RELEASE AND CHARACTERIZATION OF BETA-GALACTOSIDASE FROM LACTOBACILLUS PLANTARUM

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

FIRAT KARA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

DECEMBER 2004

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Canan Özgen

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Assoc. Prof. Dilek Sanin

Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science

Asst. Prof. Ayşegül Çetin Gözen	Assoc. Prof. Dr. Candan Gürakan
Co-Supervisor	Supervisor

Examining Committee Members

Prof. Dr. Meral Yücel	(METU, BIO)	
Assoc. Prof. Dr. Candan Gürakan	(METU, FDE)	
Prof. Dr. Faruk Bozoğlu	(METU, FDE)	
Prof. Dr. Alev Bayındırlı	(METU, FDE)	
Prof. Dr. Sedat Dönmez	(AU, FDE)	

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Kara Fırat

Signature :

ABSTRACT

RELEASE AND CHARACTERIZATION OF BETA-GALACTOSIDASE FROM LACTOBACILLUS PLANTARUM

Kara, Fırat

M.S., Department of Biotechnology

Supervisor : Assoc. Prof. Dr. Candan Gürakan

Co-Supervisor : Asst. Prof. Ayşegül Çetin Gözen

December 2004, 122 pages

The enzyme, β-galactosidase (E.C.3.2.1.23) has been used for dairy industry for removing lactose from milk and milk by-products.

In this study, three strains namely *L. plantarum* NCIMB 1193, *L. plantarum* DSM 20246 and *L. plantarum* E081 were used for β-galactosidase release by sonication method. The peak of the total enzyme activity was found to be corresponding to late logarithmic or early stationary phase of all strains.

As a disruption method sonication was used for the release of β-galactosidase. Meanwhile, the sonication time was optimized for each strain. The peak of the enzyme activity was observed between 210 seconds and 270 seconds of sonication period. It was also found that sonication did not decrease the viability of *L.plantarum* NCIMB 1193 significantly. Liquid nitrogen cell disruption method was also used to compare the results with those obtained by sonication method.

For characterization β-galactosidase, cell-free crude extract of sonicated cell culture of *L.plantanrum* NCIMB 1193 was used. Optimum pH found as 7.2, and optimum temperature range was found between between 35^0 C to 40^0 C. K_m and V_{max} values were found as 3.47 mM and 1.721 (μmol / min per mg protein) respectively from Lineweaver-Burk plot. K_m and V_{max} values were found as 4.064 mM and 1.863 (μmol / min per mg cell-free crude extract) respectively from Eadie-Hofstee plot. The number of ligand binding sites (n_{app}) on a molecule of β-galactosidase was found as 1.03 which indicates that the number of ligand binding sites on the enzyme is one.

Key Words: β-galactosidase, ONPG, sonication, lactose , *L. plantarum*

ÖZ

LACTOBACILLUS PLANTARUM'DAN BETA-GALAKTOSİDAZIN SALGILANMASI VE KARAKTERİZASYONU

Kara, Fırat
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Yöneticisi : Doç. Dr. Candan Gürakan
Ortak Tez Yöneticisi : Y. Doç. Ayşegül Çetin Gözen

Aralık 2004, 122 sayfa

β-galaktosidaz enzimi (E.C.3.2.1.23), sütte ve süt ürünlerinde laktozun tüketilmesinde süt endüstrisinde kullanılmaktadır.

Bu çalışmada, β-galaktosidazın sonikasyon yöntemiyle salgılanmasında *L. plantarum* NCIMB 1193, *L. plantarum* DSM 20246 ve *L. plantarum* E08 suşları kullanılmıştır. Bu üç organizmanın da enzim aktivitesinin en yüksek olduğu yer geç-logaritmik büyüme fazına ya da erken durgun faza denk geldiği bulunmuştur.

Hücre parçalama yöntemi olan sonikasyon, β-galaktosidazın salgılanması için kullanıldı. Aynı zamanda, her bir suş için sonikasyon zamanın optimizasyonu gerçekleştirildi. Enzimin aktivitesinin en yüksek değerleri 210 saniye ve 270 saniye sonikasyon süreleri arasında bulundu. Buna ek olarak sonikasyonun, *L.plantarum* NCIMB 1193 suşunun yaşayan hücre sayısını önemli bir miktarda azaltmadığı bulundu. Ayrıca, sonikasyon yöntemiyle elde edilen sonuçlarla karşılaştırmak üzere sıvı nitrojen hücre parçalama yöntemi kullanıldı.

Sonike edilmiş *L.plantanrum* NCIMB 1193'ün hücre içermeyen özütü, β-galacktosidazın karakterizasyonu için kullanıldı. β-galaktosidazın optimum pH değeri 7.2 olarak ve optimum sıcaklığı da 35^0C-40^0C arasında bulundu. Lineweaver-Burk grafiği çizilerek, K_m ve V_{max} değerleri sırasıyla 3.47 mM ve 1.721 (µmol / dakika mg hücre içermeyen özütteki protein miktarı) olarak bulundu. Eadie-Hofstee grafiği çizilerek, K_m ve V_{max} değerleri sırasıyla 4.064 mM ve 1.863 (µmol / dakika mg hücre içermeyen özütteki protein miktarı) olarak bulundu β-galaktosidaz molekülündeki ligand bağlanma yerinin sayısı 1.03 olarak bulundu. Bu enzim üzerindeki ligand bağlanma yerlerinin sayısının bir olduğu anlamına gelmektedir.

Anahtar Kelimeler: β-galactosidaz, ONPG, sonikasyon, lactoz, *L. plantarum*

To My Grandmother

ACKNOWLEDGEMENTS

I convey my appreciation to Assoc. Prof. Dr. Candan Gürakan who as my supervisor has offered me research guidance when necessary and has believed in me and allowed me to work freely whenever possible.

I would like to thank Prof. Dr. Meral Yücel, Prof. Dr. Faruk Bozoğlu and Prof. Dr. Alev Bayındırlı, Prof. Dr.Haluk Hamamcı, Assist. Dr. Ayşegül Çetin Gözen, Prof. Dr. Sedat Dönmez for their valuable guidance, suggestions and comments. The technical assistance of Aytekin Güler, Elif Doğan, and Fatma Çoşkun is gratefully acknowledged.

I would like to thank Ceren Aksoy, Sevgin, Esra, Ayşem Batur, Gül Sarıbay, Laktoz Group Members and all people who are working in Biotechnology Laboratory of Food Engineering Department for their patience and technical support I would like to thank all the assistants in Food Engineering Department for their friendship.

I would like to thank to Assist. Dr. Alev Yemenici for her guidance, support suggestions and comments.

My special thanks go to Yeşim Kantaş for her valuable guidance, friendship, and support during the days of hard study.

I would like to thank Ufuk Dalmış for his love, patience, support, and friendship.

I would like to thank my family for their support, encouragement and patience.

This study was supported by BAP. Grant No 2004-07-02-00-40

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

BOD	Biological Oxygen Demand
BSA	Bovine serum albumin
CAP	Catabolite activator protein
CCR	Carbon catabolite repression
CDW	Cell Dry Weight
CFU	Colony forming unit
Ed.	Edition
E081	*Lactobacillus plantarum* E081
Glu	Glutamic acid
GRAS	Generally regarded as safe
L	*Lactobacillus*
LAB	Lactic acid bacteria
Lac	Lactose-
Lb	*Lactobacillus*
Lc	*Lactococcus*
MMRS	Modified MRS
PEP	Phoshoenolpyruvate
PTS	Phosphotransferase systems
ONP	O-nitro Phenol
ONPG	O-Nitrophenyl-β-D-galactopyranoside
subsp.	Subspecies
X-gal	5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside
ε	Extinction coefficient
1193	*Lactobacillus plantarum* NCIMB 1193
20246	*Lactobacillus plantarum* DSM 20246

CHAPTER I

INTRODUCTION

The lactose-hydrolysing enzyme, β-galactosidase (β-D-galactoside galacto hydrolase, E.C.3.2.1.23, trivially lactase) has long been accepted as an important enzyme for dairy industry. β-galactosidase catalyse two reactions: it catalyses hydrolysis of lactose, the milk sugar into glucose and galactose and in some cases β-galactosidase is able to catalyse transglycosylation reactions. In dairy industry β-galactosidase has been used to prevent crystalization of lactose, to improve sweetness, to increase the solubility of the milk product. Moreover, it has been used to produce low lactose containing food products for low lactose tolerance people and for the utilization of cheese whey, which would otherwise be an environmental pollutant (Sani et al. 1999; Rouwenhorst et al. 1989). If an economical attractive method of hydrolysis is developed and a suitable microorganism sourse is found, more uses for lactose and dairy products containing lactose will become feasible (Bury and Jelen 2000; Shah and Jelen 1991).

