, the most merciful. Praise to Allah the almighty and only God that provided me with everything including health and knowledge.

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GENERAL INTRODUCTION

α-Galactosidase (EC 3.2.1.22, α-D-galactoside galactohydrolase), which is commonly known as melibiase, is found in most lactic acid bacteria (LAB) such as *Lactobacillus* and B*ifidobacterium* species. Not only is it capable of hydrolyzing the α-1,6-glycosidic linkage of galactosides releasing galactose, but it also possesses the transgalactosylation activity to synthesize galacto-oligosaccharidesgosaccharides. Various food industries routinely apply both reaction activities. As a result, the unique properties of α-Galactosidase are readily studied and exploited by the food industry.

One of the major microflora to be found in the human gastrointestinal (GI) tract is LAB. Many dairy and pharmaceutical products that contain LAB have been developed and consumed for a long time due to the health benefits they promise. Some of the most important health benefits they offer include the reduction of harmful bacteria and toxic compounds in the intestine, prevention of dental decay, reduction of total cholesterol and lipid in serum, and relief of constipation. These live probiotic bacteria, which are known to improve the microbial balance of the human GI tract, have been used for a long time as a health-beneficial supplement to dairy products. LAB can also be used to promote the number of beneficial bacteria in the human intestine. By supplying growth factors such as oligosaccharides, it is possible to stimulate the growth of beneficial bacteria in the human intestines. It has been shown that so-called Prebiotics such as Galactooligosaccharides induce this growth stimulating effect on probiotic bacteria.

The primary objectives of this research included purification and characterization of α-galactosidase from *Lactobacillus helveticus* and analyzing the hydrolytic activity on different substrates; melibiose, raffinose, and stachyose which are the most significant flatulence causing soy sugars.

CHAPTER 1

LITERATURE REVIEW

ALPHA-GALACTOSIDASE

1.1 ALPHA-GALACTOSIDASE: GENERAL INFORMATION

1.1.1 Terms and Nomenclature

The hydrolases (EC 3) are proteins that catalyze the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. The E.C. classification for these enzymes generally classifies them firstly by the nature of the bond hydrolyzed, then by the nature of the substrate, and lastly by the enzyme.

Glycosylases (EC 3.2) are classified under hydrolases. Some of them can also transfer glycosyl residues to oligosaccharides, polysaccharides and other alcoholic acceptors. The glycosylases are subdivided into those hydrolysing *O*- or *S*-glycosyl compounds (EC 3.2.1 and EC 3.2.3), i.e. glycosides, and those hydrolysing *N*-glycosyl compounds (EC 3.2.2).

Alpha-galactosidases (EC 3.2.1.22) (Dey and Pridham, 1972) are proteins that hydrolyse the terminal α-1,6 linkages (Cristofaro *et al.*, 1974) of non reducing α -galactose residues in α -D-galactosides.

Since the systematic name of the enzyme always includes hydrolase, alpha-galactosidase has α-D-galactoside galactohydrolase (Dey and Pridham, 1972) as a

systematic name. The recommended name is formed by the name of the substrate with the suffix -ase. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme. Based on these information, melibiase is the recommended name for this enzyme because it breaks down melibiose. Also, alpha-galactosidase has other names as, α-D-galactosidase and α-galactosidase A.

1.1.2 Substrates

α-Galactosidase has a wide range of substrate specificities including galactomannans, glycoconjugates (McCleary and Matheson,1974; Davis *et al.*,1997), raffinose (β-D-fructofuranosyl-O-α-D-galactopyranosyle-1,6-α-D-glucopyranoside), stachyose (galactopyranosyle-1,6-α-D-glucopyranoside-1,6-α-D-glucopyranoside), (Steggerda *et al.*, 1996) and melibiose (Dey and Pridham, 1972) (6-O-α-D-galactosypranosyl-D-glucose) (Tzortzis *et al.*, 2004).

Although it is well known that α-galactosidase breaks down smaller sugars as raffinose faster than higher sugars, there are studies showing the opposite (Dey, 1981).

P-nitrophenyl-α-D-galactopyranosdie (PNP-α-Gal) is the synthetic substrate to check the enzyme activity *in vitro*. In a study performed on the ability of the enzyme isolated from *Thermoaerobacterium polysaccharolyticum* (King *et al.*, 2002) to break down p-nitrophenol analogue substrates; α-D-galactopyranoside, β-D-galactopyranoside, β-D-glucopyranoside, β-D-lactoside, β-D-maltoside, and β-D-xylopyranoside, there was no activity because α-galactosidase is specific for the α-1,6-galactosyl bonds.

1.2 HYDROLYTIC ACTIVITY

1.2.1 Mechanism of action of alpha-galactosidase

α-Galactosidase hydrolyzes α-1,6 linkages of α-galactosides (Figure 1.1) producing galactose. The mechanism of hydrolytic activity of α-galactosidase from sweet almond (Dey, 1969) and *Vicia faba* (Dey and Pridham, 1969b) showed that a carboxyl group and an imidazole group are involved in the catalysis and two step mechanism has been proposed based on the results (Dey, 1969). The aglycon is split by the action of both carboxyl and imidazolium groups at the enzyme active site and it is followed by the intake of the acceptor molecule (R`OH) which can be water or sugar, leading to the formation of the final product through a transition state (Figure 1.2).

Another study done by Mathew and Balasubramaniam (1987) on the chemical modification of α-galactosidase showed the presence of two carboxyl groups; tyrosine and tryptophan with the absence of an imidazole group at or near the active site of α-galactosidase. Thus a new mechanism was proposed based on the chemically modified enzyme as shown in (Figure 1.3).

Swain and Brown (1952) demonstrated that a multifunctional catalyst was much more efficient than a mixture of compounds having the same catalytic group.

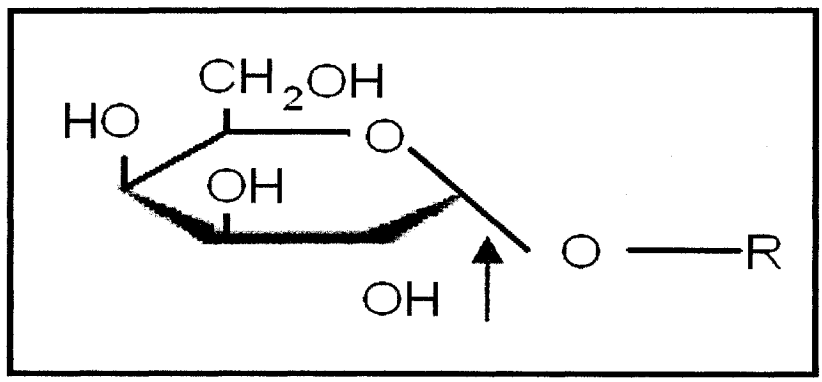
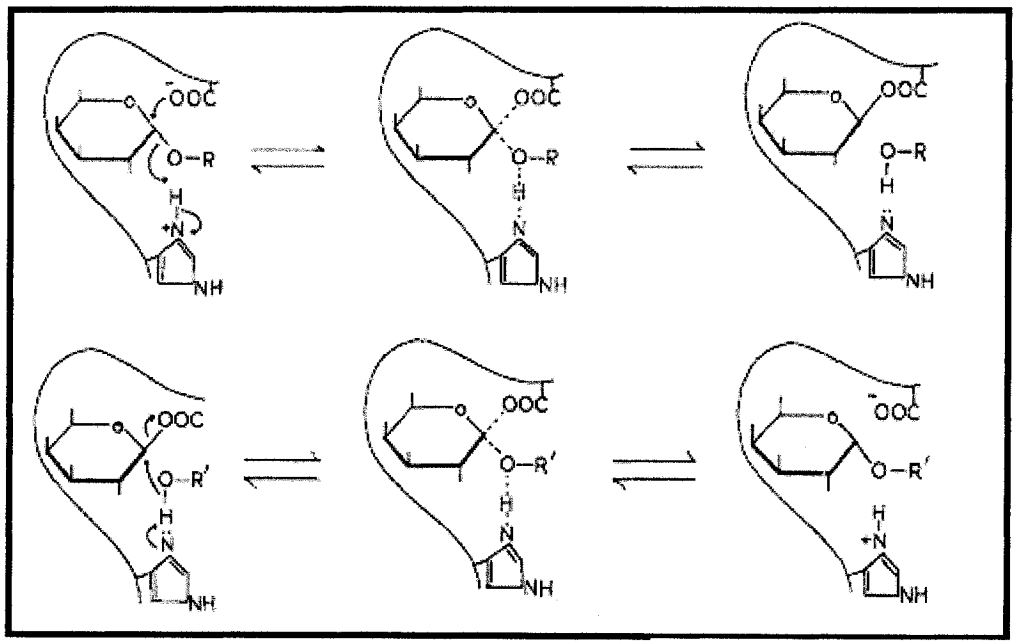
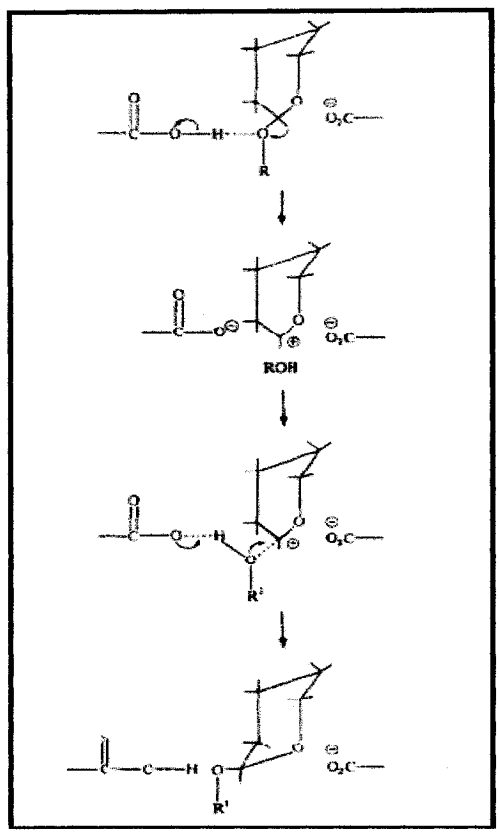


Figure 1.1 General structure of α-galactoside showing α-galactosidase cleavage point (Arrow).



Adapted from; Dey, 1969

Figure 1.2 Two-step mechanism of action of α-galactosidase.



Adapted from; Mathew & Balasubramani,1987

Figure 1.3 Mechanism of action of chemically modified α-galactosidase.

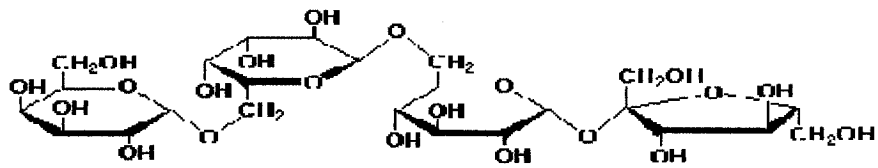
1.2.2 Alpha-galactosidase benefits

Raffinose family oligosaccharides (RFO) consists of a linear chain of galactosyl residues attached to the glucose part of sucrose through α-1,6-glycosidic linkage (Avigad and Dey, 1997). They are considered as antinutritional factor because their α–galactosidic linkages are not digested by humans and monogastric animals (Baucells *et al.*, 2000) due to lack of α-galactosidase responsible for breaking down these bonds.

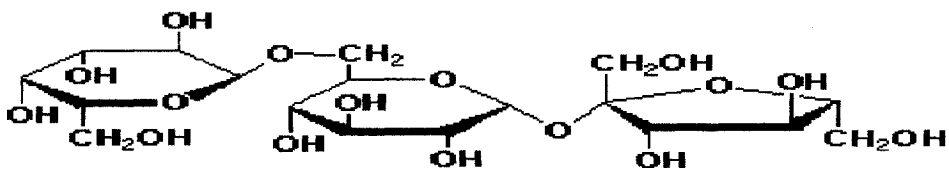
These oligosaccharides as raffinose and stachyose will enter the colon undigested and they will be fermented by anaerobic microorganisms such as *Clostridium* sp. and *Bacteroids* sp. (Rakis, 1981) leading to flatulence due to the production of carbon dioxide, hydrogen and methane (Nnanna and Phillips, 1990).

Flatulence is a very old and serious problem. Even a little increase in the pressure of the rectal gases will lead to a number of symptoms such as headache, dizziness, slight mental confusion, reduced ability to concentrate and slight edema (Cristofaro *et al.*, 1974). Also it might lead to dyspepsia, intestinal constipation and diarrhea (Thananunkul *et al.*, 1976).

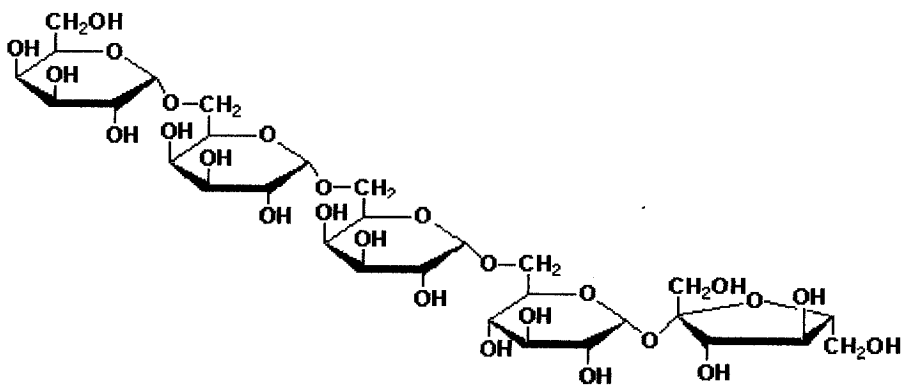
Pretreatment of these food types with α-galactosidase has the ability to take care of this situation (King *et al.*, 2002) by degrading the raffinose family of sugars such as raffinose, stachyose and verbascose (Figure 1.4) in food and feed materials (Mulimani and Ramalingam, 1995).



Stachyose



Raffinose



Verbascose

Figure 1.4 Stachyose, raffinose, and verbascose structure.

1.2.3 Assay methods

A number of methods have been developed to determine α-galactosidase activity using melibiose or other α-galactosides as substrates. The hydrolytic products, glucose and galactose can be determined by colorimetric methods (Dubois *et al.,* 1956) or by specific enzymatic reactions.

A chemically modified substrate (PNP-α-Gal) is commonly used for the determination of α-galactosidase activity. The hydrolysis of PNP-α-Gal at the non reducing end of the α-galactosidic bond, produces galactose and p-nitrophenyl which is yellow in color and absorbs maximally at 420 nm. One unit of α-galactosidase activity is defined as the amount of enzyme producing 1 μmol of p-nitrophenyl per minute under the assay conditions.

Measuring the amount of galactose liberated was compared with the hydrolysis of ortho-nitrophenyl-β-D-galactoside (ONPG). ONPG has replaced PNP-α-Gal by being a control substrate because p-nitrophenyl was an inhibitor of the galactose dehydrogenase used to measure galactose released (Bhalla and Dalling, 1984). Enzymatic activity was also determined from the amount of glucose formed during melibiose hydrolysis using glucose oxidase, peroxidase and chromogen (Hough and Jones, 1962).

Another method used to measure different sugars and oligosaccharides if present at the same time is High Performance Liquid Chromatography (HPLC) with refractive index detector (Prisino, 1983).

Some sugars as melibiose, mannitriose and raffinose like sugars were determined using gas chromatography (Delente and Laden burg, 1972; Cruz *et al*, 1981).

Others (Tanaka *et al,* 1975; Kusakabe *et al,* 1990; Kaneko *et al,* 1991) have used thin layer chromatography (TLC) to detect hydrolysis products of α-galactosidase activity from different substrates.

1.2.4 Sources and characteristics

α-Galactosidases are widely distributed in intra- or extra cellular forms including animals (Ohshima *et al.,*1997), plants (Bulpin *et al.,* 1990), microorganisms (Dey and Pridham, 1969a) such as fungi (Shibuya *et al.,* 1998) and bacteria (Li *et al.,* 1997) are shown in Table 1.1.

High activities have been found in the cytoplasm of epithelial cells of Brunner's glands in the intestine of rats, and in the blood cells as well as in bone marrow of some animals (Koppel *et al,* 1953). Both Gram positive and Gram negative bacteria have been found to produce α-galactosidase activity. Although most of them produce it intracellularly, there are some reports on extracellular activity in some organisms. α-Galactosidase from plant sources have been reported from the seeds (Dey and Pridham, 1969b), leaves and other tissues (Burns, 1990).