Enzyme preparations used to produce lactose free products are highly purified proteins. The more the protein purified, the higher its cost become. Therefore the cost of the lactose-reduced milk is about 80% higher than the regular unhydrolysed milk (Bury and Jelen 2000). Cell lysis by sonication has been shown to increase the β-galactosidase activity several times (Shah and Jelen 1991; Toba et al. 1990; Wang et al. 1996; Wang and Sakakibara 1997; Feliu et al. 1998). Shah and Jelen (1991) proposed the use of the sonicated dairy cultures to produce a relatively impure source of β-galactosidase for a potentially more economical process of lactose hydrolysis (Bury and Jelen 2000).

The commercial enzymes used for lactose hydrolysis are β-galactosidase of diverse

origins (Jurado et al. 2002). Posible sources of the enzyme are: plants, animal organs, bacteria, yeasts (intracellular enzyme), fungi and moulds (extracellular enzyme) (Gekas and Leiva 1985). Among them bacterial sources are preferable because of ease of fermentation, high activities of the enzyme and good stability (Sani et al. 1999). Lactic acid bacteria (LAB) which constitute a diverse group of lactococci, streptococci, and lactobacilli have become a focus of scientific studies for three particular reasons (Somkuti et al. 1998): a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects b) LAB are generally regarded as safe (GRAS) so the enzyme derived from them might be used without extensive purification (Vasiljevic and Jelen 2002) c) some strains have probiotic activity such as improved digestion of lactose (Vinderola and Reinheimer 2003).

1.1 Lactic Acid Bacteria

Residues of cheese in an Egyptian pot dating from 2300 BC and passages in the Bible (Anonymous) indicate that lactic acid bacteria have been used for the fermentation and preservation of human food stuffs for at least 4-5 millennia (Davidson et al. 1995).

Members of the lactic acid bacteria have been defined on the basis of cell morphology, DNA base composition, and type of fermentative metabolism (Madigan et al. 1997). Recently application of molecular genetic techniques to determine the relatedness of food-associated lactic acid bacteria has resulted in significant changes in their taxonomic classification. The lactic acid bacteria associated with foods, now include species of the genera *Carnobacterium*, *Oenococcus, Tetragenococcus, Vagococcus, Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus* (Davidson et al. 1995;

for references, see Stiles and Holzapfel 1997).

The lactic acid bacteria are gram-positive, usually non-motile, nonsporulating, catalase negative, cocci, coccobacilli, or rods bacteria that produce lactic acid as a major or sole product of fermentative metabolism. Lactic acid bacteria have less than 55 mol% G+C content in their DNA (Stiles and Holzaphel et al. 1997). Therefore they are called low G-C gram positive bacteria. Members of this group lack porphyrins and cytochromes, do not carry out electron transport phosphorylation, and hence obtain energy only by substrate level phosphorylation. Lactic acid bacteria are aerotolerant anaerobes. Most lactic acid bacteria obtain energy only from the metabolism of sugars and related fermentable compounds and hence are usually restricted to habitats in which sugars are present.

One important difference between subgroups of the lactic acid bacteria lies in the nature of the products formed during the fermentation of sugars. One group, called homofermentative, produces virtually a single fermentation product, lactic acid, whereas the other group, called heterofermentative, produces mainly ethanol and CO_2 as well as lactate. The differences observed in the fermentation products are determined by the presence or absence of the enyzme aldolase, one of the key enzyme in glycolysis. Heterofermenters can not break down fructose bisphosphate to triose phosphate. Instead they oxidize glucose 6-phosphate to 6-phosphogluconate and then decarboxylate this to pentose phosphate, which is broken down to triose phosphate and acetylphosphate by means of the enzyme phosphoketolase. In Figure 1 homofermentative and heterofermentative mechanism is shown.

In heterofermenters, triose phosphate is converted ultimately to lactic acid with the production of 1 mol of ATP, while the acetylphosphate accepts electrons from the NADH generated during the production of pentose phosphate and is thereby converted to ethanol without yielding ATP. Because of this, heterofermentors produce only 1 mol of ATP from glucose instead of the 2 mol produced by

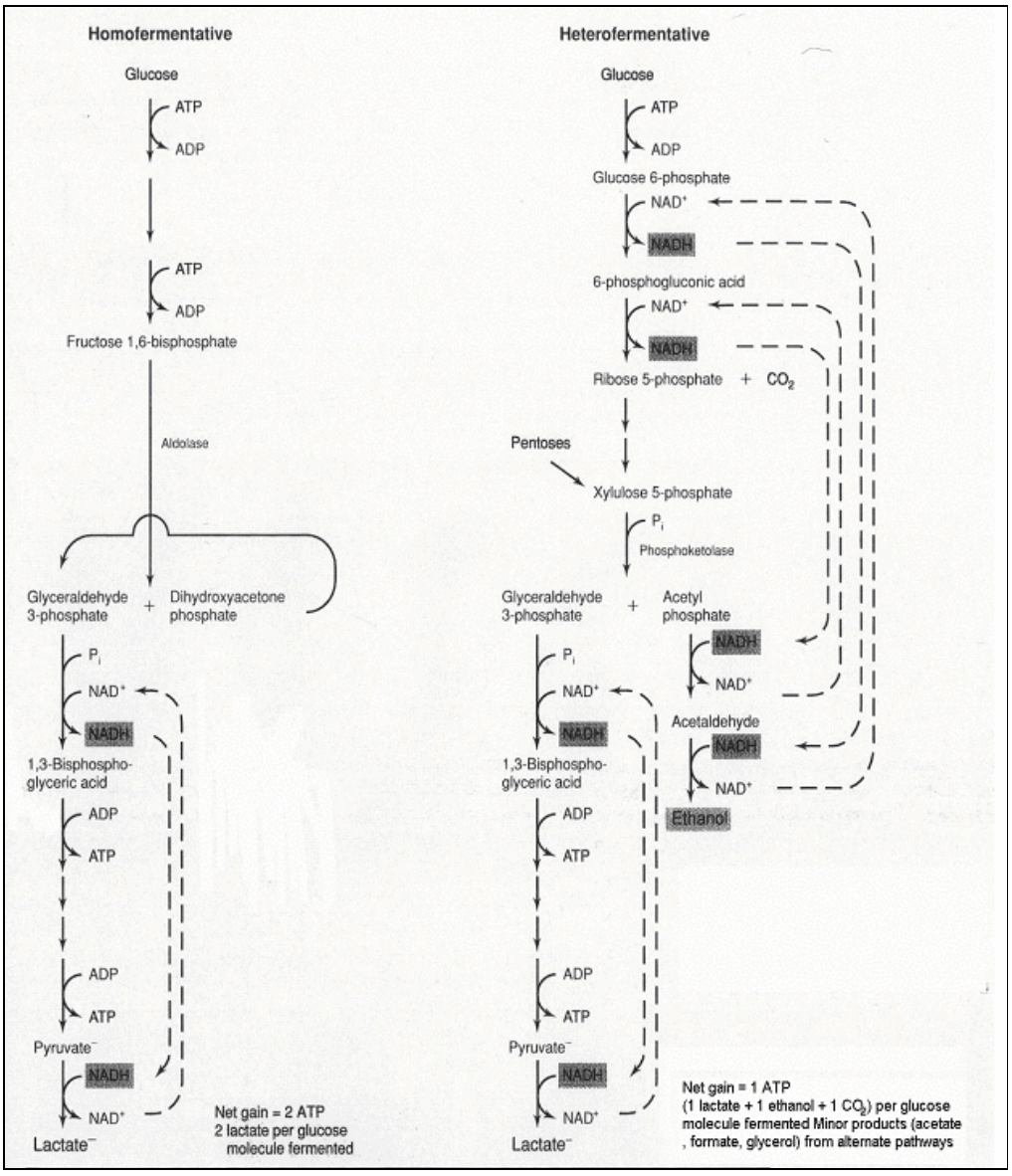

Figure 1. The fermentation of glucose in homofermentative and heterofermentative lactic acid bacteria. No ATP is made in reactions leading to ethanol formation (Madigan 1997).

homofermenters. This difference in ATP yield from glucose is reflected in the fact that homofermenters produce twice as much cell mass as heterofermenters from the same amount of glucose (Madigan et al. 1997).