α-Galactosidases from different sources have different properties such as pH, temperature, kinetics and activity. Because some people may be allergic to crude enzymes isolated from one source, looking for more sources of α-galctosidases would be useful. In this case, a person allergic to one extract could choose another (Bryant *et al.,* 2004).

Table 1.1 Sources of α-galactosidase.

PLANTS		BACTERIA	
Acer pseudoplatanus	Dey & Pridham, 1972	*Aerobacter aerogenes*	Dey & Pridham, 1972
Brassica oleracea	Dey & Pridham, 1972	*Aspergillus awamori*	McGhee *et al.*, 1978
Cajanus indicus	Dey & Dixon, 1974	*Aspergillus ficuum*	Zapater *et al.*, 1990
Canavalia ensiformis	Dey & Pridham, 1972	*Aspergillus flavipes*	Ozsoy & Berkkan, 2003
Castanea sativa	Dey, 1981	*Aspergillus nidulans*	Rios *et al.*, 1993
Citrullus battich	Itoh *et al.*, 1986	*Aspergillus niger*	Kaneko *et al.*, 1991
Citrullus lanatus	Itoh *et al.*, 1986	*Aspergillus oryzae*	Khare *et al.*, 1994
Citrullus vulgaris	Dey & Pridham, 1972	*Aspergillus paxillus*	Dey & Pridham, 1972
Cocos nucifera	Mujer *et al.*, 1984	*Aspergillus tamarii*	Civas *et al.*, 1984
Coffea arabica	Carchon & DeBruyne, 1975	*Azotobacter vinelandii*	Wong, 1990
		Bacillus sp.	Li *et al.*, 1997
Coffea canephora	Carchon & DeBruyne, 1975	*Bacillus amyloliquefaciens*	Overbeeke *et al.*, 1990
		Bacillus subtilis	Overbeeke *et al.*, 1990
Cucumis sativus	Smart & Pharr., 1980	*Bacillus stearothermophilus*	Friddjonsson *et al.*, 1999a
Cucurbita pepo	Gaudreault & Webb 1983	*Bacteroides ovatus*	Gherardini *et al.*, 1985
Cyamopsis tetragonoloba	Fellinger *et al.*, 1991.	*Bifidobacterium adolescentis*	Leder *et al.*, 1999
Glycine max	Hobbs *et al.*, 1995	*Bifidobacterium breve*	Xiao *et al.*, 2000
Glycine soja	Secova *et al.*, 1988	*Bifidobacterium bifidum*	Hughes & Hoover, 1995
Helianthus annuus	Kim *et al.*, 2003	*Bifidobacterium longum*	Hughes & Hoover, 1995
Hordeum sp.	Dey & Pridham, 1972	*Bifidobacterium angulantum*	Hughes & Hoover, 1995
Hordeum vulgare	Chrost *et al.*, 2004	*Clostridium josui*	Jindou *et al.*, 2002
Lens culinaris	Dey *et al.*, 1983	*Clostridium sporogenes*	Dybus & Aminoff, 1983
Lens esculenta	Dey & Wallenfels, 1994	*Clostridium stercorarium*	Suryani *et al.*, 2003
Lupinus angustifolius	Plant & Moore, 1982	*Corynebacterium murisepticum*	Nadkarni *et al.*, 1992
Lycopersicon esculentum	Bassel *et al.*, 2001	*Diplococcus pneumoniae*	Dey & Pridham, 1972
Medicago sativa	Itoh *et al.*, 1979	*Escherichia coli*	Dey & Pridham, 1972
Ophryoscolex caudatus	Dey & Pridham, 1972	*Geobacillus stearothermophilus*	Pederson & Goodman, 1980
Oryza sp.	Fujimoto *et al.*, 2003	*Lactobacillus acidophilus*	Huges & Hoover, 1995
Oryza sativa	Fujimoto *et al.*, 2003	*Lactobacillus fermentum*	LeBlanc *et al.*, 2004
Phaseolus aureus	Dey & Pridham, 1972	*Lactobacillus lactis*	Leenhouts *et al.*, 1995
Phaseolus vulgaris	Dhar *et al.*, 1994	*Lactobacillus plantarum*	Silvestroni *et al.*, 2002
Pisum sativum	Dey & Pridham, 1972	*Lactobacillus reuteri*	Tzortzis *et al.*, 2004
Prunus amygdalus	Dey & Pridham, 1972	*Lactococcus raffinolactis*	Boucher *et al.*, 2003
Prunus dulcis	Malhotra & Dey, 1967	*Micrococcus sp.*	Akiba & Horikoshi, 1976a
Raphanus sativus	Dey & Pridham, 1972	*Pseudomonas fluorescens*	Halstead *et al.*, 2000
Ricinus communis	Dey & Pridham, 1972	*Ruminococcus gnavus*	Hata & Smith, 2004
Saccharum officinarum	Chinen *et al.*, 1981	*Streptococcus bovis*	Dey & Pridham, 1972
Spinacia oleracea	Dey & Pridham, 1972	*Streptococcus mutans*	Aduse *et al.*, 1991
Stachys affinis	Ueno *et al.*, 1980	*Streptococcus pneumoniae*	Rosenow *et al.*, 1999
Trifolium repens	Williams *et al.*, 1978	*Streptomyces olivaceus*	Oishi & Aida, 1975
Trigonella foenum -graecum	Dey & Pridham, 1972	*Thermoanaerobacterium Polysaccharolyticum*	King *et al.*, 2002
Triticum aestivum	Smiley *et al.*, 1976	*Thermotoga maritime*	Millar *et al.*, 2001
Triticum sp.	Dey & Pridham, 1972	*Thermotoga neapolitana*	Millar *et al.*, 2001
Verbascum thapsus	Bom *et al.*, 1998	*Thermus brockianus*	Fridjonsson *et al.*, 1999b
Vicia dumetorum	Dey & Pridham, 1972	*Thermus sp.*	Ishiguro *et al.*, 2001
Vicia faba	Dey & Pridham, 1969a	*Thermus thermophilus*	Fridjonsson & Mattes, 2001
Vicia sativa	Dey & Pridham, 1972		
Vigna radiate	Dey, 1984		
Vigna uniguiculata	Alani *et al.*, 1989		

Table 1.1 Sources of α-galactosidase (continued).

FUNGI		ANIMALS	
Absidia griseola	Masoud et al., 2002	*Bos taurus*	Dey & Pridham, 1972
Calvatia cyanthiformis	Li & Shetlar, 1964	*Dictyostelium discoideum*	Kilpatrick & Striling, 1976
Candida javanica	Cavazzoni *et al.*, 1987		
Cephalosporium acremonium	Zaprometova &Ulezlo, 1988	*Epidinium ecaudatum*	Dey & Pridham, 1972
		Eudiplodinium maggii	Dey & Pridham, 1972
Circinella muscae	Zaprometova &Ulezlo, 1988	*Haliotis corrugate*	Kusomoto *et al.*, 2000
Corticium rolfsii	Kaji &Yoshihara, 1972	*Helix pomatia*	Dey & Pridham, 1972
Kluyveromyces lactis	Hensing *et al.*, 1995	*Melolontha melolontha*	Dey & Pridham, 1972
Monascus pilosus	Wong *et al.*, 1986	*Polyplastron multivesculatum*	Dey & Pridham, 1972
Mortierella vinacea	Shibuya *et al.*, 1997	*Spodoptera frugiperda*	Grossmann & Terrra, 2001
Penicillium duponti	Wiley, 1976		
Penicillium purpurogenum	Shibuya *et al.*, 1998	*Tenebrio molitor*	Grossmann & Terrra, 2001
Penicillium sp.	Malanchuk *et al.*, 2001		
Phanerochaete chrysosporium	Brumer *et al.*, 1999	*Trichomonas foetus*	Dey & Pridham, 1972
Pichia pastories	Zhu *et al.*, 1995		
Pycnoporus cinnabarinus	Ohtakara *et al.*, 1984		
Saccharomyces carlsbergensis	Lazo *et al.*, 1978		
Saccharomyces cerevisiae	Ulezlo *et al.*, 1980		
Saccharomyces sp.	Dey & Pridham, 1972		
Thermomyces lanuginosus	Puchart *et al.*, 2000		
Torulaspora delbrueckii	Oda & Tonomura, 1996		
Trichoderma hamatum	Thornton, 2005		
Trichoderma reesei	Zeilinger *et al.*, 1993		
Zygosaccharomyces mrakii	Oda & Fujisawa, 2000		

α-Galactosidases from different sources have different pH and temperature profiles (Rios *et al.,* 1993) and multiple forms within the same source may have different pH and temperature profiles.

Kinetic properties of α-galactosidase differs with the source of the enzyme and substrate used (Table 1.2). The Km and Vmax values are determined by Michaelis-Menton, Eadie-Hofstee and Lineweaver-Burk plots.

Different studies showed that different compounds such as sugars, metals and other chemicals have different inhibitory effects on α-galactosidases isolated from different sources. Example of this case appears with raffinose and melibiose when the enzyme is isolated from *Vicia faba,* they showed no inhibitory effect (Dey and Pridham, 1972), but they inhibited the enzyme activity of *Aspergillus niger* being the source of the enzyme (Rios *et al.,* 1993). Also the inhibition by other sugars such as D-glucose, melibiose, raffinose, L-arabinose and D-fucose also showed depends on the sources of α-galactosidases used for the hydrolysis (Dey and Pridham, 1972).

Metal ions behave differently on the enzyme from different origins (Table 1.3). Examples of these were shown by the effect of Cu^{+2} and Co. Cu^{+2} acts as a strong inhibitor on α-galactosidase from *Torulaspora delbrueckii* (Oda and Tonomura, 1996), where it was a weak inhibitor when the source of the enzyme was T*hermoanaerobacterium polysaccharolyticum* (King *et al.*, 2002). In case of Co, it has no effect on α-galctosidase from grape flesh (Kang and Lee, 2001), whereas it is a strong inhibitor on the enzyme from *Torulaspora delbrueckii* (Oda and Tonomura, 1996).

Other examples of reported metals are Zn^{+2} (Kang and Lee, 2001), Fe^{+2}, Mn^{+2}, Ca^{+2}, and Mg^{+2} (Kang and Lee, 2001; King *et al.*, 2002), Ag^{+2} and Hg^{+2} that are strong inhibitors (Oda and Tonomura, 1996).

α-Galactosidases have been isolated, purified and characterized using a wide range of techniques, after which some of them have been cloned (Zhu and Goldstein, 1994; Davis *et al.,* 1997) and expressed in various hosts such as *Escherichia coli* (Koppel *et al.,* 1953), yeasts as well as fungi. Some of these methods are ammonium sulfate precipitation and chromatographic techniques which depend on the ionic strength (fast performance liquid chromatography-FPLC), molecular weight (Ultrafiltration, Gel filtration), hydrophobicity (Hydrophobic interaction) and ligand affinity of α-galactosidase (Affinity chromatography).

Molecular weight of the purified enzyme was measured by the use of sodium-dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weight markers that can be visualized by the use of Coomassie Brilliant Blue or silver stain. Also it can be determined by Native-PAGE or size exclusion chromatography (gel filtration) using different columns with different molecular weight markers (King *et al.*, 2002).

Table 1.2 The Km values of α-galactosidase from different sources and substrates.

SUBSTRATES	Km (mM)	REFERENCES
Aspergillus ficuum		Zapter *et al.*, 1990
m-Nitrophenyl-α-D-galactopyranoside	0.72	
o-Nitrophenyl-α-D-galactopyranose	0.84	
p-Nitrophenyl-α-D-galactopyranose	1.46	
Aspergillus niger		Ademark *et al.*, 2001
p-Nitrophenyl-α-D-galactopyranose	0.22/0.24/0.27	
Raffinose	0.5	
Melibiose	0.39/1.0	
Aspergillus tamari		Civas *et al.*, 1984
Melibiose	3.7/3.8	
o-Nitrophenyl-α-D-galactoside	1.3/2.3/3.8	
Raffinose	27.7/71.4	
Stachyose	35.5/72.0	
Aspergillus favips		Ozsoy & Berkkan, 2003
p-Nitrophenyl-α-D-galactopyranoside	1.89	
Bacillus sp.		Akiba & Horokoshi, 1976b
Melibiose	7.9	
p-Nitrophenyl-α-D-galactoside	1.0	
Raffinose	24.1	
Bacteroid ovatus		Gheradini *et al.*, 1985
Melibiose	20.8	
o-Nitrophenyl-β-D-galactopyranose	0.2/0.3	
p-Nitrophenyl-β-D-galactopyranose	0.2/0.4	
Raffinose	5.9/98.1	
Stachyose	0.3/8.5	
Bifidobacterium breve		Xiao *et al.*, 2000
Melibiose	2.14	
Raffinose	0.66	
Cocos nucefera		Mujer *et al.*, 1984
p-Nitrophenyl-α-D-galactoside	0.35	
Corticium rolfsii		Kaji & Yoshihara, 1972
o-Nitrophenyl-α-D-galactopyranose	0.26	
p-Nitrophenyl-α-D-galactopyranose	0.16	
Corynebacterium murisepticum		Nadkarni *et al.*, 1992
Melibiose	2.0	
p-Nitrophenyl-α-D-galactopyranose	0.17	

Table 1.2 The Km values of α-galactosidase from different sources and substrates (continued).

SUBSTRATES	Km (mM)	REFERENCES
Cucorbita pepo		Gaudreault & Webb, 1983
m-Nitrophenyl-α-D-galactoside	1.0	
o-Nitrophenyl-α-D-galactoside	1.4	
p-Nitrophenyl-α-D-galactoside	1.0	
Raffinose	36.4	
Stachyose	4.5	
Cucumis sativas		Smart & Pharr, 1980
Melibiose	3.0/3.5	
p-Nitrophenyl-α-D-galactoside	0.22/0.32	
Raffinose	5.0/6.5	
Stachyose	10/30	
E.coli		Kawamura *et al.*, 1976
Melibiose	2.33	
p-Nitrophenyl-α-D-galactoside	0.12	
Raffinose	3.65	
Geobacillus stearothermophilus		
p-Nitrophenyl-α-D-galactoside	0.38	Pederson & Goodman, 1980
p-Nitrophenyl-α-D-galactopyranose	0.23/0.47/ 0.53/0.59	
Melibiose	19	Fridjonsson *et al.*, 1999a
Raffinose	17.4	
Glycine max		
Melibiose	5.53	Guimaraes *et al.*, 2001
p-Nitrophenyl-α-D-galactopyranoside	0.76/1.55	
p-Nitrophenyl-α-D-galactoside	0.15/0.39	Campillo & Shannon, 1982
Raffinose	5.34	Millar *et al.*, 2001
Micrococcus sp.		Akiba & Horikoshi, 1976b
Melibiose	1.5	
p-Nitrophenyl-α-D-galactoside	0.47	
Raffinose	1.6	
Monascus pilosus		Wong *et al.*, 1986
p-Nitrophenyl-α-D-galactoside	0.8	
Lens culinaris		Corchete & Guerra, 1987
p-Nitrophenyl-α-D-galactoside	0.26/0.29/0.31	
Lens esculenta		Dey & Wallenfels, 1994.
p-Nitrophenyl-α-D-galactoside	0.26	
Raffinose	40	
Lupinus angustifolius		Plant & Moore, 1982
p-Nitrophenyl-α-D-galactoside	0.28/0.56	

Table 1.2 The Km values of α-galactosidase from different sources and substrates (continued)

SUBSTRATES	Km (mM)	REFERENCES
Lycopersicon esculentum		Pressey, 1984
Methyl alpha-D-galactoside	5.3/8.4	
p-Nitrophenyl alpha-D-galactoside	0.22/0.54	
Raffinose	1.8/2.7	
Stachyose	2.4/3.8	
Pycnoporous cinnabarinus		Ohtakara *et al.*, 1984
Melibiose	0.8	
p-Nitrophenyl-α-D-galactoside	0.31	
Raffinose	2.16	
Prunus dulicus		Dey & Pridham, 1972
Ethyl α-D-galactoside	6.25	
m-Chlorophenyl-α-D-galactoside	8.33	
m-Cresyl α-D-galactoside	8.33	
m-Nitrophenyl α-D-galactoside	1.57	
Melibiose	2.14	
Methyl α-D-galactoside	10.9	
n-Propyl α-D-galactoside	6.25	
o-Cresyl α-D-galactoside	4.54	
o-Nitrophenyl α-D-galactoside	0.33	
p-Cresyl-α-D- galactoside	4.76	
p-Nitrophenyl α-D-galactoside	0.53	
p-Nitrophenyl β-L-arabinoside	33.3	
Phenyl-α-D-galactside	5.0	
Raffinose	12.5	
Saccharomyces carlsbergensis		Lazo *et al.*, 1978
Melibiose	6.0	
p-nitrophenyl-α-D-galactoside	6.0	
Spodoptera frugiperda		Grossmann & Terra, 2001
Melibiose	1.7	
p-Nitrophenyl-α-D-galactopyranoside	12	
Raffinose	4.8	
Stachyose	7.2	
Stachys affinins		Ueno *et al.*, 1980
p-Nitrophenyl-α-D-galactoside	0.42	
Plateose	6.9	
Raffinose	11.8	
Teneibrio molitor		Grossmann & Terra, 2001
Melibiose	6.3	
p-Nitrophenyl-α-D-galactopyranoside	3.2	
Raffinose	4.4	
Stachyose	3.8	
Thermoanaerobacterium Polysaccharolyticum		King *et al.*, 2002
p-Nitrophenyl-α-D-galactopyranoside	0.29	

Table 1.2 The Km values of α-galactosidase from different sources and substrates (continued).