1.1.1 *Lactobacillus* Strain

Lactobacilli are members of lactic acid bacteria**.** They are typically rod-shaped, varying from long and slender to short, bent rods. Most species are homofermentative, but some are heterofermantative (Madigan et al., 1997). The current taxonomic status of the lactobacilli based on the classical phenotypic subdivision in Table 1 was derived from different reviews (for references, see Stiles and Holzapfel 1997). In Table 1, group 1 includes the obligately homofermentative lactobacilli that ferment glucose exlusively to lactic acid and do not ferment pentoses of gluconate. Group two includes the facultatively heterofermentative lactobacilli that ferment hexoses to lactic acid and may produce gas from gluconate but not from glucose. They also ferment pentoses. Group three includes the obligately heterofermentative lactobacilli that ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. The production of gas from glucose is, a characteristic feature of obligately heterofermentative bactereia (for references, see Stiles and Holzapfel 1997).

The lactobacilli are strictly fermentative and have complex nutritional requirements. They grow in and are associated with many different habitats (Table 2) (for references, see Stiles and Holzapfel 1997). Lactobacilli belong to the normal mucosal flora of the human mouth and intestine. The most common lactobacilli in the mucosae of healty subsects were *Lactobacillus plantarum*, *L. rhamnosus*, and *Lactobacillus paracasei* subsp. *paracasei*, which were isolated from 52%, 26%, and 17%, of the individuals, respectively (both from rectal and oral mucosae). Most *L.*

Table 1. Major divisions within the genus *Lactobacillus* based on phenotypic characteristics (for references, see Stiles and Holzapfel 1997).

Group 1	Group 2	Group 3
Obligate Homofermenters	Facultative Heterofermenters	Obligate Heterofermenters
Lb. acidophilus *Lb. amylophilus* *Lb. amylovorus* *Lb. aviarius* subs.*araffinosus* subs. aviarius *Lb. crispatus* ***Lb. delbrueckii*** subs. ***bulgaricus*** subs ***delbrueckii*** subs. ***lactis*** ***Lb. farciminis*** *Lb. gallinarum* ***Lb. gasseri*** ***Lb. helveticus*** Lb. jensenii ***Lb. johnsonii*** ***Lb. kefiranofaciens*** ***Lb. kefirgranum*** ***Lb. mali*** ***Lb. ruminis*** ***Lb. salivarius*** subsp. salicinus subsp. salivarius *Lb. sharpeae*	***Lb. acetotolerans*** Lb. agilis ***Lb. alimentarius*** ***Lb. bifermentans*** ***Lb. casei*** ***Lb. coryniformis*** subsp. coryniformis subsp. torquens ***Lb. curvatus*** Lb. graminis Lb. hamsteri ***Lb. homohiochii*** Lb. intestinalis Lb. murinus ***Lb. paracasei*** subsp. ***paracasei*** subsp. ***tolerans*** ***Lb. paraplantarum*** ***Lb. pentosus*** ***Lb. plantarum*** ***Lb. rhamnosus*** ***Lb. sake***	***Lb. brevis*** ***Lb. buchneri*** ***Lb. collinoides*** ***Lb. fermentum*** ***Lb. fructivorans*** *Lb.fructosus*[a] ***Lb. hilgardii*** ***Lb. kefir*** ***Lb. malefermentans*** Lb. oris ***Lb. panis*** ***Lb. parabuchneri*** ***Lb. parakefir*** ***Lb. pontis*** ***Lb. reuteri*** ***Lb. sanfrancisco*** ***Lb. suebicus*** ***Lb. vaccinostercus*** ***Lb. vaginalis***
.		[a] *Lb. fructosus* classified with the *Leuconostoc* group of lactic acid bacteria
Bold face, lactobacilli of importance in foods and as probiotics		

plantarum strains of intestinal origin can adhere to cell lines of intestinal origin because they possess a mannose binding adhesin(for references, see Molin 2001).

Lactobacilli are used as starter cultures for several varieties of cheese, fermented plant foods, fermented meats, in wine and beer production, sourdough bread and silage (for references, see Stiles and Holzapfel 1997). Lactobacilli are used extensively in the dairy industry for the manufacture of Bulgarian buttermilk, yogurt, Kefir, Koumiss, and Swiss, Emmental, and Italian cheese; they are present in Cheddar cheese mostly as contaminants. The lactobacilli produce a greater range of end products than do the lactic acid (group N) streptococci (traditional starter for Cheddar cheese manufacture) and appear to be less susceptible to attack by bacteriophages (Hickey et al. 1986). There is also another area where lactobacilli are extensively used. It is known that lactobacilli, which have been used in food products, have beneficial effect on health of human and animals. In other words they have probiotic potential. As a definition, probiotics are live microbiol food supplements, which benefit the health of consumers by maintaining, or improving their intestinal balance (Mattila-Sandholm et al. 2002). In the following two sections the general characteristics of *Lactobacillus plantarum* and its potential to be used as probiotics will be discussed.

1.1.1.1 *Lactobacillus plantarum*

Lactobacillus plantarum are catalase negative gram positive bacteria. Cell walls of *Lactobacillus plantarum* contain either ribitol or glycerol teichoic acid. They can be isolated from dairy products, and environments, silage, sauerkraut, pickled vegetables, sour dough, cow dung, and the human mouth, intestinal tract, and stools, and from sewage (for references, see Stiles and Holzapfel 1997).

Table 2. Habitats of the genus *Lactobacillus* (Stiles and Holzapfel 1997)

Humans
Oral cavity
Intestinal tract
Vagina
Other habitats
Plants and plant materials
Soil, water, sewage and manure
Food fermentations (milk, meat, and vegetable)
Cereal products
Silage
Food spoilage
Beer
Fruit and grain mashes
Marinated fish
Sugar processing
Milk
Meat and meat products
Fermented bevetages

Generally *Lactobacillus plantarum* strain do not grow at 45^0 C. However some strains have been reported to grow at 45^0 C, the general ability to grow 15^0 C serves as conformation for the allocation of *Lactobacillus plantarum* to the streptobacteria. *Lactobacillus plantarum* is used as a starter organism in some fermented sausages and cereal products; it is a part of the adventitious LAB growing in fermented vegetable and meat products and it is a spoilage organism in citrus juice, wine and some cheeses (for references, see Stiles and Holzapfel 1997).

1.1.1.2 Use of *Lactobacillus* Strain as Probiotic

Probiotics are live microbiol food supplements, which benefit the health of consumers by maintaining, or improving their intestinal balance (Mattila-Sandholm et al. 2002).

Lactobacilli have become a focus of scientific studies as a potential probiotic microorganism for five particular reasons: (a) lactose maldigesters may consume some dairy products, fermented with lactobacilli, with little or no adverse effects such as yogurt (b) lactobacilli are generally regarded as safe so the enzyme (β-galactosidase) derived from them might be used without extensive purification (Vasiljevic and Jelen 2002) (c) they have ability to adhere to host cells (d) they have ability to exlude or reduce pathogenic bacteria by producing acids, hydrogen peroxide and bacteriocins (Chang et al. 2001) (e) they have resistance to bile and they have ability to tolerate low pH values (for references, see Vinderola and Reinheimer 2003).

The strain *Lactobacillus plantarum* 299V, originates from the human intestinal mucosa, has been shown in rats to decrease traslocation, improve mucosal status, improve liver status, improve the immunologic status of the mucosa, and reduce

mucasal inflammation. In humans, *Lactobacillus plantarum* 299V can increase the concentration of carboxylic acids in feces and decrease abdominal bloating in patients with irriversible bowel disease (Molin 2001).