SUBSTRATES	Km (mM)	REFERENCES
Thermomyces lanuginosus		Puchart *et al.*, 2000
4-Methylumbilleferyl-α-D-galactoside	0.5	
Melibiose	2.4	
Thermotoga maritima		Millar *et al.*, 2001
Melibiose	15.11	
p-Mitrophenyl-α-D-galactopyranoside	0.075	
Raffinose	2.1	
Thermotoga neapolitana		Millar *et al.*, 2001
Melibiose	15.11	
p-Mitrophenyl-α-D-galactopyranoside	0.74	
Raffinose	5.32	
Thermus sp.		Ishiguro, *et al.*, 2001
p-nitrophenyl-α-D-galactopyranoside	4.7	
Trichoderma reesei		Zeilinger, *et al.*, 1993
Melibiose	1.3	
p-nitrophenyl-α-D-galactoside	1.2	
Raffinose	3.8	
Stachyose	1.8	
Vicia faba		Dey & Pridham, 1972
6-bromo-2-naphthyl-α-D-galactoside	0.62/1.8	
Ethyl-α-D-galactoside	8.0/8.93	
galactinol	0.13/0.69	
m-chlorophenyl-α-D-galactoside	0.83/1.17	
m-cresyl.α-D-galactoside	1.38/2.0	
m-nitrophenyl-α-D-galactoside	2.5/10	
Melibiose	0.77/0.96	
Methyl-α-D-galactoside	7.13	
n-propyl-α-D-galactoside	5.88/6.13	
o-cresyl-α-D-galactoside	0.78/1.33	
o-nitrophenyl-α-D-galactoside	0.69/1.14	
p-aminophenyl-α-D-galactoside	0.87/0.95	
p-cresyl-α-D- galactoside	1.0/1.54	
p-nitrophenyl-α-D-fucoside	4.76/5.88	
p-nitrophenyl-α-D-galactoside	0.38/0.45	
p-Nitrophenyl-β-L-arabinoside	12.5/14.3	
Phenyl-α-D-galactoside	1.11/1.25	
Raffinose	4.0/5.0	
Stachyose	5.26/7.5	
Vigna unguiculata		Alani, *et al.*, 1989
p-nitrophenyl alpha-D-galactoside	1.5/2.2/5.3	
Raffinose	1.6/5.0	
Stachyose	11.0/15.0	

Table 1.3 Different α-galactosidase inhibitors from different sources.

ORGANISM	INHIBITORS	REFERENCES
Aerobacter aerogenes	Iodacetamide, N-ethylmaleimide, P-chloromercuribenzoate	Dey & Pridham, 1972
Aspergillus ficuum	Ag^{+}, Hg^{+2}, Zn^{+2},Cu^{+2}, D-Galactose, D-Glucose, D-Mannose, Fructose,	Zapater *et al.*, 1990
Aspergillus niger	Galactose, Mannose	Ademark *et al*, 2001
Aspergillus tamarii	Ag^{+}, Hg^{+2}, Zn^{+2}, Cd^{+2}, Co^{+2},Cu^{+2}	Civas *et al.*, 1984
Bacillus sp.	Ag^{+}, Hg^{+2},NaN_3, Ni^{+2}, Zn^{+2} P-chloromercuribenzoate, Pb^{+2}	Akiba &Horikoshi, 1976b
Bifidobacterium breve	Cu^{+2}, Hg^{+2}	Xiao *et al.*, 2000
Citrullus battich	Ag^{+}, Hg^{+2},	Itoh *et al.*, 1986
Cocos nucifera	D-Galactose, Iodoacetic acid, Myo-inositol	Mujer *et al.*, 1984
Corticium rolfsii	Ag^{+}, Hg^{+2}	Kaji & Yoshihara, 1972
Cucurbita pepo	Ag^{+}, Cu^{+2}, Hg^{+2},Mg^{+2}, Mn^{+2}, Ni^{+2}, Zn^{+2}, Co^{+2} D-Galactose	Gaudreault & Webb, 1983
Diplococcus pneumoniae	Iodacetamide, N-ethymaleimide, P-chloromercuribenzoate	Dey & Pridam, 1972
Glycine max	α-D-Galactose, Melibiose, SDS	Guimaraes *et al.*, 2001
Lactobacillus fermentum	D-Galactose, Fructose, Sucrose, P-chloromercuribenzoate, Hg^{+2}	Schuler *et al.*, 1985 Garro *et al.*, 1993
Lens culinaris	D-Galactose, Myo-inositol	Corchete & Guerra, 1987
Lupinus angustifolius	Ag^{+}, Hg^{+2} ,D-Galactose	Plant & Moore, 1982
Medicago sativa	Ag^{+}, Hg^{+2}	Itoh *et al.*, 1979
Micrococcus sp.	Ag^{+}, Cu^{+2},Hg^{+2}, Ni^{+2}, Pb^{+2} ,Zn^{+2}, Co^{+2} P-chloromercuribenzoate	Akiba & Horikoshi, 1976b
Monascus pilosus	Ag^{+}, Cu^{+2},Hg^{+2}	Wong *et al.*, 1986
Mortierella vinacea	Hg^{+2}	Dey & Pridham, 1972
Oryze sativa	Hg^{+2}, PCMB	Kim *et al.*, 2002
Prunus amygdalus	Ag^{+}, Cu^{+2},Hg^{+2}	Dey & Pridham, 1972
Pycnoporus cinnabarinus	Ag^{+}, Hg^{+2},D-Galactose, Melibiose, P-chloromercuribenzoate	Ohtakara *et al.*, 1984
Saccharomyces carlsbergensis	D-fucose, D-Galactose, D-Glucose, L-Arabinose, Melibiose	Lazo *et al.*, 1978

Table 1.3 Different α-galactosidase inhibitors from different sources (continued).

ORGANISM	INHIBITORS	REFERENCES
Saccharum officinarum	Ag^{+}, Hg^{+2}, D-Galactose, D-Ascorbic acid, Melibiose, P-chloromercuribenzoate	Chinin, *et al.*, 1981
Spodoptera frugiperda	1-ethyl-3-(dimethylaminopropyl)carbodiimide, γ-1,4-galactonolactone, Melibiose, Raffinose, Stachyose, D-Galactose, Tris	Grossman & Terra, 2001
Stachys affinis	D-fucose, D-Galactose, L-Arabinose, Myo-inositol	Ueno, *et al.*, 1980
Streptomyces olivaceus	D-fucose, D-Galactose, D-Xylose, Melibiose, L-Arabinose, N-ethyl-maleimide, Iodacetamide	Oishi & Aida, 1975
Tenibrio moliter	1-ethyl-3-(dimethylaminopropyl)carbodiimide, γ-1,4-galactonolactone, Melibiose, Raffinose, Stachyose, D-Galactose, Tris	Grossman & Terra, 2001
Thermoanaerobacterium polysaccharolyticum	Hg^{+2}, Ag^{+2}, D-Mannose, D-Glucose	King, *et al.*, 2002
Thermotoga maritima	Fe^{+2}	Millar, *et al.*, 2001
Thermus sp.	Ag^{+}, Hg^{+2}, PCMB	Ishiguro, *et al.*, 2001
Trichoderma reesi	β-D-Galactose	Golubev, *et al.*, 2004
Vicia faba	Hg^{+2}, Ag+ D-Mannose, D-Glucose	Dey & Pridham, 1972

1.2.5 Alpha-galactosidase applications

α-Galctosidase has a great potential in food processing and medical applications.

1.2.5.1 Food processing applications

In sugar industry, this enzyme is able to break down raffinose –the major impurity in sugar production (Liang *et al.*, 1989) which inhibits the normal crystallization of sucrose in molasses.

Cane and beet molasses are the major substrates in alcohol production. Although sucrose is the predominant sugar, beet molasses contain 0.5-5.2% raffinose which can not be degraded leading to incomplete use of the substrate sugar.

Using themostable enzymes in industry has an advantage in improving reaction rates at higher temperatures (Singleton *et al.*, 1973; Vieille *et al.*, 1996). Thermostable α-galactosidase was isolated and used to sugar beet oligosaccharides at moderately high temperatures (Delente *et al.*, 1974; Ganter *et al.*, 1988). Thermostable α-galactosidase from extreme themophiles was also investigated to be used in the petroleum and soy processing industries.

Several microorganisms are known to produce α-galactosidase. The selection of the carbon source in relation to its cost and efficiency is an important point to consider for industrial production (Akiba and Horikoshi, 1976a).

In soybean industry, human consumption of soy products has been hampered by the presence of non digestible oligosaccharides such as raffinose and stachyose (Leske, 1993) which has a degree of polymerization of two or more. They are soluble in 80% ethanol but can not be broken down by pancreatic and brush border enzymes (Quigley

et al., 1999). The absence of α-galactosidase in human intestinal tract prevents the utilization of raffinose, stachyose, and verbascose (Steggerada *et al.*, 1996) thus induceing flatulence which will greatly affect the use of soy products as a major source for humans and animals (Suarez *et al.*, 1999).

Many attempts have been carried out to eliminate the oligosaccharides from soybean and derived products by soaking (Kawamura, 1976), germination (Kim *et al.*, 1973), fermentative processes (Mital *et al.*, 1975) and ultrafiltration.

Enzyme treatment by microbial α-galactosidase might be a promising solution for the elimination of these oligosaccharides (Sugimoto and Van Buren, 1970). Lactic acid bacteria have been consumed in fermented foods by human beings for centuries. They are able to hydrolyze the galacto-oligosaccharides into digestible carbohydrates during vegetable fermentations because of the action of α-galactosidase (Fuller, 1989).

1.2.5.2 Medical applications

Erythrocytes containing B-antigen are coverted to universal donor cells of type O, and blood group AB to A by the action of α-galactosidase (Lenny *et al.*, 1995; Zhu and Goldstein, 1995).

The B-antigen belongs to the ABO blood group system which was discovered by Landsteiner (1901). It is an immunodominant monosaccharide found on complex oligosaccharides linked to membrane proteins and lipids. It is highly immunogenic and it is abundant on red blood cells.

B-like antigens are omnipresent in nature, and a person who lacks them produce potent and highly specific complement fixing antibodies. H-antigen, blood type O, is the

precursor of blood type B and is weakly immunogenic. When the cells have this antigen only, they are universally transfusable. Since the transfusion of immunologically incompatible blood may result in fatal hemolytic transfusion reactions, red blood cells type B have limited compatibility (Hobbs *et al.,* 1995).

Some α-galactosidases were reported in converting the blood group B-antigen on erythrocyte membranes to H-antigen (blood type O) through hydrolysis of terminal immunodominant residues (Kubo, 1989).

This enzyme can also replace therapy in Fabry disease, is x-linked lysosomal storage disorder resulting from deficient activity of exogalactosidase α-gal A. This enzyme is needed to metabolize lipids, fat-like substances that include oils, waxes and fatty acids. When a mutation in the gene controlling this enzyme happens, it leads to deposition of neutral glycosphingolipids with terminal α-galactosyl residues especially globotriaosylceramide (GL-3) in the plasma and cellular lysosomes throughout the body. With age, progressive deposition of GL-3 in the endothelial cells of the kidney, heart and the brain cells leads to death due to renal failure, myocardial infarction or heart failure or stroke (Desnick *et al.*, 2001).

Enzyme replacement therapy with α-galactosidase may be effective in slowing the progression of the disease.

1.3 TRANSGALACTOSYLATION

α-Galactosidase, like many hydrolases, is able to catalyze the hydrolysis and synthesis (transgalactosylation reaction), which results in galacto-oligosaccharides (GOS) and complex oligosaccharides. A general formaula of (galactose)n-glucose, containing usually 2-10 galactose residues represents GOS. Complex oligosaccharides are polymeric carbohydrates made up of heterologous residues. These residues could be alcohols or derivatives of sugar.

The primary factors to be investigated involved the nutritional concerns of the indigestibility of the GOS, and Its low solubility in whey. This low solubility results in the crystallization problem. There are two main reasons for the continued interest towards transgalactosylation activity. One reason is the recognition of oligosaccharide moieties of glycoproteins and glycolipids with biological activity (Lowe *et al.*, 1990), which leads to the efforts of synthesizing them. The other reason is the identification of the bifidogenic properties of some GOS, which make the development of synthesizing them using α-galactosidase enticing for researchers.

1.3.1 Assay methods

Analyzing GOS (transgalactosylation products) involves more complex examination than the hydrolytic products glucose and galactose because this analysis requires the techniques of chromatography such as GC and HPLC for quantitative analysis and determining the sugar units of GOS. In order to identify the structure and the glycosidic linkages, one must utilize mass spectroscopy and nuclear magnetic resonance. Often, chromogenic substrates, like PNP-α-Gal are used in order to define the units of

transgalactosylation activity for the synthesis of di-, tri-, and/or other oligosaccharides under any given condition.

1.3.2 Properties of oligosaccharides

Generally, Oligosaccharides can be defined as glycosides that contain between three and ten sugar moieties. Nevertheless, It is remarkable to note that many disaccharides have similar properties to larger polysaccharides, and are often significant components of food-grade oligosaccharide products. Consequently, disaccharides such as lactulose are considered as oligosaccharides (Rodney, 1998). Food-grade oligosaccharides are, generally, mixtures that contain different degrees of polymerized oligosaccharides.

Since oligosaccharides enhance quality of foods and gives health benefits, they are considered to be very attractive food ingredients. Depending on how the oligosaccharides are formed their specific properties can be very different. However, there are some properties that have been found to be common to almost all oligosaccharides. For instance, the sweetness of the oligosaccharide depends on its structure and molecular mass (Crittenden and Playne, 1996). Oligosaccharides are also normally water soluble and mildly sweet. Their sweetness is usually lower than that of sucrose. This reduced sweetness is an asset in food production when food producers require a smaller amount of sweetness to enhance other food flavors. The higher molecular weight of oligosaccharides imparts a greater viscosity in comparison with mono and disaccharides. As a result, food products containing oligosaccharides will have improved body and will feel better in the mouth of the consumer. Another important fact

is that they can be used to change the freezing temperature of frozen foods, and to regulate the amount of browning that arises from Maillard reactions in heat-processed food. Since oligosaccharides have a high moisture-retaining capacity, they are able to prevent excessive drying. Their low water activity is convenient for controlling microbial contamination (Nakajima and Nishio, 1993). Though it is evident that numerous physiochemical characteristics belong to oligosaccharides, their greatest importance for the food industry lies in the sometimes-overlooked fact that they have a plethora of beneficial physiological properties to offer. While starch and simple sugars are utilized by mouth microflora to form acid or polyglucans, the currently available food-grade oligosaccharides are not used in this manner. Thus, they are used as low-carcinogenic sugar substitutes in confectionery, chewing gums, yogurts and drinks. Humans also do not digest many of these oligosaccharides (Tomomatsu, 1994). Researchers have recently labeled oligosaccharides as being one of several 'prebiotics' that are able to encourage the growth of beneficial microflora (Gibson and Roberfroid, 1995).

There are 12 classes of food-grade oligosaccharides being commercially produced at this moment worldwide. With additional study and subsequent understanding of the fonctional properties of oligosaccharides, we can expect to see an increase in the volume and diversity of these products. Nakajima and Nishio (1993) and Playne (1994) have reviewed and detailed the production methods for various oligosaccharides.

1.3.3 Types of oligosaccharides and formation mechanisms

1.3.3.1 Galacto-oligosaccharides

Researchers have noted that the establishment of bifidus microflora in the intestines of breast-fed infants necessitates the presence of galacto-oligosaccharides in the milk of a lactating mother (Smart 1993). While Mother Nature is capable of producing galacto-oligosaccharides in the case of a nursing mother, it can also be manufactured commercially in the laboratory. It is commonly produced artificially in the laboratory from lactose by using the galactosyl transferase activity of β-galactosidase, which dominates lactose hydrolysis at high lactose concentrations (Smart, 1993; Hung *et al.,* 2001). Considering the recent emphasis on its' importance for human health, it is likely that research will continue to seek ways of improving and understanding the production process.

1.3.3.2 Lactulose

Lactulose is the oligosaccharide that is produced in the largest quantity. It is made from lactose in the same way that galacto-oligosaccharides are. Manufacturers use an alkali isomerization process in order to convert the glucose moiety in lactose into a fructose residue. Humans are not able to digest the disaccharide, lactulose, which is created during this process. Consequently, the preferential growth of bifidobacteria is encouraged in the colon (Modler *et al.*, 1990; Tamura *et al*, 1993).

1.3.3.3 Lactosucrose

Lactosucrose is the third bifidogenic oligosaccharide that is produced using lactose as a raw material. This trisaccharide is made up of a lactose molecule to which a fructose moiety is joined at the glucose residue by a β-2,1 glycosidic bond. It is made with a mixture of lactose and sucrose using the transfructosylation activity of the enzyme.

1.3.3.4 Fructo-oligosaccharides

In terms of their production volume, Fructo-oligosaccharides represent one of the major classes of bifidogenic oligosaccharides. The health and safety benefits of these substances as food ingredients have been reviewed by Spiegel, *et al.* (1994). The two different ways of manufacturing them result in slightly different end products. The transfructosylation activity of the enzyme β-fructofuranosidase is used in the first method to produce, fructo-oligosaccharides from the disaccharide sucrose. A high concentration of the starting material is required for efficient transglycosylation during the production of fructo-oligosaccharides (Park and Almeida, 1991; Van Balken, 1991).

1.3.3.5 Palatinose (isomaltulose)

Palatinose, which is also referred to as isomaltulose, is produced from sucrose using an immobilized isomaltulose synthase (EC 5.4.99.11). One of the most obvious benefits of this disaccharide is that it does not promote tooth decay. It can also be used as a low-carcinogenic sweetener. Since it is digested in the small intestine of humans cannot act as a prebiotic. By contrast, Palatinose oligosaccharides, formed by the intermolecular

dehydration of palatinose, do survive the passage to the colon, enabling them to stimulate the growth of bifidobacteria (Nakajima and Nishio, 1993).

1.3.3.6 Glycosyl sucrose ('Coupling Sugar')

Using the enzyme cyclomaltodextrin glucanotransferase, trisaccharide glycosyl sucrose ('Coupling Sugar') can be fabricated from the disaccharides maltose and sucrose (EC 2.4.1.19). Similar to most oligosaccharides, glycosyl sucrose can be used as a substitute sweetener since it is about half as sweet as sucrose. The most significant advantage that oligosaccharides offer in lieu of using sucrose is the fact that its use has proven to reduce dental caries (Nakamura, 1984).

1.3.3.7 Malto-oligosaccharides

Due to the fact that Malto-oligosaccharides are hydrolyzed and absorbed in the small intestine and don't make it to the colon intact, it is not believed that they increase the numbers of bifidobacteria in the human colon. Although they may not increase the bifidobacteria in the human colon, Nakajima and Nishio reported in 1993 that the consumption of maltotetraose-rich corn syrup has proven in human trials to reduce the levels of intestinal putrefactive bacteria such as *Clostridium perfrigens* and members of the family Enterobacteriaceae. Thus malto-oligosaccharides may prove useful in improving conditions of the colon.

1.3.3.8 Isomalto-oligosaccharides

Isomalto-oligosaccharides are similar to malto-oligosaccharides in that they are produced using starch as the raw material just as are. Yet, there is evidence that points to an important difference between the two, namely, that these oligosaccharides result in a bifidogenic response (Kaneko *et al.*, 1994). Isomalto oligosaccharides consist of α-D-glucose residues linked by α-1,6-glycosidic bonds. The isomalto-oligosaccharide mixture also contains oligosaccharides with both α-1,6 and α-1,4 linked glucose like the trisaccharide panose. Using a combination of immobilized enzymes one can produce them in a two-stage reactor. The first stage involves liquefying the starch using α-amylase (EC 3.2.1.1). In the second stage the liquefied starch is processed in an operation involving reactions catalyzed by both β-amylase (EC 3.2.1.2) and α-glucosidase (EC 3.2.1.20). After the β-amylase hydrolyses the liquefied starch to maltose, the transglucosidase activity of α-glucosidase then produces isomalto-oligosaccharides.

1.2.3.9 Cyclodextrins

Cyclodextrins can be defined as cyclic α-1,4 linked malto-oligosaccharides that are made up of 6-12 glucose units. The starch digested by the action of cyclomaltodextrin glucanotransferase is what forms these Cyclodextrins. By incorporating various organic compounds into the cavity of their cyclical structure, oligosaccharides are capable of forming inclusion complexes. Desirable changes in the physical and chemical properties of the incorporated compound can result from this reaction. There are many practical applications for cyclodextrins in the food production industry, including the emulsification of oils and fats, protection from oxidation and photodegradation of

substrates, and the stabilization of deliquescent or volatile compounds in foods. They are also used to mask the bitter taste of various drugs and foods.

1.3.3.10 Gentio-oligosaccharides

Gentio-oligosaccharides are produced from glucose syrup by enzymatic transglucosylation, and they consist of several glucose residues linked by β-1,6 glycosidic bonds. Since oligosaccharides are not hydrolyzed in the stomach or small intestine, manufacturers advertise them as being able to promote the growth of bifidobacteria (Nakakuki, 1991) and lactobacilli.

1.3.3.11 Soybean oligosaccharides

Soybean oligosaccharides are different from other oligosaccharides in that they can be taken directly from the source material. It is not necessary to fabricate them using a costly enzyme manufacturing process. One of the valuable by-products that results from the production of soy protein isolates and concentrates is soybean whey. It contains the oligosaccharides raffinose and stachyose, as well as sucrose, glucose and fructose. Concentrating the sugars that are extracted from the soybean whey can make soybean-oligosaccharide syrup. Although they can reach the intestine and stimulate the growth of bifidobacteria, raffinose and stachyose are indigestible by the human GI and are, thus, flatulence inducing substances (Oku, 1994).

1.3.3.12 Xylo-oligosaccharides

These oligosaccharides, which promote the growth of bifidobacteria in the colon, represent only a small proportion of the total oligosaccharide market (Modler, 1994). They are employed primarily in the production of prebiotic drinks. Xylan, which is mainly extracted from corncobs, provides the raw material for xylo-oligosaccharide synthesis. The controlled activity of the enzyme endo-l,4- β-xylanase is used to hydrolyzed xylan to xylo-oligosaccharides (EC 3.2.1.8).

1.3.4 Applications of oligosaccharides

Traditionally, the food industry has used oligosaccharides for many different utilitarian purposes. For example, they have been used as sweeteners for the production of desserts such as jellies and ice creams. They are also widely used in bakery products like biscuits, breads and pastries. One can find oligosaccharides used in spreads such as jams and also in the production of infant milk formulas. Another domain that uses oligosaccharides is production of odors (Kitahata *et al.*, 1992) and surfactants (Ismail *et al.*, 1999). They use oligosaccharides to make emulsifiers, stabilizers.

In addition to their natural value, oligosaccharides are valued as being an immuno stimulating agents. They are regarded as prebiotic compounds that can help the colonic microflora in the GI establish homeostasis (Gibson and Roberfroid, 1995). This healthy balance in the GI is achieved by raising the levels of bifidobacteria and lactobacilli. Less desirable bacteria are allowed to perish (Fuller and Gibson, 1998). The enzyme with broad acceptor specificity catalyzes transglycosylation (Dey and Pridham, 1972).

Certain unique glycosides that are contained in glycoproteins have important cellular functions. Some of these functions include blood type determination (Hedbys *et al.*, 1989), recognition between cells, and differentiation of cells. Oligosaccharides play an important role in the working of certain diseases because of the effect that they have on the surface of diseased cells. A few of the more significant diseases that Oligosaccharides can be associated with include Acquired Immune Deficiency Syndrome (AIDS), Cancer and diabetes (Osborn and Khan, 2000).

1.4 LACTOBACILLI

The genus *Lactobacillus, which* belongs to the family lactobacteriaceae, has been subdivided into three different subgenera. The subgenre include Thermobactrium whose members are homofermentative, with a maximum growth temperature of 45-50°C; Streptobacterium, which are also homofermentative, producing lactic and acetic acid, CO_2, and ethanol.

The lactobacilli, which are Gram positive and non-spore forming cells, can appear as Gram negative cells with age and low pH. During the logarithmic phase of growth, they can take the shape of rod shaped cells of varying lengths that commonly form chains. Motility rarely occurs in cells having peritrichoious flagella, and their metabolism is primarily by saccharolytic fermentation. Lactate, which is excreted into the medium, forms the primary end product. The bacteria are cultured under anaerobic or microaerophilic conditions. Lactobacilli are very fastidious microorganisms that have complex nutritional requirements. For instance, the optimal growth temperature for Lactobacilli is usually found between 30-40°C with a range that goes from 2-53°C. The

optimal PH for growth can be found between 5.2-6.2, though some can develop at a slower rate between 5.2-6.2. Depending on the species and strain, growth usually ceases between PH 4.0 and 3.6 (Kandler and Weiss, 1986).

Due to the fact that they are able to produce α-galactosidase, Lactic acid bacteria (LAB) such as *L. plantarum, L. fermentum, L. brevis, L. buchneri,* and *L. reuteric* are able to hydrolyze α-galactooligosaccharides (α-GaOS) into digestible carbohydrates (Garro *et al.,* 1996). Many researchers have become interested in studying LABs recently since LABs hydrolyze GaOS sugars are also involved in the production of exopolysaccharides which can be used as prebiotics.

Researchers have described α-GaOS, especially raffinose and stachyose, as prebiotic substrates capable of supporting the growth of probiotic bacteria in the colon (Benno *et al.*, 1987). It is generally considered that most food-grade LAB microorganisms are safe for humans. They are also generally regarded as being able to contribute to the taste, smell or preservation of food products (Geel-Schuten *et al.*, 1999). In order for their theraputic benefits to be maximized, a sufficient number of viable microorganisms must be present during the entire shelf life of the product.

There is a great interest at the moment in adding various species of lactobacilli (mainly from *L. acidophilus* and *L. casei* group) to fermented milks. As a result, many products have been formulated that contain these lactobacilli (Kneifel and Pacher, 1993). After being ingested, these microorganisms should pass the biological barriers such as stomach acids and bile in the intestine and be able to implant in the intestinal tract (Kailasapathy and Rybka, 1997). As part of the common intestinal microflora of humans and other animals, LAB known to help increase resistance to common intestinal disorders

associated with microbial pathogenesis such as gastroenteritis (Casas and Dobrogosz, 2000). They succeed in increasing resistance by fortifying the normal microflora through their fermentation products or by production of α-galactosidases that can break down carbohydrates and provide the energy for the growth of other bacteria (Sandine, 1979).

L. helveticus, the organism of my interest, was isolated from milk and cheese starter cultures, and is well known in stimulating the immune system, controlling diarrhea, reduction of lactose intolerance and inhibiting unfriendly bacteria.

1.5 PROBIOTICS AND PREBIOTICS

The Nobel Prize winner Ilya Metchinikoff (1908) was one of the first to study the probiotic effects of soured milk, which was found to contain bacteria that may have positive effects on intestinal health. Early research efforts were mainly focused on organisms in fermented milk products such as yogurt. It was suggested that intestinal isolates might have better effects than non-intestinal organisms (Fuller, 1992). More recently, researchers have concentrated their research on probiotics and their role in health improvement and prevention of intestinal disturbances.

Probiotic bacteria must possess a very specific set of characteristics (Havenaar and Huis in't Veld, 1992). These characteristics include ability of the bacterium to survive the acidic conditions of the GI tract and then colonize in the intestine. They will then easily reproduce and remain viable during processing and storage. No pathogenic, toxic, mutagenic, or carcinogenic reaction to the organism, its fermentation products or cell components must occur. Finally, the bacterium should be antagonistic towards

carcinogenic and pathogenic microorganisms, and it must also be genetically stable with no plasmid transfer mechanism.

There are several beneficial roles for probiotic strains that have been identified or researched. A Few examples of these benefits include enhancement of intestinal microflora, improving colonization resistance and/or prevention of diarrhea as well as the systemic reduction of serum cholesterol and reduction of faecal enzymes. Other benefits include potential mutagens which may induce tumors, metabolism of lactose and reduction of lactose intolerance, enhancement of immune system response, improved calcium absorption and synthesis of vitamins and predigestion of proteins (Havenaar and Huis in't Veld, 1992; Lee and Salminen, 1995).

Due to the survivability and colonization difficulties that are related to probiotics, the prebiotic approach appears to be a promising alternative. Prebiotics exploit selective enzyme production by gut microorganisms that may offer health benefits to the host. Non-digestible carbohydrates have received the most attention in research as prebiotics. Certain carbohydrates, oligo- and poly-saccharides, occur naturally and meet the criteria of prebiotics. Among other potential prebiotics are some peptides, proteins and certain lipids (Gibson and Robertfroid, 1995).

Certain non-digestible carbohydrates have a number of functional effects on the GI tract which have been used to validate emptying, modulation of GI tract transit times, improved glucose tolerance, reduced fat and cholesterol absorption through binding of bile acids, augmented volume and water carrying capacity of intestinal contents. Modulation of microbial fermentation with increased short chain fatty acid (SCFA) production and decreased PH and ammonia production are also known effects of

prebiotics (Robertfroid, 1996). As a result of these effects, host health can be improved by reducing constipation and diarrhea, cardiovascular and intestinal cancer.

Some oligosaccharides are defined as bifidogenic factors; serving resistance to digestive enzymes they pass into the large intestine where they become available for the fermentation by large intestine. These oligosaccharides are utilized by bifidobacteria as carbon and energy sources (O'Sullivan, 1996).

1.6 SYNBIOTICS

Synbiotic is defined as a food product that contains both probiotics and prebiotics that affect the host by improving the survival and implantation of live microbial dietary supplement in the gastrointestinal tract. This concept involves a useful probiotic, combined with an appropriate dietary vehicle, and a suitable prebiotic. Both the structure of the carbohydrate and the bacterial species present in the ecosystem are crucual factors for management of the gut microflora. This leads to the increase of probiotic survival in the hostile environment of the colon by offering a readily available growth substrate. (Gibson and Roberfroid, 1995).

CHAPTER 2.0

PURIFICATION, CHARACTERIZATION AND HYDROLYTIC ACTIVITY OF ALPHA-GALACTOSIDASE FROM *LACTOBACILLUS HELVETICUS* ATCC 10797

2.1 ABSTRACT

α-Galctosidase (EC 3.2.1.22) was purified from *Lactobacillus helveticus* ATCC 10797 by ammonium sulphate precipitation and fast performance liquid chromatography system using ion exchange and gel-filtration columns. This enzyme was purified to only 9.44 fold over the crude extract with a recovery of 1.8%. The km of 3.83 mM and Vmax of 416.44 μmol/min/mg protein were calculated from PNPG. The molecular mass was estimated to be 188 kDa by gel-filtration, but 90 kDa by SDS-PAGE, indicating two similar molecular weight subunits. The optimum temperature for enzyme activity was 37 °C, but with a stability below 30 °C. The optimum pH was at 6 with a stability of pH 4-8 range.

This enzyme was activated by 10 mM monovalent ions such as K^+, NH_4^+, Li^+ and CS^+, while the activity was inhibited by divalent ions such as Cu^{+2}, Zn^{+2}, Fe^{+2}. About 40% of the enzyme activity was inhibited with 100 mM EDTA. α-Galactosidase was inhibited by 1mM glucose and galactose, 10 mM sucrose, high

concentrations of melibiose, or raffinose and stachyose, but the least inhibitory effect was shown with fructose.

When the sugars were incubated with α-galactosidase, melibiose was hydrolyzed to glucose and galactose, raffinose to galactose and sucrose, while stachyose to galactose and sucrose with raffinose as intermediate product.