1.1.1.3 Lactose Intolerance and Malabsorbtion

1.1.1.3.1 Improvement of lactose metolism by using *Lactobacillus* strain as a probiotic

Gurr (1987) mentioned that to be efficiently absorbed from the gut, lactose must be digested into its constituent sugars, glucose and galactose, by the enzyme lactase.

The enzyme lactase is present in the gut to digest the milk lactose. In most of the world's races this enzyme is lost during the first or second decade of life and only people of Northern European origin, their overseas descendents and some isolated African and Indian communities maintain a high intestinal lactase activity throughout life. Incidence of lactase non-persistent in different population groups around the world is shown in Table 3 (Fernandes et al. 1987; Sanul 1990). It is generally believed that the amount or activity of the enzyme is not influenced by lactose in the diet.

Lactase insufficiency means that the concentration of the lactose-cleaving enzyme β-galactosidase, also called lactase, in the brush border membrane of the mucosa of the small intestine is too small. This hypolactasia causes insufficient digestion of the disaccharide lactose, a phenomenon called lactose malabsorbtion or, more precisely, lactose maldigestion. Hypolactasia and lactase maldigestion accompanied by clinical symptoms such as bloating, flatulence, nausea, diarrhea, and abdominal pain is termed lactose intolerance (de Vrese et al. 2001)

Lactase non-persistent (lactose intolerant) persons do not have the ability to synthasize lactase. It has been proposed that lactase producing bacteria can release its enzyme therefore when the lactase producers contaning products are consumed digestion of lactose can be achieved by bacterial enzyme activity (Gurr 1987). As Marteau et al. (2001) summerized that persons with lactose maldigestion experience better digestion and tolerance of the lactose contained in yogurt. At least two mechanisms, which do not exclude each other, have been shown: digestion of lactose in the gut lumen by the lactase contained in the yogurt bacteria (the yogurt bacteria deliver lactose when lyzed by bile acids) and slower intestinal delivery or transit time of yogurt compared with milk.

One of the advantages of using *Lactobacillus* strain as β-galactosidase source in milk or milk products to catalyse the hydrolysis reaction of lactose is they have been accepted as GRAS organism and their enzymes can be used in food products.

Lactase is intracellular enzyme in *Lactobacillus*. But the enzyme can be released into the medium by mechanical disruption methods. Although probiotic viability would be a reasonable measure of probiotic activity, but for improved digestion of lactose, cell viability is not required for probiotic activity (for references, see Vinderola and Reinheimer 2003). In these case, health beneficial effect has been linked to β-galactosidase activity of disrupted cells. Naidu et al., (1999) introduced the concept of "Probiotic-Active Substance", as a cellular complex of lactic acid bacteria that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independently of viability of lactic acid bacteria.

1.2 Lactase, An Overview

Lactase is trivial name of the enzyme β-D-galactosidase or more formerly β-D-

Table 3. Incidence of lactase non-persistent in different population groups around the world (Fernandes et al., 1987; Sanul 1990).

Group	No. of subjects	% lactose intolerance/ hypolactasia
U.S.A		
White	19-138	6-21
Black	20-41	70-75
Indian	3	67
Africa		
Uganda	135	72
S.Africa	38	90
Nigeria	11-48	58-99
Europe		
Greek Cypriots	17	88
Switzerland	18	17
Finland	504	17
Denmark	700	6
Czechoslovakia	17	18
Poland	21	29
Germany	55	15
Greece	16	38
Turkey (Sanul,	30	15
1990)	33- 50	6- 34
U.K		
Asia	73	100
Chinese	4	100
Korean	2	100
Japan	15	100
Malaysia	10	100
Philippines	39-140	97- 100
Thailand	67	81
Arabs	10- 100	0- 8
Australia		

galactoside galactohydrolase (Gekas and Leiva 1985). β-galactosidase was among the first hydrolyses to be discovered (Rouwenhorst et al. 1989). β-galactosidase not only catalyses the hydrolysis of β-galactosidic linkage, but also catalyses transglycosylation reactions. β-D-Galactopyranosides, such as lactose, are thereby converted to galactooligosaccharides by the mechanism described by Prenosil et al.(1987).

The most commonly found natural substrate for this enzyme is lactose, the main sugar of milk and several dairy products (Ladero et al. 2002). Lactose, however, is not the only substrate and not always the best. Some enzymes in this group hydrolyze alpha-L-arabinosides; some animal enzymes also hydrolyze beta-D-fucosides and beta-D-glucosides (Pomeranz 1964).

1.2.1 Hydrolysis Mechanism

Wallenfels and Malhotra (1960; 1961) were described the mechanism of lactose hydrolysis by using lactase obtained from *E.coli*. Also in several review articles the mechanism of lactose hydrolyis and galactosyl transfer reaction have been decribed (Richmond 1981; Mahoney 1990; Zhou and Chen 2001). The reaction mechanism proposed in these articles was that the active side of β-galactosidase contains the cyteine and histidine amino acids which function as proton donor and proton accepter, respectively. Cysteine contains the sulphydryl group (acting as a general base) acted as proton donor and histidine residues (contains imidazole group as a substructure) acted as nucleophile site to facilitate splitting of the glycosidic bond, respectively, during the enzymatic hydrolysis procedure (Richmond 1981; Hart et al. 9. ed.; Mahoney 1990; Zhou and Chen 2001). Proposed mechanism of lactose hydrolysis by β-galactosidase is represented in Figure 2. The galactosyl transfer reaction is shown in Figure 3.

Recently a new active side for β-galactosidase has been suggested and widely accepted. Glutamic acid residue was suggested as the new active site. The β-galactosidase from a variety of microbiol origins has two glutamic acid residues (such as Glu^{482} and Glu^{551}) as the proton donor and the nuclephile/base at the same time in the enzymatic reaction. The reaction mechanism is shown in Figure 4. The first step is the enzyme-galactosyl complex formation and simultaneous glucose liberation. In the second step, the enzyme-galactosyl complex is transferred to an acceptor containing a hydroxyl group. While in a diluted lactose solution, water (rather than other sugars such as glucose, lactose) can be more competitive to be an acceptor, therefore galactose is formed and released from the active side. On the other hand, in high lactose content solution, lactose molecule has more changes to act as the acceptor, binding with the enzyme-galactose complex to form oligosaccharides (Zhou and Chen 2001).

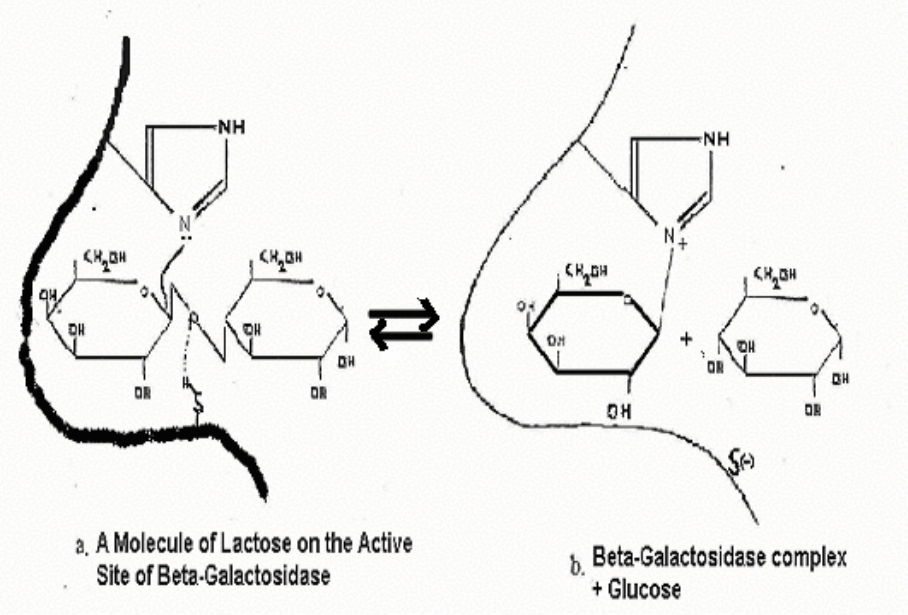

Figure 2. Proposed mechanism of lactose hydrolysis by β-galactosidase. Sulfuhydryl group acted as proton accepter and imidazole group acted as proton donor (Richmond 1981).