2.2 INTRODUCTION

Today reduction of harmful bacteria and toxic compounds in the intestine, prevention of dental caries, reduction of total cholesterol and lipid in serum, and relief of constipation are all possible due to many LAB-containing dairy and pharmaceutical products. These products have long been developed and consumed thanks to their promising health-promoting characteristics (Crittenden and Playne, 1996). Live probiotic bacteria, which are improving the microbial balance of the human GI tract, have been used to supplement dairy products for several decades. Another way to increase the number of beneficial bacteria in the human intestine is to stimulate their growth by supplying growth factor such as oligosaccharides. So-called prebiotics or galactooligosaccharides, have been shown to employ this growth stimulating effect on probiotic bacteria (Kang and Lee, 2001).

Most lactic acid bacteria (LAB) such as lactobacilli and bifidobacteria containe α-Galactosidase (EC 3.2.1.22, α-D-galactoside galactohydrolase). This so-called melibiase is known not only to hydrolyze the α-1,6-glycosidic linkage of galactosides releasing galactose, but also known to possess the transgalactosylation activity to synthesize galacto-oligosaccharides. Both reaction activities are well recognized and applied in numerous food industries.

2.3 MATERIALS AND METHODS

2.3.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma (Oakville, Ontario) unless otherwise mentioned. Bio-Rad reagent for protein assay was puschased from Bio-Rad (Mississauga, ON). *Lactobacillus helveticus* ATCC 10797 was purchased from The American Type Culture Collection, (Rockville,MD). The culture media was purchased from Difco laboratories (Detroit, MI).

2.3.2 Preparation of alpha-galactosidase

L. helveticus was grown on MRS media (De Man *et al.*, 1960) at 37 °C for 48 h. After *L. helveticus* cells were harvested by centrigugation (10,000 rpm, 20 min,4°C), the supernatant was discarded and the pellet was washed twice with 20 ml sodium phosphate buffer (50 mM, pH 7.0) and centrifuged (8,000 rpm, 20 min). After the supernatant was discarded again and pellet was dissolved in 20 ml sodium phosphate buffer (50 mM, pH 7.0), cells were broken using a French pressure cell (SLM instruments, Inc; American Instrument Company, Urbana, illinois), and then centrifuged (4,000 rpm, 40 min, 4°C). The supernatant was collected as the crude enzyme.

2.3.3 Enzyme activity and protein assay

The enzyme activity was measured using p-nitrophenyl-alpha-D-galactopyranoside (PNPG), following a modified method of Scalabrini *et al.*, (1998). The enzyme (20 µl) was reacted with 500 µl PNPG (10 mM) in sodium phosphate buffer (50

mM, pH 7.0) at 37 °C for 10 min and the reaction was stopped by the addition of equal amount of 1.0 M sodium carbonate. The released p-nitrophenol was quantitatively determined by measuring optical density at 420 nm. One unit of the enzyme was defind as the amount of the enzyme that releases 1μmol of p-nitrophenol from the substrate PNPG per ml per min under the assay conditions. P-nitrophenol was used as a standard (Fig. 2.1)

Protein concentrations were determined by the Bio-Rad protein assay method. The enzyme (5 μl) was reacted with 800 μl water and 200 μl Bio-Rad protein assay reagent (Bio-Rad, Mississauga, ON) for 5 min. Absorbance was measured at 595 nm. Bovin serum albumin (Sigma; Oakville, Ontario) was used as a standard (Fig. 2.2)

2.3.4 Enzyme purification

2.3.4.1 Ammonium sulfate precipitation

The enzyme was fractionated by salting out with solid ammonium sulfate to 70%.

After the mixture was stirred for 18 h at 4 °C, it was centrifuged (15,000 rpm, 20 min, 4 °C). The supernatant was removed and the pellet was redissolved in sodium phosphate buffer (50 mM, pH 7.0) and dialyzed overnight against sodium phosphate buffer (50 mM, pH 7.0) for further purification.

2.3.4.2 First Anion-exchange chromatography

Portions (5 µl) of nuclease (Benzoase; Novagen; Mississage, Ontario) were first added to the partially purified enzyme, kept at 15 °C for 30 min. Then it was directly applied to anion-exchange chromatography. The partially purified enzyme was applied to anionic-exchange chromatography column (Mono Q HR 5/5, Amersham Pharmacia Biotech. Inc.; Montreal, QC) using FPLC system (Amersham Pharmacia Biotech. Inc.) and equilibrated with Buffer A (50 mM sodium phosphate buffer, pH 7.0).

Elution was performed with a linier gradient of (0-30 %) of 1 M sodium chloride in buffer A at a flow rate of 0.25 ml/min. Fractions (1 ml) were collected and tested for enzyme activity.

After several runs, fractions exhibiting enzyme activity were pooled, dialzed against sodium phosphate buffer (50 mM, pH 7.0) and concentrated using 100 kDa centricon ultrafiltration membranes (Millipore Corporate Headquarters, MD) and applied to second anionic-exchange chromatography.

2.3.4.3 Second Anion-exchange chromatography

The pooled fractions of the first anion-exchange chromatography were concentrated with 100 kDa centricon ultrafiltration membranes (Millipore Corporate Headquarters, MD) and applied to a second anionic-exchange chromatography column (Mono Q HR 5/5, Amersham Pharmacia Biotech. Inc.; Montreal, QC) using FPLC system (Amersham Pharmacia Biotech. Inc) and equilibrated with Buffer A (50 mM sodium phosphate buffer, pH 6.0).

Elution was performed with a linier gradient of (0-30 %) of 1 M sodium chloride in buffer A at a flow rate of 0.25 ml/min. Fractions (1 ml) were collected and tested for enzyme activity.

After several runs, fractions exhibiting enzyme activity were pooled, concentrated using 100 kDa centricon ultrafiltration membrane (Millipore Corporate Headquarters, MD) and applied to gel filtration column.

2.3.4.4 Gel filtration

The pooled fractions from anion-exchange chromatography were applied to a gel filtration column (Superose-12 HR 10/30, Amersham Pharmacia Biotech. Inc.; Montreal, QC) and equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride. Elution was performed at a flow rate of 0.5 ml/min, and 1ml fractions were collected.

After several runs of chromatography, fractions exhibiting enzyme activity were pooled, concentrated with 100 kDa centricon ultrafiltration membranes (Millipore Corporate Headquarters, MD) and stored at 4 °C for further SDS-PAGE analysis.

2.3.5 Gel electrophoresis

2.3.5.1 SDS-PAGE

To determine the purity of purified protein, the purity of the enzyme at each purification step was examined by the method of Laemmli (1970) using 10% Bis-Tris gel (Invitrogen, CA). To estimate the molecular mass, a broad range of prestained protein

markers (6-191 kDa) (See blue, Invitrogen, CA) was applied to the same gel and the gel was stained with Coomassie brilliant blue R-250.

2.3.6 Determination of native molecular mass

The native molecular mass of purified alpha-galactosidase was determined by gel filtration chromatography (Superose 12 HR 10/30) using combination of standard proteins of low and high molecular weight gel filtration kit (catalase, 232 kDa; aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa) purchased from Amersham Pharmacia Biotech Inc. (Montreal. QC).

The mobile phase was 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at a flow rate of 0.5 ml per min.

2.3.7 Characterization of alpha-galactosidase

2.3.7.1 Effect of temperature

The optimum temperature was measured by incubating 20 µl enzyme with 500 µl of 10 mM PNPG in 50 mM sodium phosphate buffer (pH 7.0) at different temperatures in the range of 20-45 °C for 10 min. The reaction was stopped with 500 µl sodium carbonate and the activity was measured at 420 nm.

Thermal stability of the enzymes was determined by incubating enzyme at different desired temperatures (20-40 °C) for 2 h. Aliqouts (20 µl) were taken from the enzyme at 20 min intervals, and the activity was measured at 37 °C using the same assay method.

2.3.7.2 Effect of pH

Three buffer systems; citrate buffer (50 mM, pH 3-5), sodium phosphate buffer (50 mM, pH 6-8) and sodium carbonate buffer (50 mM, pH 9-11) were used to determine the pH stability and optimum pH for the enzyme activity.

The optimum pH was measured by incubating 20 µl of the enzyme with 500 µl PNPG of 10 mM prepared in different buffers at 37 °C for 10 min and the reaction was stopped by the addition of 500 µl sodium carbonate. The activity was measured at 420 nm.

To determine the pH stability, the appropriate amounts of enzymes were pre-incubated at specified pH for 2 h at 15 °C and then substrate solutions were added and incubated for 10 min to determine the enzyme activity.

2.3.7.3 Effect of metal chelators and other inhibitors

The purified enzyme solution (20 µl) was incubated in the presence or absence of the selected monovalent ions which are K^+, NH_4^+, Li^+, CS^+ and Rb^+ or divalent ions which are Zn^{+2}, Cu^{+2}, Fe^{+2}, Mn^{+2} and Ca^{+2} and other inhibitors (EDTA) at a final concentration of 1, 10 and 100 mM with 10 mM PNPG in sodium phosphate buffer (pH 7.0) at 37 °C for 10 min. The enzyme activity without effectors was used as control.

2.3.7.4 Effect of sugars

A number of selected sugars were incubated with the enzyme at a final concentration of 1, 10 and 100 mM with 10 mM PNPG in sodium phosphate buffer (pH 7.0) at 37 °C for 10 min. The enzyme activity without effectors was used as control.

2.3.7.5 Enzyme kinetics

The kinetic constants (Km and Vmax) were determined with PNPG concentrations ranging from 0.1 to 10 mM in sodium phosphate buffer (50 mM, pH 7.0) at 37 °C. The reaction was stopped by adding same volume of 1 M sodium carbonate.

The Lineweaver-Burk plot was constructed by using a least-square, best-fit Michaelis-Menten equation (Lineweaver and Burk, 1934) and the kinetic constants was computed from the slope and intercept of the regression line.

2.3.8 Alpha-galactosidase hydrolytic activity

Portions (40 µl) of 1 % substrate (melibiose, raffinose, stachyose) were incubated with 20 µl of the purified enzyme and 40 µl sodium phosphate buffer (50 mM, pH 7.0) at 15 °C for 48 h. Samples were taken at several intervals and boiled for 10 min at 90 °C to stop the enzymatic reaction.

To check hydrolytic activity of the enzyme, 10 µl of different samples were loaded on thin layer chromatography (TLC) plate (Silica gel 60 without fluorescence indicator, Fluka; Sigma, Oakville, Ontario). Solvent system used was 1-propanol:water (80:20 v/v). Then the plates were dried and sprayed with 75 % sulfuric acid in alcohol and heated for 5 min at 140 °C to detect the products. Melibiose, stachyose, raffinose, galactose, glucose and sucrose at 1 % concentration were used as standards for the identification of the product.

2.4 RESULTS

2.4.1 Purification and gel electrophoresis

The crude enzyme was purified using ammonium sulphate precipitation and by anion-exchange chromatography and gel filtration using fast performance liquid chromatography system. It was found to be produced intracellularly. This enzyme was purified to 9.44 fold over the crude extract with a recovery of 1.8%. Overall purification results are summarized in Table 2.1.

The homogeneity of the purified enzyme during the purification steps was verified with SDS-PAGE and the molecular weight was estimated to be 90 kDa (Figure 2.3). The molecular mass of α-galactosidase determined by gel filtration was 188 kDa (Figure 2.4).

2.4.2 Effect of temperature

Optimum temperature for the enzyme activity (Figure 2.5) was found to be 37 °C, while the enzyme showed 100 % stability at 20 and 25°C for 2 h (Figure 2.6), however at 30 °C, the enzyme lost about 25% of the activity after 2 h. At higher temperatures, the activity showed a significant decrease as the temperature increases.

2.4.3 Effect of pH

Optimum pH for the enzyme activity was 6.0 (Figure 2.7), while the enzyme showed 100 % stability at pH 6.0. The enzyme was completely unstable at the extreme

pHs of 3, 9, 10 and 11. For pHs of 4, 5, 7 and 8, the enzyme retained 40, 80, 91, and 77 % of the activity, respectively after 2 h incubation in these buffers (Figure 2.8).

2.4.4 Effect of metal chelators and other inhibitors

When 1mM monovalent ions were used, no major differences in enzyme activity were found. Whereas the enzyme was activated when the concentration used was 10 mM of Li^+, K^+, CS^+, and NH_4^+. There was no significant effect with Rb^+ at all concentrations. By increasing the ions concentration to 100 mM, there was no significant increase in activity with K^+ and NH_4^+, but there was a little decrease in activity when Li^+, CS^+ and Rb^+ effect was tested (Figure 2.9).

Among the tested divalents, Zn^{+2}, Cu^{+2} were the strongest inhibitors of the enzyme activity, even with 1 mM concentration. Fe^{+2} is another inhibitor for the enzyme activity with significant loss at a concentration of 10 mM. Although Mn^{+2} and Ca^{+2} activated the enzyme at 1 and 10 mM, they inhibited the enzymatic activity completely when their concentration was 100 mM. Mg^{+2} appeared to be an activator at concentration of 10 mM (Figure 2.10). No inhibition was observed with 1 and 10 mM of EDTA, but the inhibition was strong at 100 mM concentration (Figure 2.11).

2.4.5 Effect of sugars

Enzyme activity was decreased with increasing concentration of sugars. Sucrose, glucose and galactose acted as strong inhibitors for enzymatic activity with even 1 mM. Others such as melibiose, stachyose and raffinose also inhibited enzyme activity at a

concentration of 100 mM. Fructose had the least inhibitory effect among sugars tested (Figure 2.12).

2.4.6 Enzyme kinetics

The Km and Vmax values of the purified enzyme with PNPG were determined from Lineweaver Burk plot. The Km value was 3.83 mM and Vmax was 416.66 μM/min/mg protein (Figure 2.13).

2.4.7 Hydrolytic activity

After 6 h incubation of different substrates with the purified enzyme, the hydrolysis reaction started. After 48 h of incubation, the substrates were hydrolyzed completely. Melibiose was hydrolyzed to galactose and glucose (Figure 2.16). Raffinose was hydrolyzed to galactose and sucrose (Figure 2.15). Stachyose was hydrolyzed to galactose and sucrose with raffinose as intermediate product (Figure 2.14).

Table 2.1 Summary of the purification steps of α-galactosidase

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Recovery Yield (%)
Crude enzyme	320.40	83,093.60	259.34	1.00	100
Ammonium sulfate precipitation (0-70%)	297.60	80.853.20	271.70	1.05	97.3
First Mono Q [a]	54.40	39,635.20	724.60	2.81	47.7
Second Mono Q [a]	10.00	11,112.80	1,111.30	4.30	13.4
S-12 [b]	0.61	1,493.33	2,448.10	9.44	1.8

a) Anion-exchange chromatography column.
b) Superose-12 gel filtration column.

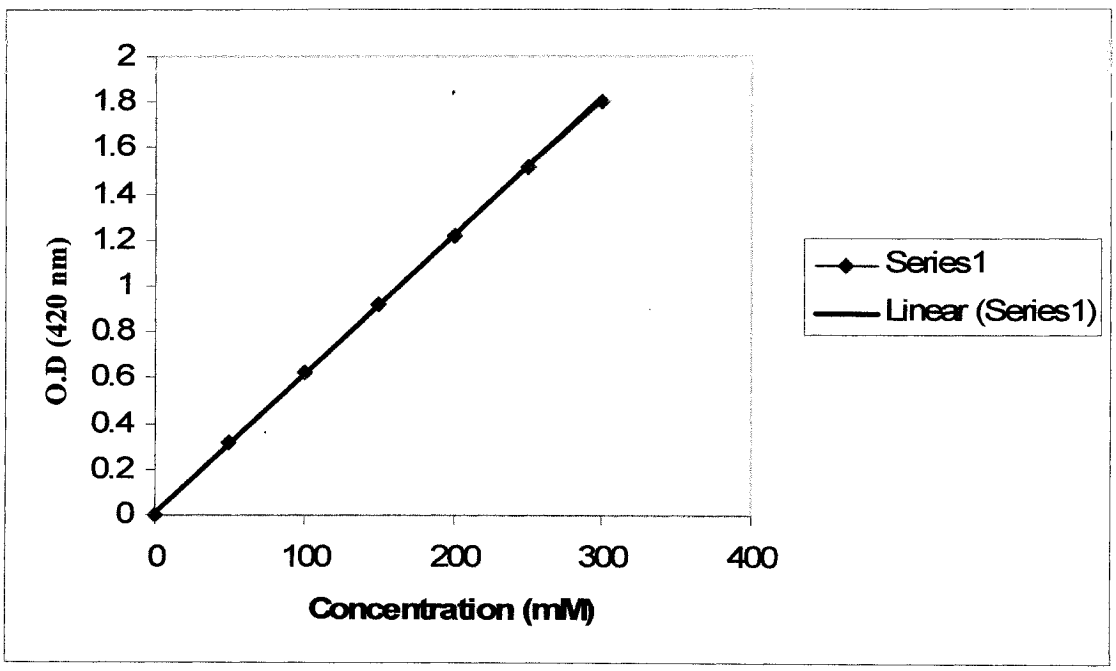


Figure 2.1 Standard curve of para-nitrophenol (PNP)

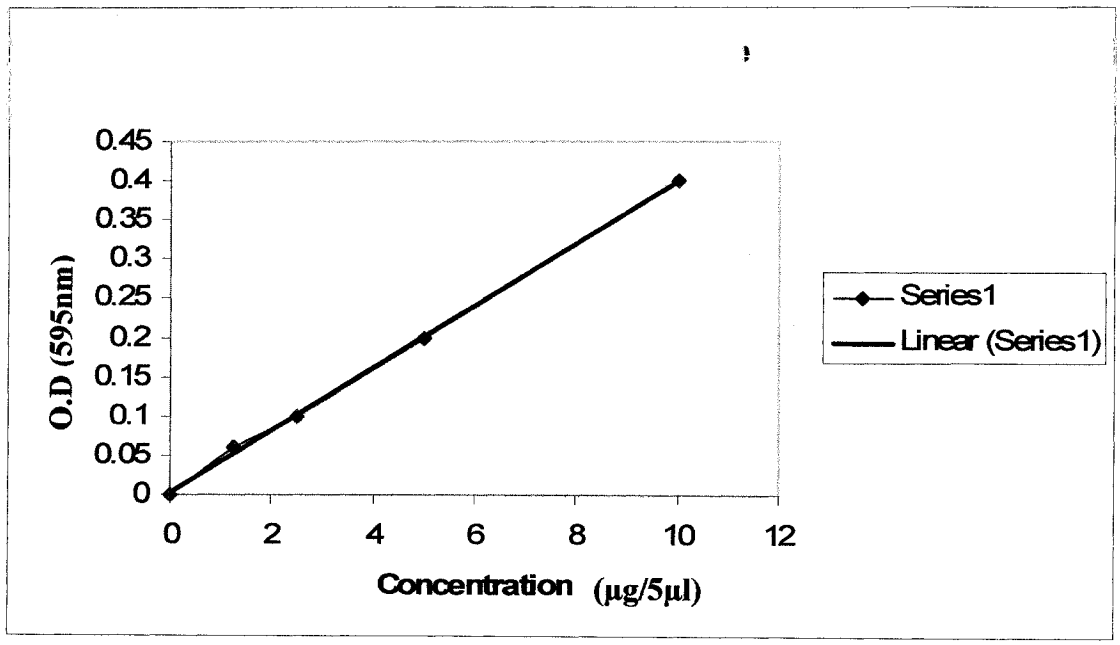


Figure 2.2 Standard curve of bovine serum albumin (BSA).

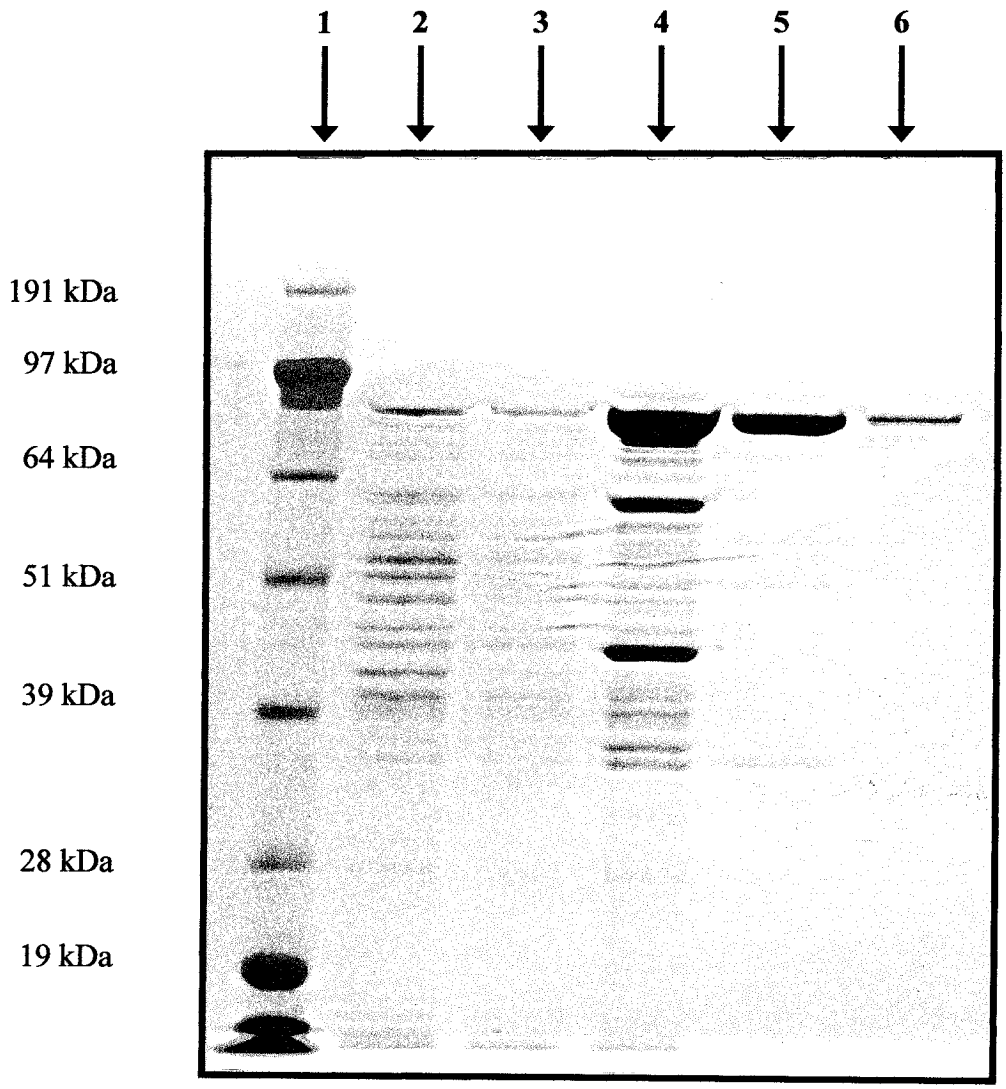


Figure 2.3 SDS-PAGE analysis on the fractions from *L. helveticus* during purification steps. Lane 1, Molecular Weight marker; Lane 2, Crude enzyme; lane 3, 0-70% Ammonium sulfate precipitation; lane 4, 1st Mono Q; lane 5, 2nd Mono Q; lane 6, Gel filtration.

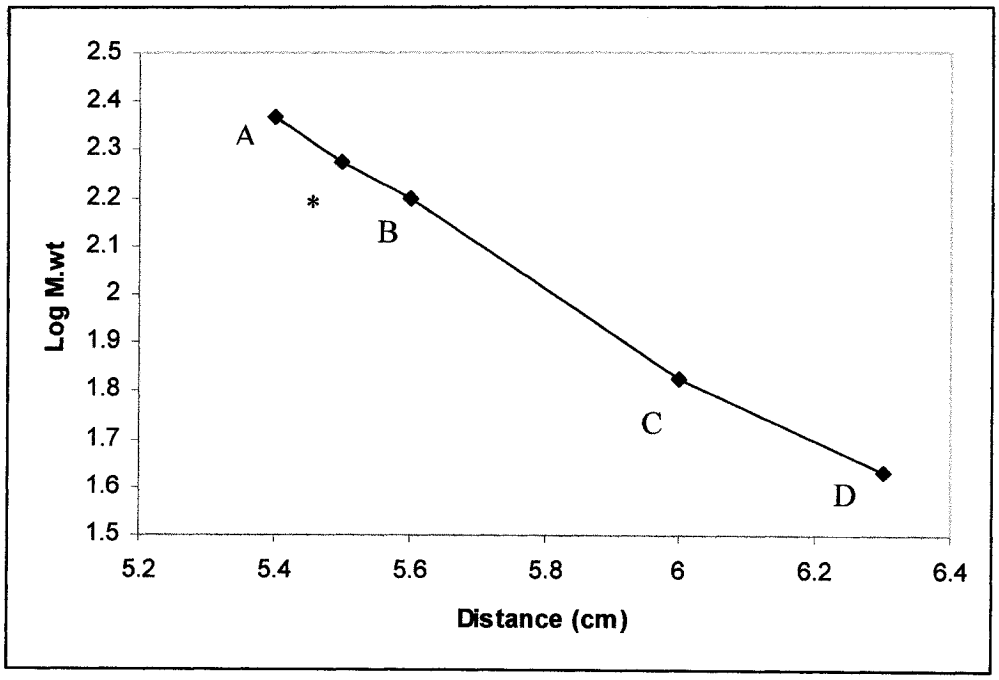


Figure 2.4 Determination of molecular mass of α-galactosidase by gel filtration. A, Catalase (232 kDa); B, Aldolase (158 kDa); C, Albumin (67 kDa); D, Ovalbulmin (43 kDa); * Purified enzyme (188 kDa).

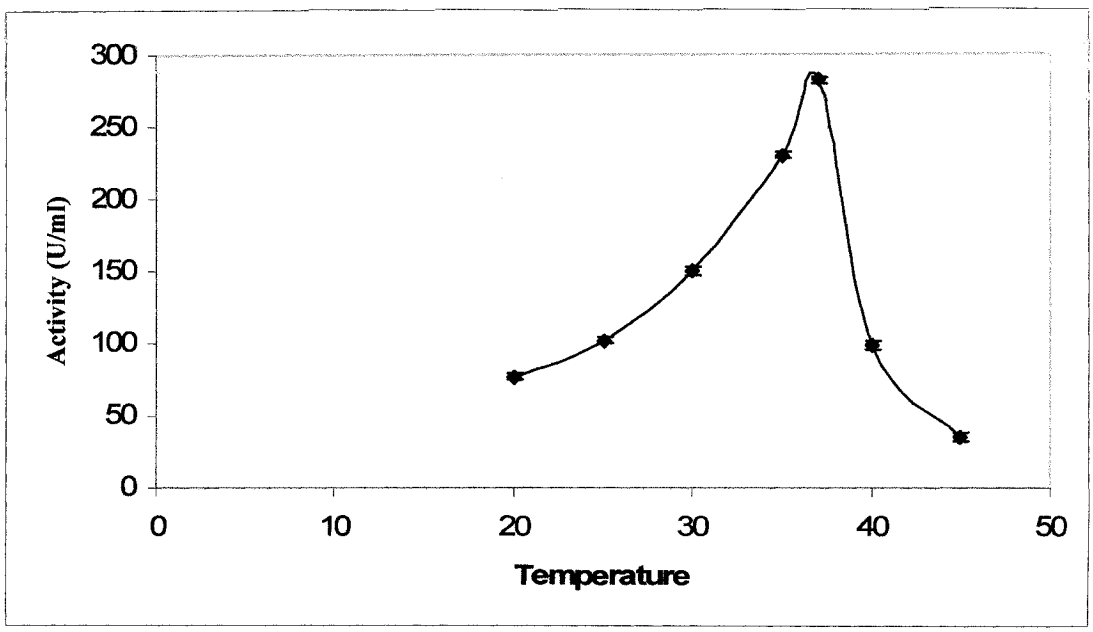


Figure 2.5 Activity of α-galactosidease with PNPG at different temperatures. Enzyme activities were the means of triplicates.

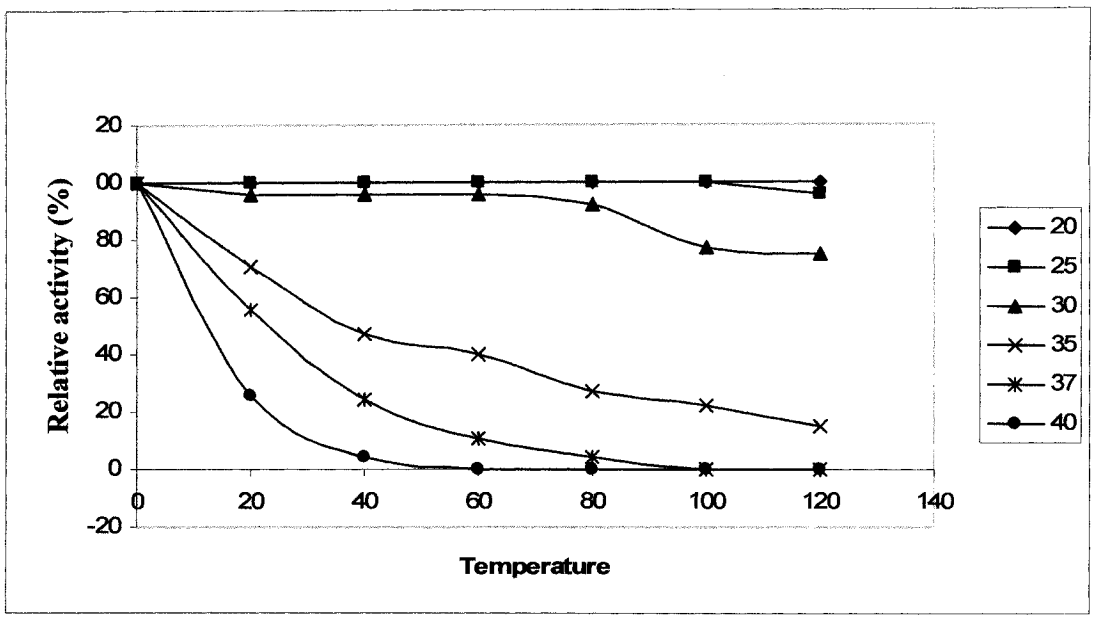


Figure 2.6 Thermal stability of α-galactosidase using PNPG at different temperatures. Enzyme activities were the means of triplicates. Untreated enzyme was used as control.

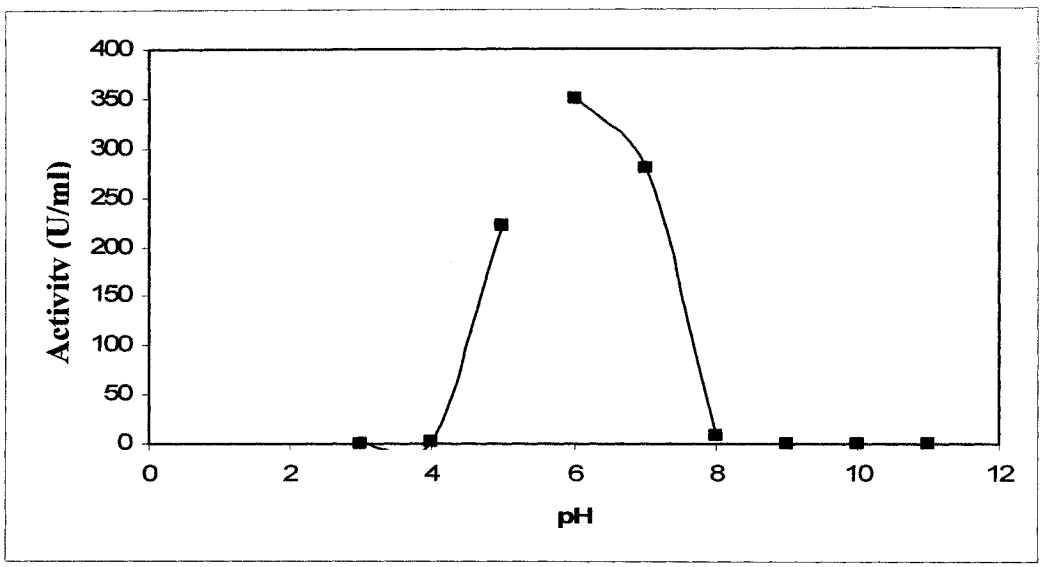


Figure 2.7 α-Galactosidase activity with PNPG at different pH values. Citrate buffer (50 mM, pH 3-5); Sodium phosphate buffer (50 mM, pH 6-8); Sodium carbonate buffer (50 mM, pH 9-11). Enzyme activities were the means of triplicates.