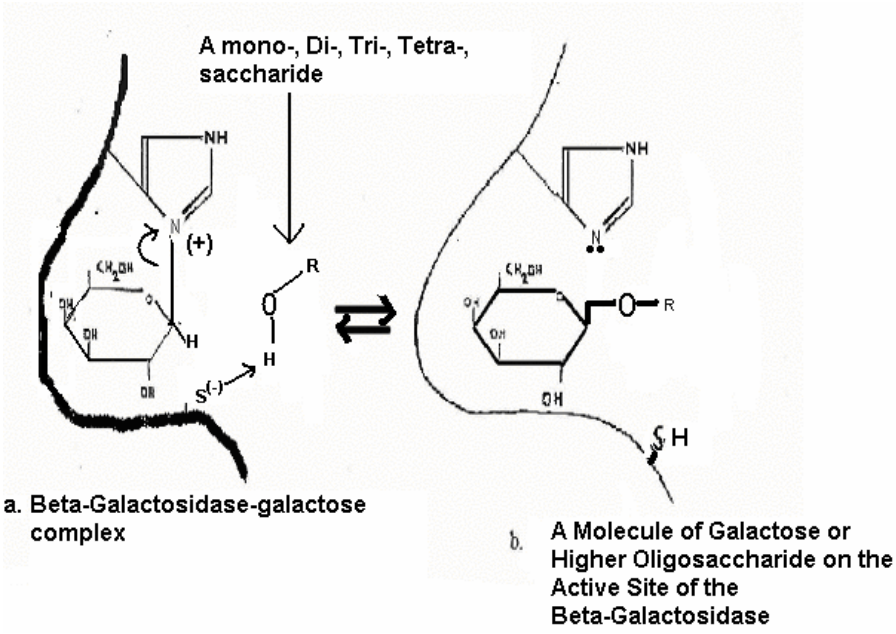

Figure 3. Proposed mechanism of galactosyl transfer reaction catalyzed by β-galactosidase (Richmond 1981).

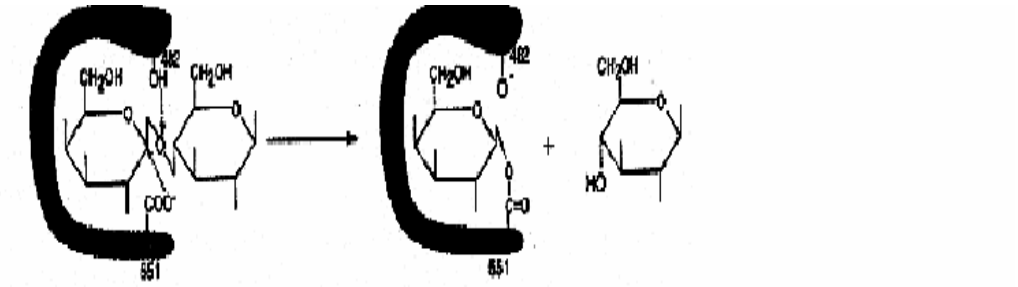

Figure 4**.** Schematic mechanism of the lactose hydrolysis by β-galactosidase. This mechanism has been suggested recently. Glutamic acid functions at active sites as the proton donor (Glu^{482}) and the nuclephile/base (Glu^{551}) in the enzymatic reactions (Zhou and Chen 2001).

Although the enzymes derived from various microbial origins have different properties, such as molecular weight, protein chain length, and the position of the active site; it has been found recently that β-galactosidase from different sources have the same amino acid residue, glutamic acid, as their catalytic site, as shown in Table 4 (Zhou and Chen 2001).

Table 4. Active sites and other physical properties of β-galactosidase from various microbial origins (Zhou and Chen 2001)

Enzyme origin	*K. lactis*	*E.coli*	*E.coli*	*A. niger*
Molecular weight (Da)	117618	116351	118016	119160
Length (AA)	1025	1023	1031	1006
Proton donor	Glu ^{482}	Glu ^{461}	Glu^{449}	Glu^{200}
Nucleophile/base	Glu ^{551}	Glu^{537}	Glu^{512}	Glu^{298}

1.2.2 The Activation or Inhibition of β-Galactosidase from Different Sources by Mono- and Divalent Cations

Mono- and divalent cations effect on lactase has been well documented (Kreft and Jelen 2000; Greenberg and Mahoney 1982; Vasiljevic and Jelen 2002; Garman et al. 1996). Divalent cations such as magnesium and manganase may enhance the β-galactosidase activy, while monovalent cations may have a positive or negative effect(Pivarnik and Rand 1992; Garman, Coolbear and Smart 1996; Kreft and Jelen 2000).

Garman and others (1996) studied 6 species of lactic-acid bacteria. The rate of hydrolysis of lactose by β-galactosidase activity from each of the lactic acid bacteria studied was in all cases enhanced by Mg^{+2}, while the effect of K^{+} and Na^{+} differed

from strain to strain. Mg^{+2} has been found to be a major activator of β-galactosidase hydrolytic activity in *S .thermophilus* (Greenberg and Mahoney 1982), *Lactobacilli*, and bifidobacteria (Garman et al. 1996). Greenberg and Mahoney (1982) proposed that both monovalent and divalent cations were required for maximum activity of partialy purified β-galactosidase from *Streptococcus thermophilus* but not for stability. The effects of K^+ and Na^+ on enzyme activity varied greatly between species. Becker and Evans (1969) suggested that association of monovalent cations with a β-galactosidase was on the basis of ionic radius with Na^+ being more tightly bound than K^+, and that both ions affected acitivity by inducing conformational changes in the enzyme structure.

Ca^{+2} is a known inhibitor of β-galactosidase, almost all of the calcium in milk is bound to casein and therefore not free in solution. Therefore it does not inhibit β-galactosidase activity (for references, see Garman et al. 1996).

1.2.3 Mechanism of Lactose Transport in Lactic Acid Bacteria

Most bacterial cells have the capacity to utilize several carbohydrates as carbon and energy source and posses various transport proteins and catabolic enzymes for the metabolism of the different carbohydrates (Poolman 2002). The sytems by which the carbohydrate molecules are transported can be subdivided into 3 classes that differ in their mechanism of energy coupling: (a) primary transport systems, (b) secondory transport systems and (c) phosphotransferase systems (PTS) (Poolman 1993). These different mechanisms of transport have been observed for a wide variey of sugars but only those that mediate lactose(galactoside) transport in lactic acid bacteria will be described in detail.

In case of lactose transport, generally secondary transport systems and PEP–PTS are used in lactic acid bacteria.(for references, see Hickey et al. 1986). This two mechanisms use different enyzmes to catalyse the hydrolization reaction of lactose. β-galactosidase enzyme is the marker enzyme for secondory transport system, on the other hand P-β-galactosidase is marker enzyme of phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (Hickey et al. 1986; Poolman 2002; Postma et al. 1993). In the secondary transport system, lactose is not chemically modified. But in PEP-PTS case, translocation of lactose across the cytoplasmic membrane is coupled to the chemical modification of the molecule, ie. transport followed with phosphorylation of the lactose by PTS.

1.2.3.1 Primary Transport Systems

Primary transport systems use the energy driven by the hydrolysis of an energy-rich chemical bond (eg. ATP; Figure 5) for translocation of a sugar. In the lactic acid bacteria, the ATP-binding cassette transporters are the most abundant class of transporters in primary transport systems. ATP-binding cassette transporters are used not only to accumulate substrates and compatible solutes, but also to excrete unwanted products such as drugs (Poolman 2002). The number of ATP molecules hydrolyzed per solute taken up by the transport ATPases is most likely 1-2 which makes these transporters energetically expensive as compared to the ion-linked transporters, exchange systems and PTS. Downstream of generally accepted (putative) lactose transport ATPase genes of *Lc lactis*, two translationally coupled genes (*lacL* and *lacM*) have been found to encode a funtional β-galactosidase (LacZ) of *S thermophilus*, *Lb bulgaricus* and *E.coli* (for references, see Poolman 1993).

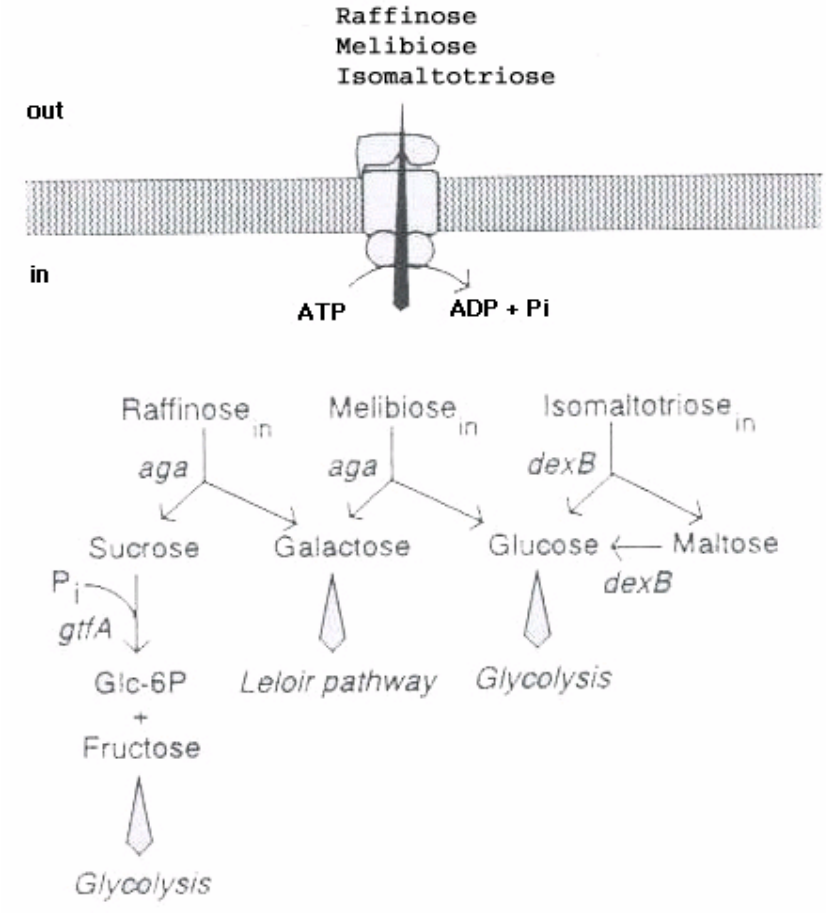

Figure 5**.** Sugar transport ATPase and multiple sugar metabolisms. aga, α-galactosidase; *dexB,* dextran glucosidase; gtfA, sucrose phosphorylase (Poolman 1993)

1.2.3.2 Secondary Transport System

In the secondary transport system the translocation of a sugar molecule is supplied by the sugar concentration gradient, and, if another molecule is co- or counter transported with the carbohydrate, the (electro-) chemical gradient of this coupling molecule, *ie* acumulation is achieved by the downhill movement of another molecule (Poolman 1993) (Figure 6).

In *Lactobacillus delbrueckii* subsp. *bulgaricus* as well as in *Streptococcus thermophilus* (*S thermophilus*), lactose is known to be transported by the secondary transport system (for references, see Delcour et al. 2000; Poolman 1993). These lactose transporters turn out to be specific not only for lactose (β-galactoside) but also for melibiose (α-galactoside), galactose (mono-saccharide) and to a lesser extent raffinose (trisaccharide). The genes encoding the lactose (galactoside) transport proteins (LacS) of *S thermophilus* and *Lb bulgaricus* have been cloned, characterized and functionally expressed in E coli (for references, see Poolman 1993). The LacS permease works in antiport with the galactose (non-metabolizable in most *St* strains) internally released from lactose by β-galactosidase, and also (at a lower efficiency) in symport with H^+ (Delcour et al. 2000). It was recently suggested that the galactoside transporter of *S thermophilus* is a strict lactose/galactose antiporter is not correct. Although the lactose/galactose exchange reaction may be favoured under many conditions, the exchange mode simply reflects partial steps, forward and backward reactions with no net proton translocation, of a complete translocation cycle involving sugar and proton uptake on one side and release on the other side of the membrane, and reorientation of loaded and unloaded substrate binding sites. The lactose transport genes (*lac*S) of *S thermophilus* and *Lb bulgaricus* are organized in an operon that also contains the β-galactosidase gene (*lac*Z) (for references, see Poolman 1993).

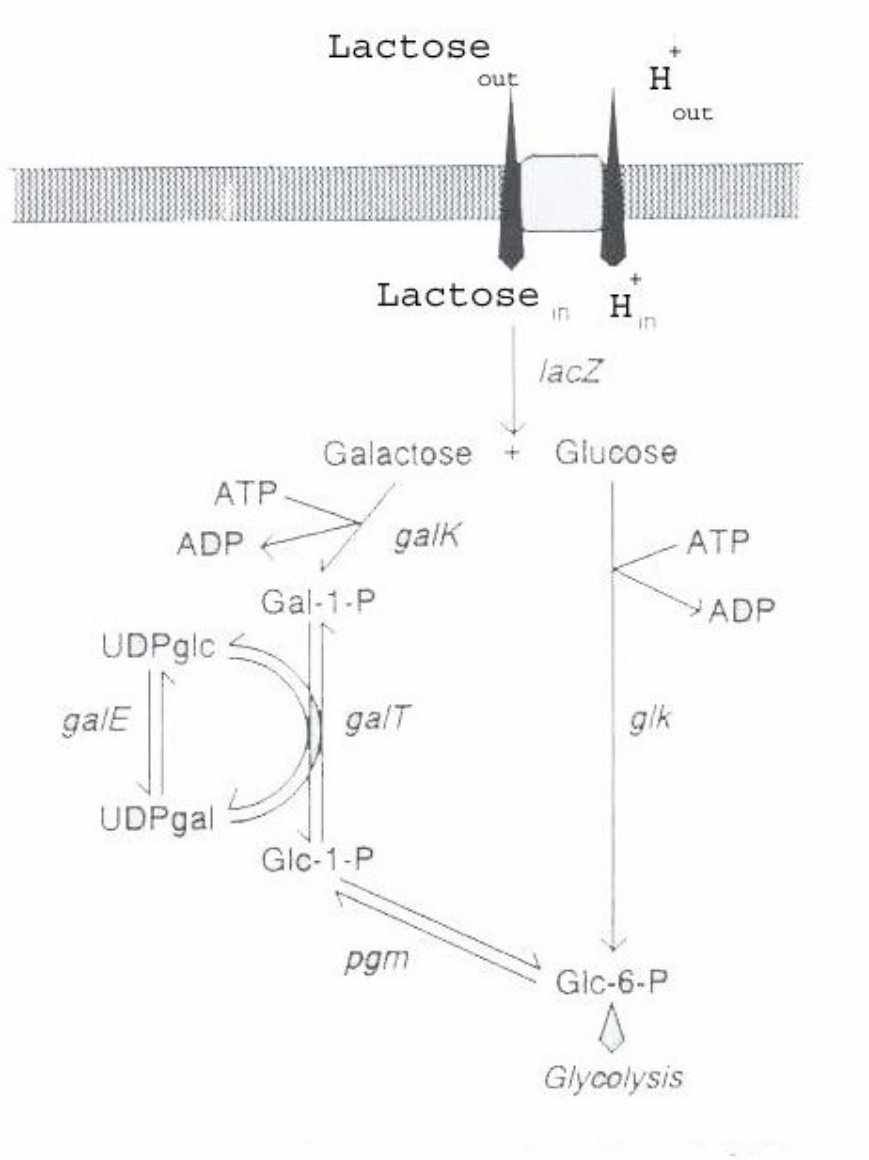

Figure 6. Galactoside-H^+ symport and Leloir pathway. *lacZ*, β-galactosidase; *galK,* galactokinase; *galT,* UDPglucose: galactose 1-phosphate uridylyl transferase; *galE,* UDPglucose 4-epimerase; *pgm,* phosphoglucomutase; *glk*, glucokinase. (Poolman 1993).