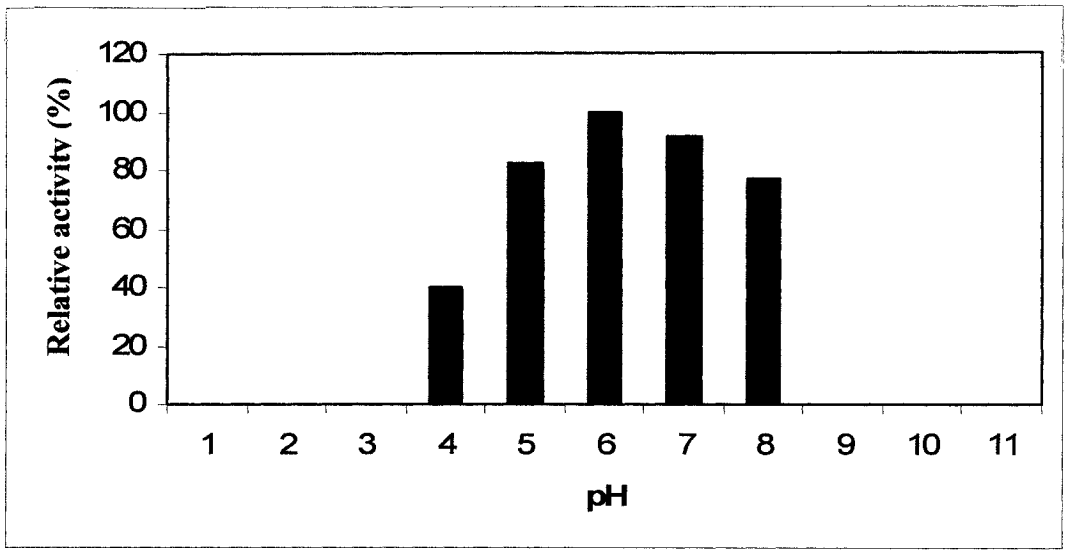


Figure 2.8 pH stability of α-galactosidase with PNPG after 2 h incubation with different buffers at 15°C. Citrate buffer (50 mM, pH 3-5); Sodium phosphate buffer (50 mM, pH 6-8); Sodium carbonate buffer (50 mM, pH 9-11). Enzyme activities were the means of triplicates. Un treated enzyme was used as control.

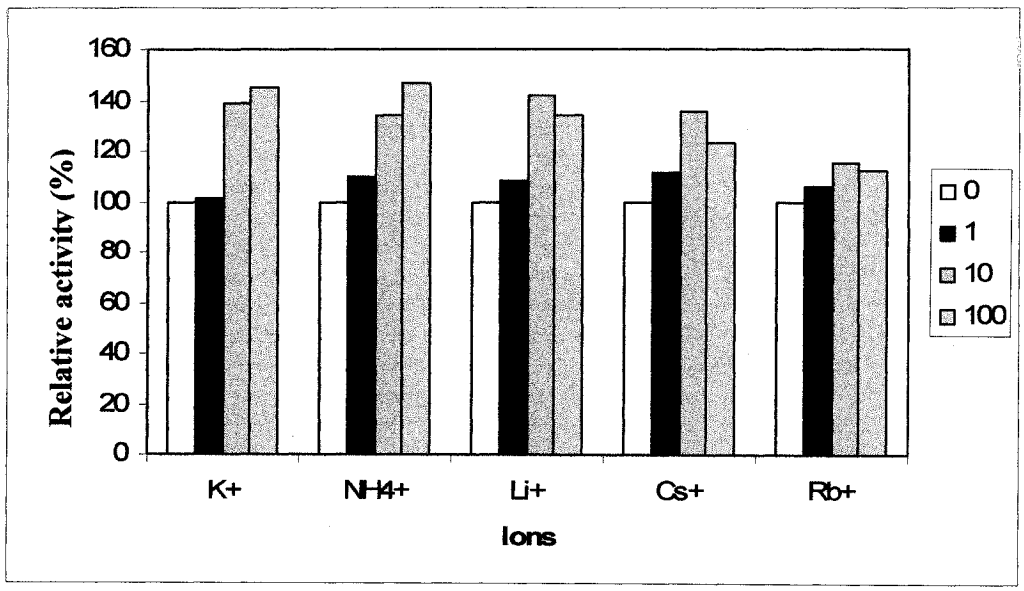


Figure 2.9 Effect of monovalent ions on α-galactosidase activity with PNPG using final concentration of 1, 10 and 100 mM. Enzyme activities were the means of triplicates. Enzyme without effecter was used as a control.

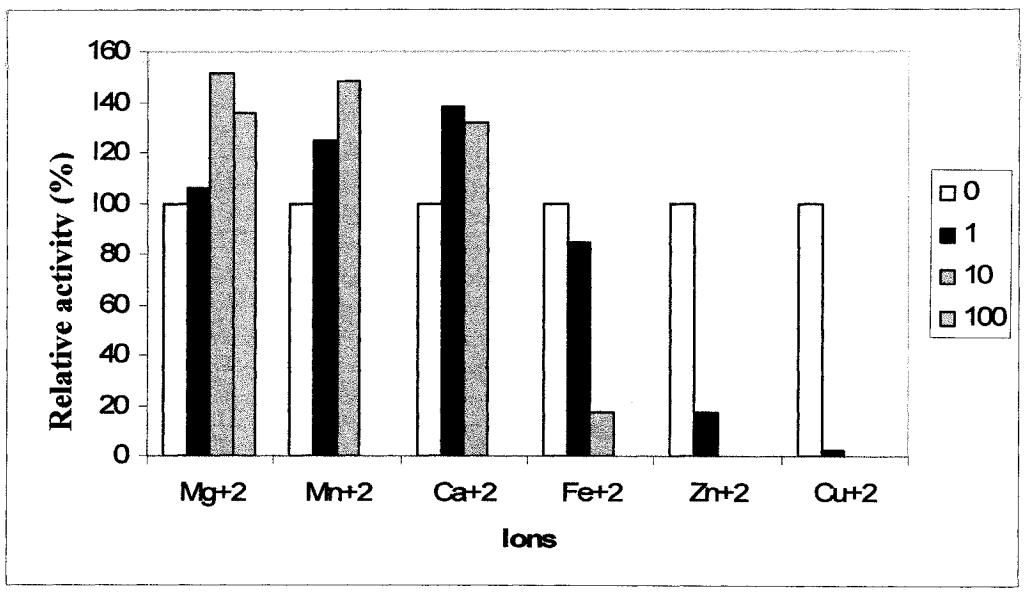


Figure 2.10 Effect of divalent ions on α-galactosidase activity with PNPG using final concentration of 1, 10 and 100 mM. Enzyme activities were the means of triplicates. Enzyme without effecter was used as a control.

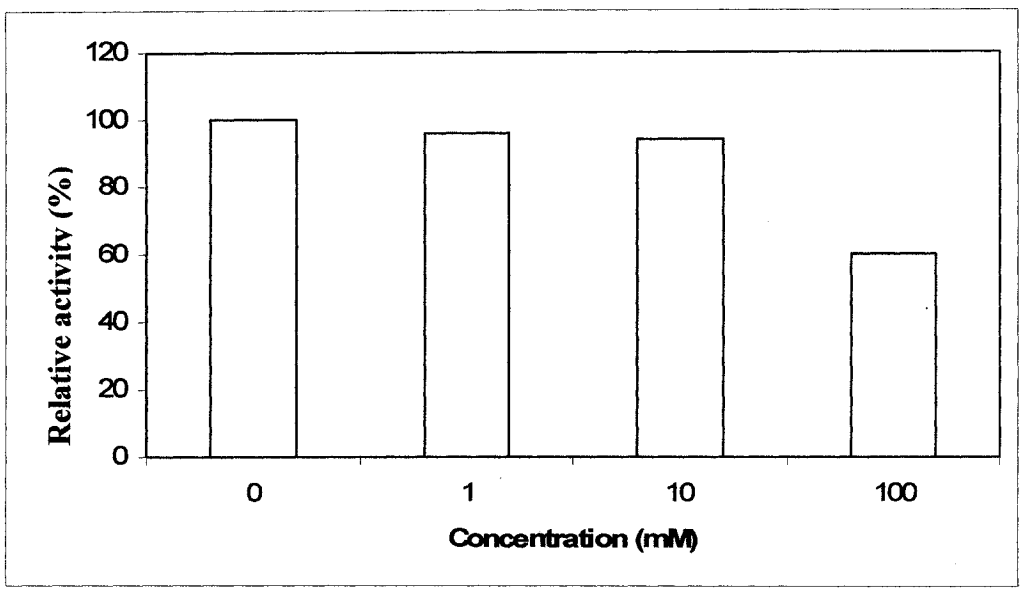


Figure 2.11 Effect of EDTA on α-galactosidase activity with PNPG using final concentration of 1, 10 and 100 mM. Enzyme activities were the means of triplicates. Enzyme without effecter was used as a control.

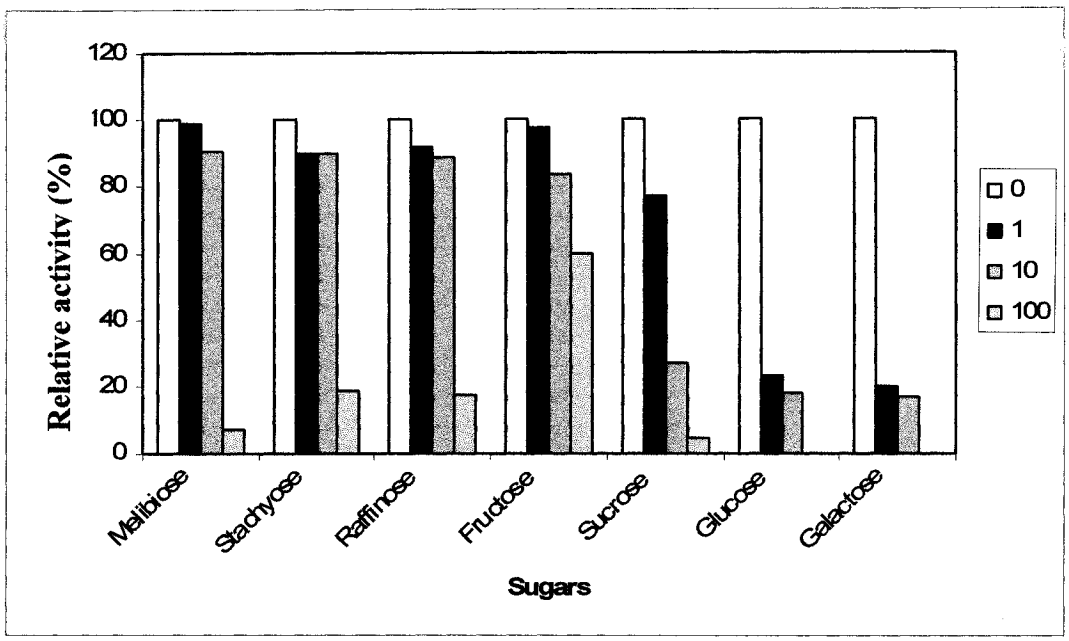
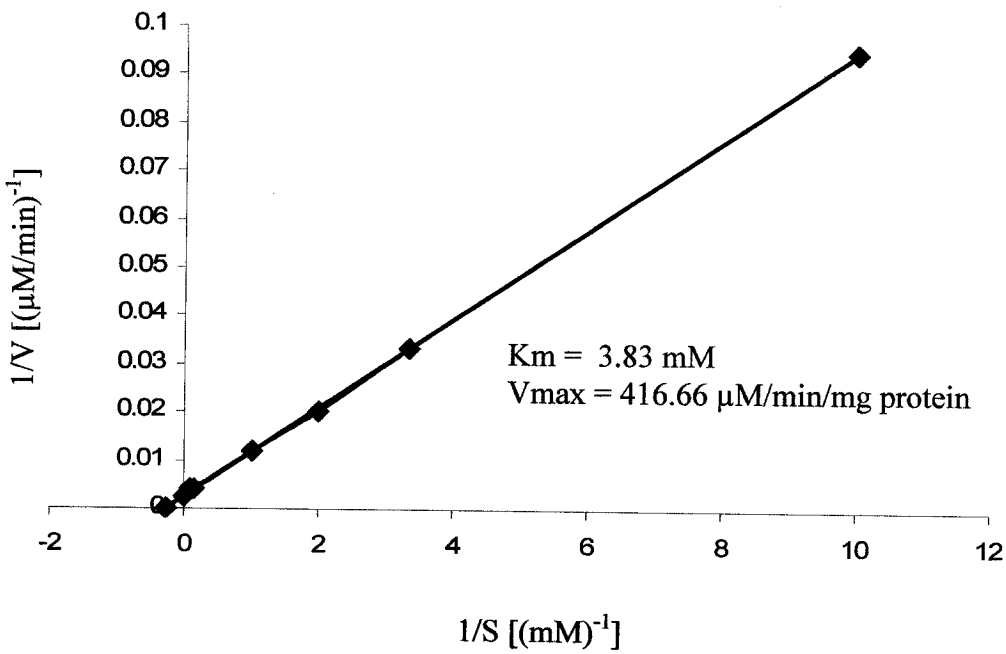


Figure 2.12 Effect of different sugars on α-galactosidase activity with PNPG using final concentration of 1, 10 and 100 mM. Enzyme activities were the means of triplicates. Enzyme without effecter was used as a control.



2.13 Lineweaver-Burk plot of α-galactosidase activity with PNPG.

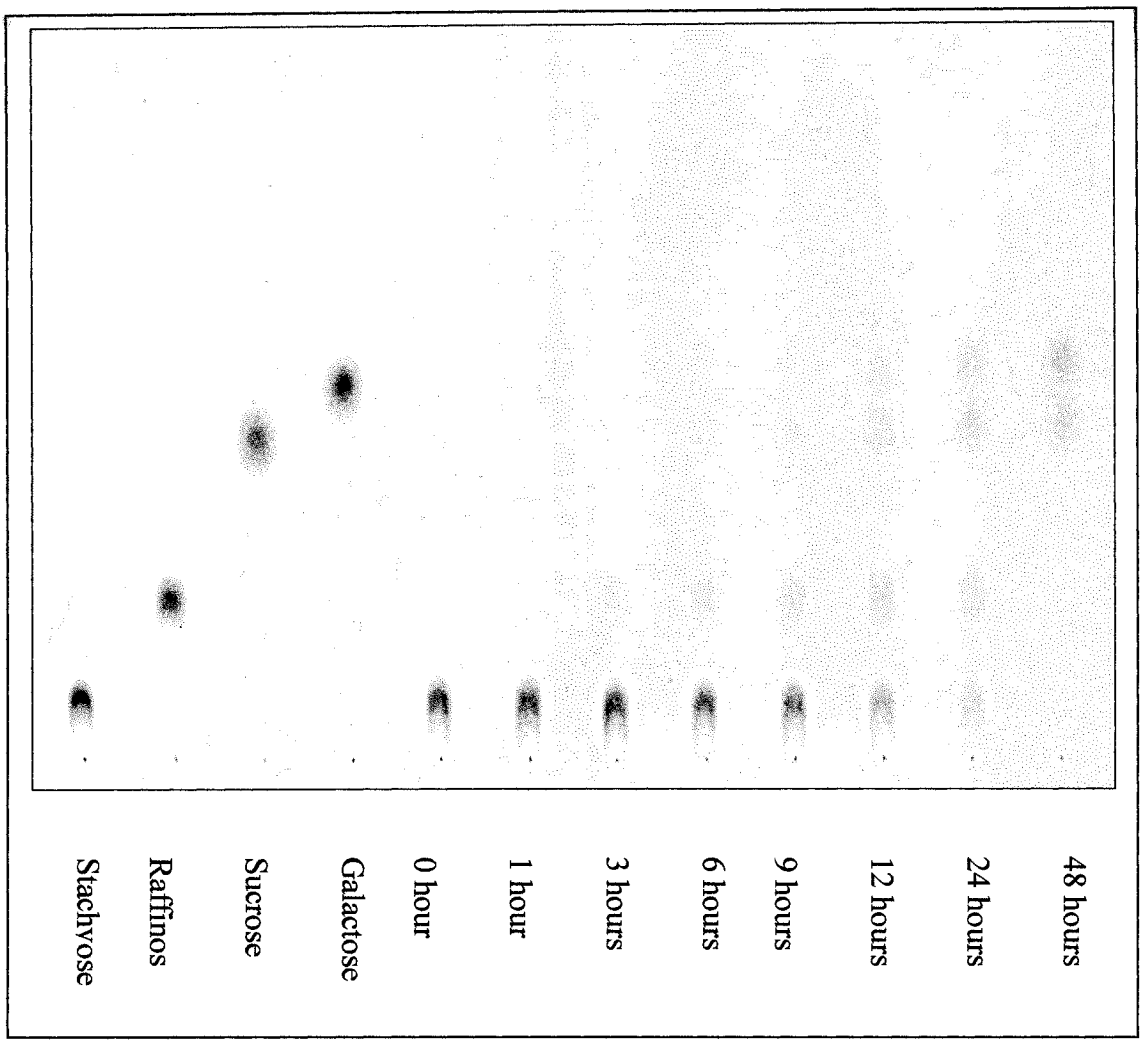


Figure 2.14 1 % of stachyose (40 µl) hydrolysis with α-galactosidase (20 µl) at 15 °C for 48 h. Samples of 10 µl were loaded on TLC plates (silica gel 60 without fluorescence indicator, Fluka; Sigma). Solvent system used was 1-propanol:water (80:20 v/v). Products developed by spraying 75 % sulfuric acid in alcohol and heating at 140 °C for 5 min.