1.2.3.3 Phoshoenolpyruvate Dependent Phoshotransferase System (PEP-PTS)

PEP-PTS system is involved in both the transport and phoshorylation of a large number of carbohydrates. Regardless of the organism or carbohydrate, all PTSs that have been characterized catalyze the following overall process:

Phospho(P)-enolpyruvate (in) + carbohydrate (out) --------^{PTS}---> pyruvate (in) + carbohydrate- P(in)

Carbohydrate phosphorylation is coupled to its traslocation across the membrane, the energy for these processes being provided by the glycolytic intermediate PEP. In most gram-positive bacteria and in a few plasmid containing strains of enteric bacteria, galactose and the disaccharide lactose are PTS carbohydrates (Postma et 1993). Lactose specific PTS and P-β-galactosidase (P-β-gal) has only been described in Gram-positive bacteria belonging to the genera *Staphylococcus*, *Streptoccocus*, *Lactococcus* and *Lactobacillus.* Certain fundemental differences are found between genera regarding their gene order, regulatory elements, accompanying genes and genetic location (for references, see Gosalbes et al. 1997).

This multicomponent phosphotransfer system consist of the two general cytoplasmic proteins (enzyme I (EI) and HPr (heat sensitive protein)) and two sugar-specific proteins. One of the sugar-specific pair (III^{sugar}) may be cytoplasmic or loosely associated with the cell membrane, while the other is an integral membrane protein (II^{sugar}) which recognizes, binds and mediated translocation of substrate (Thomson 1988). In Figure 7, diagrammatic representation of the lactose, PTS- glycolysis cycle in *S. lactis* is shown (Thomson 1987).

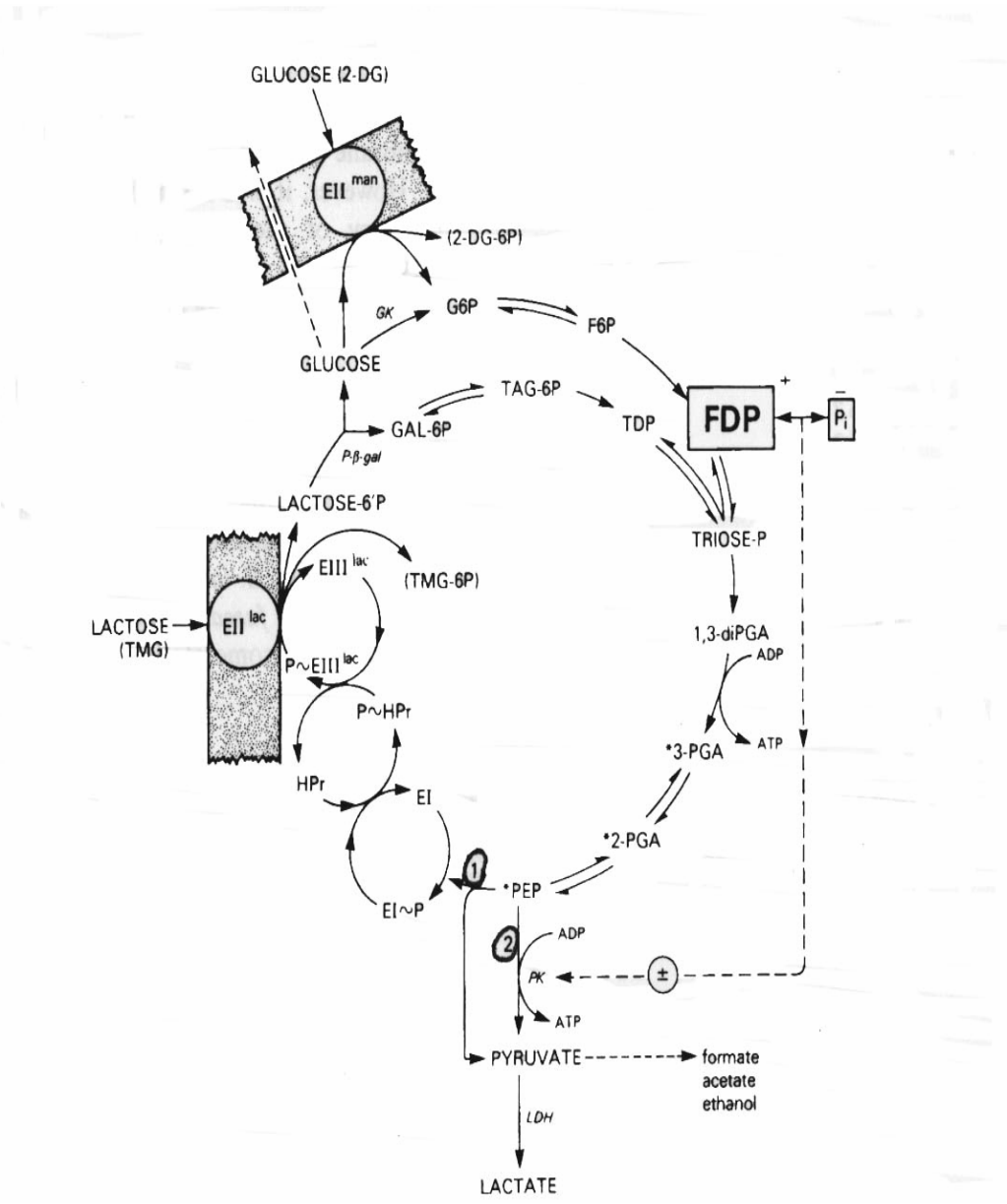

Figure 7. Diagrammatic representation of the lactose-PTS and glycolysis cycle in *S. lactis*. Number 1 and 2, show distribution of PEP to the lac-PTS or to phosphokinase (PK), respectively. The symbols (+) and (-), indicate positive or negative effectors of PK; constituent of the PEP-potential in starved cells (Thomson 1987).

1.2.3.4 Control of Sugar Metabolism

As Titgemeyer and Hillen (2002) summarized, when bacteria are exposed to a mixture of carbon sources they choose the substrate that yields a maximum profit for growth.

Two mechanism of carbon regulation operating at the protein level have been reported in members of LAB. These are inducer exclusion and inducer expulsion.

Inducer exclusion describes the allostretic inhibition of a catabolic protein, e.g. a permease, which prevents the entry or formation of the inducer for the respective catabolite genes.

The mechanism of inducer expulsion describes the rapid expulsion of a previously internalized non-preferred carbon source, e.g., the lactose-derivative methyl-β-D-thiogalactoside (TMG), when exposed to a preferred carbon source afterwards (for references, see Titgemeyer and Hillen 2002).

As it was suggested by Titgemeyer and Hillen (2002), among other control mechanisms, global transcriptional control mechanisms and the protein control mechanism of inducer exclusion are probably the most important ones.

Two principal solutions for global carbon control are currently recognized at molecular detail in the bacterial world. These have been described for species belonging to the enteric Gram-positive bacteria and the low-GC gram-positive bacteria. Both mechanisms take advantage of the components of the phosphotransferase system (PTS) to integrate C-regulatory signals. They differ at the molecular level as transmission of carbon catabolite repression (CCR) signals in enteric bacteria involves the PTS protein IIA^{Glc}. IIA^{Glc} -P triggers the activity of the

catabolite activator protein (CAP) that is required for gene activation of numerous catabolite controlled genes. Enzyme IIA^{Glc} functions also in catabolic protein regulation via inducer exclusion. Low G-C gram-positive bacteria, on the other hand, use the PTS protein Hpr to exert inducer exclusion and to trigger the global regulator CcpA to confer CCR by global gene repression/activation (Titgemeyer and Hillen 2002). The models of carbon catabolite repression are shown In Figure 8 a and 8 b (Brückner and Titgemeyer 2002).

The Figure 8a and 8b show regulatory circuits in enteric and low-GC gram-positive bacteria. The schemes highlight the equivalent roles of two PTS proteins, $EIIA^{glc}$ in enteric bacteria (Figure 8 a) and HPr in gram-positive bacteria (Figure 8 b). Their state of phosphorylation and different phophorylated forms trigger and coordinate the major responses of carbon regulation. Solid lines indicate catalytic interactions/activities and carbon flow, while dashed lines show information pathways.