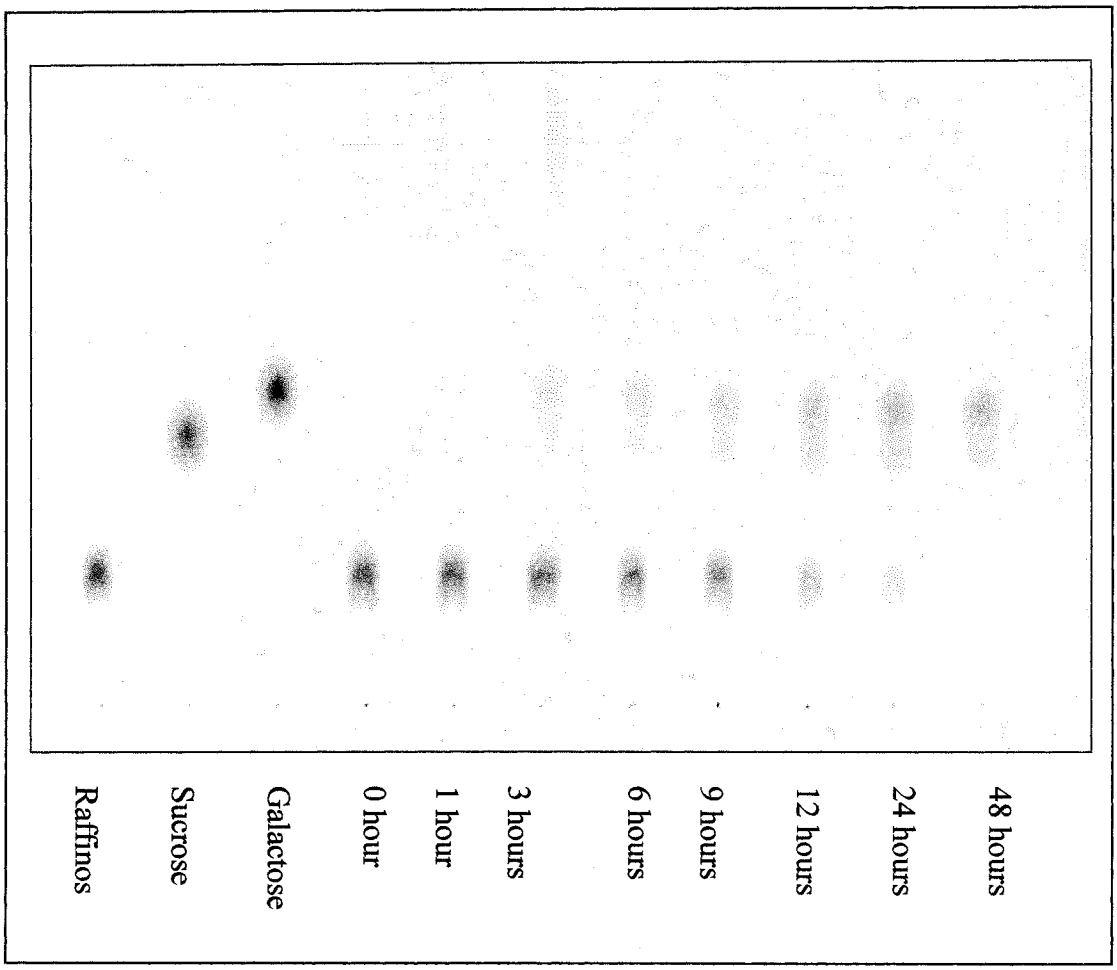


Figure 2.15 1% of raffinose (40 µl) hydrolysis with α-galactosidase (20 µl) at 15 °C for 48 h. Samples of 10 µl were loaded on TLC plates (silica gel 60 without fluorescence indicator, Fluka; Sigma). Solvent system used was 1-propanol:water (80:20 v/v). Products developed by spraying 75 % sulfuric acid in alcohol and heating at 140 °C for 5 min.

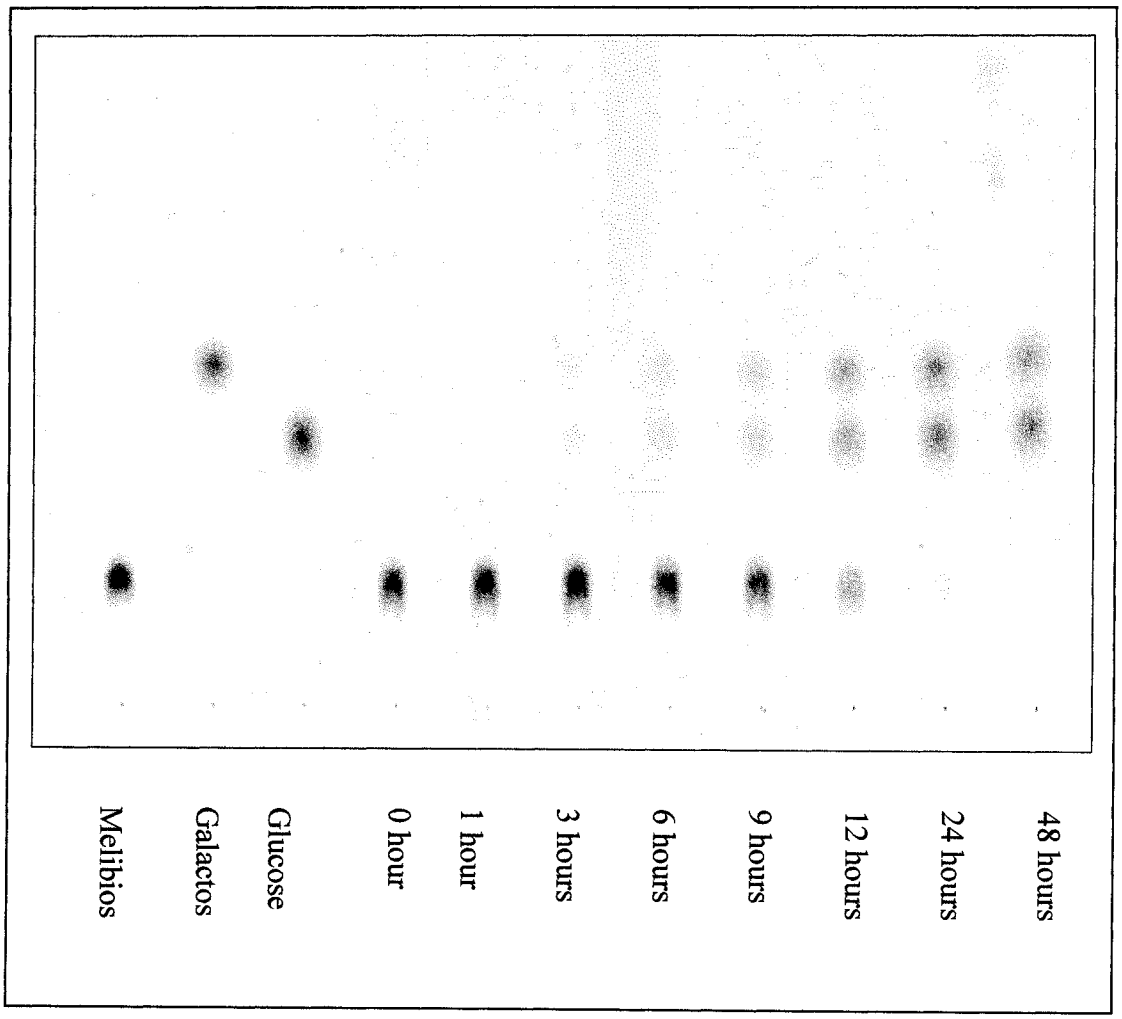


Figure 2.16 1% of melibiose (40 µl) hydrolysis with α-galactosidase (20 µl) at 15 °C for 48 h. Samples of 10 µl were loaded on TLC plates (silica gel 60 without fluorescence indicator, Fluka; Sigma). Solvent system used was 1-propanol:water (80:20 v/v). Products developed by spraying 75 % sulfuric acid in alcohol and heating at 140 °C for 5 minutes

2.5 DISCUSSION

In this study α-galactosidase was purified for the first time from *L. helveticus* ATCC 10797 and found to be produced intracellularly. This enzyme was purified to 9.44 fold over the crude enzyme with a recovery with 1.8 % (Table 2.1). The homogeneity of the purified α-galactosidase was examined by SDS-PAGE (Figure 2.3) and it's molecular mass was found to be 90 kDa. The molecular mass was determined also by Superose-12 gel filtration chromatography (Figure 2.4) and found to be 188 kDa. This value was lower than those of *Lactobacillus fermentum* (195 kDa; Garro *et al.,* 1996) *Aspergillus saitoi* (290 kDa; Sugimoto and Van Buren, 1970), but higher than those obtained from *Aspergillus awamori* (130 kDa; McGhee *et al.,* 1978) and *Pichia guilliermondii* (143 kDa; Church *et al.*, 1980). The Km of 3.83 mM and Vmax of 416.66 μM/min/mg protein were calculated with PNPG (10 mM) (Figure 2.13).

This enzyme has properties different from other α-galactosidases isolated from fungi, yeasts and plant seeds. Most fungal α-galactosidase reached their maximal activity at extremely acid pH values (2.7-4.0) and at temperature above 50°C (Li and Shelter, 1964; Suzoki *et al.,* 1970). The enzyme from *L. helveticus* ATCC 10797 exhibited the maximum activity at pH 6.0 (Figure 2.7) which is similar to those of *Lactobacillus sp.* α-Galactosidase from *L. fermentum* was found to be inactive at pH levels below 4.5, and exhibited the activity in a pH range from 5.0 to 6.5 using McIlvaine buffer. *L. fermentum* CRL 251 had the optimum pH at 5.8 (Garro *et al.*, 1993). An optimum pH between 5.5 to 5.8 has been reported for an α-galactosidase from *Lactobacillus salivarius* using 0.1 mM citrate and 0.1 mM phosphate buffer (Mital *et al.,* 1973). It can be concluded that this enzyme is suitable for enzyme processing technology, since the neutral pH is desirable to

avoid decomposition and side-reactions of the monosaccharide products. α-Galactosidase from *L. helveticus* ATCC 10797 was very stable around neutral pH values. Although the activity at extreme pH values was low, it showed the stability at pH 4 and 8 (40 and 77 %), respectively when incubated in such buffers for two hours at 15 °C (Figure 2.8).

The analysis of optimum temperature (Figure 2.5) showed that maximum activity for α-galactosidase at 37°C was different from the reported temperature for *L. fermentum* (55 °C; Garro *et al.*, 1993), but same as others like *E. coli* (Kawamura *et al.*, 1976) and *Haliotis corrugate* (Kusumoto *et al.*, 2000). α-Galactosidase from our study was stable at temperature below 30°C (Figure 2.6). The activity was rapidly decreased at higher temperatures within 20 min when the enzyme was incubated at such temperatures most likely, due to thermal denaturation effect. Based on this result, it can be concluded that α-galactosidase from this source is not suitable for an enzyme processing at elevated temperatures, since it is not very stable at high temperatures.

The enzyme activity was affected by the presence of monovalent ions (Figure 2.9) and divalent ions (Figure 2.10) differently. The enzyme was activated by monovalent ions as K^+, NH_4^+, Li^+ and Cs^+, whereas it was not affected by Rb^+. K^+ that reported to be an activator of α-galactosidase from *Vicia faba* (Dey and Pridham, 1972). Divalent ions as Cu^{+2}, Zn^{+2} and Fe^{+2} were strong inhibitors, where Mg^{+2} showed no effect on α-galactosidase from *L. helveticus* ATCC 10797 which is similar to that of *Monascus pilosus* (Wong *et al.*, 1986).

Mn^{+2} and Ca^{+2} activated α-galactosidase from *L. helveticus* ATCC 10797 with concentrations of 1 and 10 mM, while it showed no effect on α-galactosidase from *L. fermentum* (Garro *et al.*, 1996). Mn^{+2} and Ca^{+2} inhibited α-galatactosidase activity from *L. helveticus* ATCC 10797 at 100 mM concentration, while they inhibited the activity with 1 mM using the enzyme of *Thermoaerobacterium polysaccharolyticum* (King *et al.*, 2002).

The presence of chelating agent (EDTA) had no effect on the enzyme activity from *L. helveticus* ATCC 10797 at concentrations of 1 and 10 mM (Figure 2.11), which was similar to of *Monascus pilosus* (Wong *et al.*, 1986) and *L. fermentum* (Garro *et al.*, 1996) whereas the activity was decreased to certain extend with 100 mM. This result suggests that metal cations are involved in the catalytic sites of the enzyme.

Different sugars had different inhibitory effect on α-galactosidase activity from *L. helveticus* ATCC 10797 (Figure 2.12). Galactose and glucose are the strongest in inhibiting α-galactosidase activity followed by sucrose. Also α-galactosidase was inhibited strongly by melibiose, raffinose and stachyose when their final concentration is 100 mM. Fructose seems to have the least inhibitory effect. These results are similar to the reported sugars of *L. fermentum* (Schuler *et al.*, 1985) that was inhibited by galactose, fructose and sucrose. On the other hand, α-galactosidases from *Tenebrio molitor* and *Spodoptera frugiperda* (Grossmann and Terra, 2001) were inhibited by melibiose, raffinose and stachyose. Although glucose is well known as inhibitor in *Vicia faba* (Dey *et al.*, 1986) and *Aspergillus ficcum* (Zapter *et al.*, 1990), it was reported as activator for α-galactosidase from *Candida javanica* (Cavazzoni *et al.*, 1987).

Melibiose, raffinose and stachyose are commonly used substrates in examination of α-galactosidase activity. Melibiose was hydrolyzed to glucose and galactose (Figure 2.16), raffinose was hydrolyzed to galactose and sucrose (Figure 2.15), while stachyose was hydrolyzed to galactose and sucrose with raffinose as the intermediate compound (Figure 2.14) as analyzed by TLC when incubated with α-galactosidase from *L. helveticus* ATCC 10797. Similar results were found with α-galactosidase of *Monascus pilosus* (Wong *et al.*, 1986).

GENERAL CONCLUSIONS

An α-galactosidase from *L. helveticus* ATCC 10797 was purified for the first time and found to be produced intracellularly. This enzyme was purified to 9.44 fold over the crude extract with a recovery of 1.8%. The molecular mass was estimated to be 188 kDa by gel filtration, but 90 kDa by SDS-PAGE, indicating that my enzyme has two similar molecular weight subunits. The Km of 3.83 mM and Vmax of 416.66 μM/min/mg protein were calculated with PNPG (10 mM).

The enzyme was found to be stable between 20 and 30 °C and maximum activity was observed at 37 °C. It lost the activity rapidly at this temperature and above. According to these results, it was concluded that this enzyme was not suitable for enzyme processing at elevated temperatures. However, the pH studies demonstrated that this enzyme could be used for enzyme processing because the optimum activity was very close to neutralilty.

The enzyme activity was increased by the presence of monovalent inos K^+, NH_4^+, Li^+ and Cs^+, but not affected by Rb^+. On the other hand, it was decreased by the presence of divalent ions as Cu^{+2}, Zn^{+2} and Fe^{+2} at low concentrations and Mn^{+2} and Ca^{+2} at 100 mM but not affected by Mg^{+2}. EDTA has no effect at low concentrations, but at 100 mM the activity was decreased.

Galactose and glucose showed strongest inhibitory effect followed by sucrose. Melibiose, raffinose and stachyose had no effect on the enzyme activity using 1 and 10 mM, but at 100 mM they inhibited the enzymatic activity strongly. Fructose was observed to have the least inhibitory effect on the enzyme activity.

Melibiose was hydrolyzed to galactose and glucose. Raffinose was hydrolyzed to galactose and sucrose. Stachyose was hydrolyzed to galactose and sucrose with raffinose as intermediate product by the action of α-galactosidse.

The cloning and over expression of this enzyme in the future studies could be useful in the industrial applications.

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