1.2.4 Genetic Control of Lactose Metabolism

Lactose-phosphoenolpruvate-dependent phosphotransferase operon (Lac-PEP-PTS operon) and lac operon are known to play main role in utilization of lactose in lactobacilli strain.

The lac- PEP PTS operon encodes proteins that mediate the transport of lactose into the cell with concomitant phoshorylation and catalyse the hydrolyis of lactose 6-phosphate to glucose and galactose 6-phosphate. The lac-PTS is composed of Enzyme II^{lac} (LacE), an integral 55 kDa membrane protein responsible for the vectorial phosphorylation and translocation of lactose into the cell as lactose 6-phosphate, and Factor III^{lac} (lac F), a 39 kDa trimeric peripheral membrane protein.

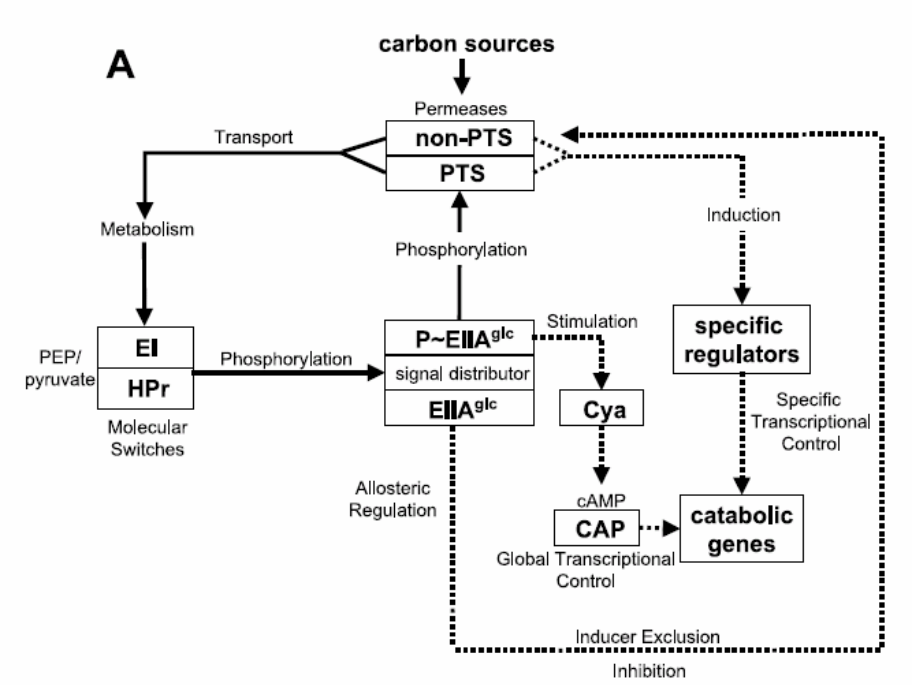

Figure 8 a. Models of carbon catabolite repression (CCR). CCR in enteric bacteria. Incoming carbon sources generate specific signals by which the activity of specific regulators is modulated. Concomitantly, metabolism of the internalized carbon sources determines the ratio of phosphoenolpyruvate to pyruvate, which influences, via EI and HPr, the phosphorylation state of the major signal distribution factor $EIIA^{glc}$. Non-phosphorylated $EIIA^{glc}$ exerts inducer exclusion of non-PTS permeases by allosteric regulation (inhibition), while phosphorylated $EIIA^{glc}$ stimulates adenylate cyclase, thereby triggering global transcriptional control by CAP (Brückner and Titgemeyer 2002).

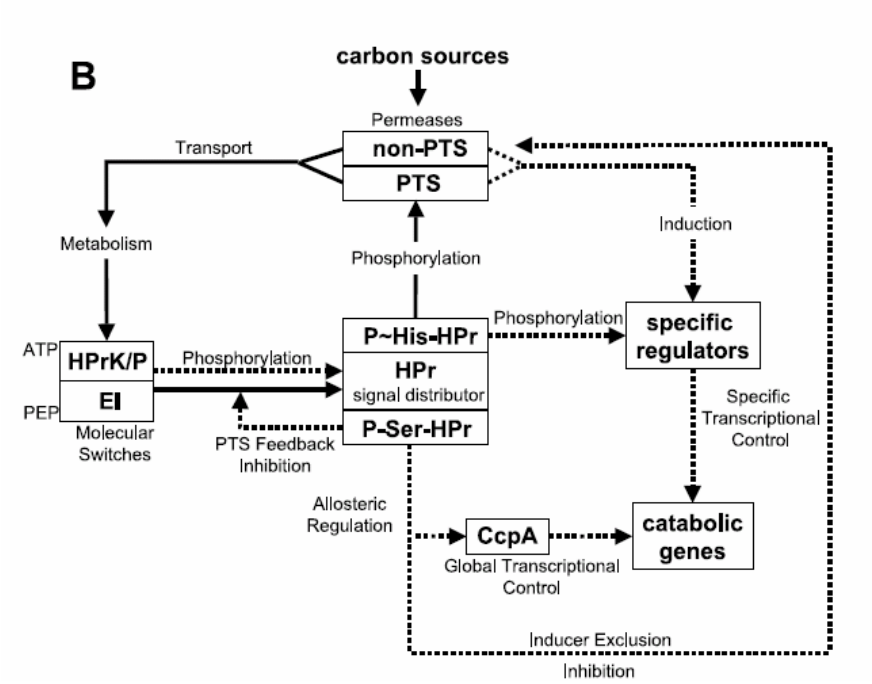

Figure 8 b**.** Models of carbon catabolite repression (CCR). CCR in low-GC Gram-positive bacteria. Besides carbohydrate–specific induction processes, incoming carbon sources generate glycolytic intermediates that stimulate HPrK/P leading to the phosphorylation of HPr at serine-46. An elevated amount of P-Ser-HPr has three consequences: (i) global transcriptional control by CcpA, (ii) inducer exclusion of non-PTS permeases, and (iii) feedback inhibition of EI –dependent phosphorylation of HPr resulting in reduced PTS transport activity and diminished activity of PRD-containing activators (Brückner and Titgemeyer 2002).

that phosphorlates Enzyme II^{lac}. The cytoplasmic lactose 6-phosphate-hydrolysing enzyme, β-D-phosphogalactoside galactohydrolase (P-β-gal), is designated LacG

The genes encoding each of these proteins in lactobacilli, streptococci and staphylococci have been isolated by molecular cloning and sequenced. Based on protein sequence homology, LacG is encoded by a member of the same gene family as *bgl*B, that encodes the β-D-phosphoglucoside glucohydrolase of *E.coli.*

Strains *of S. thermophilus* and *Lactobacillus spp.* contain a pathway for lactose metabolism that is functionally analogous to that encoded by the classical *E.coli* lac operon genes, *lac*Y and *lac* Z. The *lac* Z protein β-galactosidase of *Lactobacillus bulgaricus* is composed of two identical subunits of 114 kDa. The monomoeric molecular weight deduced from sequence data (117 kDa) agrees well with published biochemical data. Quite suprisingly for an organism used in milk fermentation, the *lac*Z gene of some strains of *Lactobacillus bulgaricus* has been observed to undergo spontaneous deletions that give rise to a Lac^- phenotype. The factors underlying this instability are as yet unclear.

It is interesting to note that the regulation of the *Lactobacillus bulgaricus* lac operon differs from that of *E.coli* and *S. thermophilus* since high levels of expression are seen during growth in media containg glucose.

Two unique *Lactobacillus casei* strains possess β-galactosidase activity in addition to the lac-PTS. The plasmid-encoded β-galactosidase gene from the 28 kbp plasmid, Plz15, resident in strain ATCC393 has been cloned into E.coli and sequenced. Active β-galactosidase isolated from *Lactobacillus casei* has a molecular weight around 220.000. The enzyme, an $\alpha_2\beta_2$ type, is comprised two identical 38 kDa and two identical 70 kDa subunits (Gasson and Vos 1994).

In *L. casei* ATTC 393 the lactose operon is located on the chromosome. This strain has the chromosomal lactose assimilation system encoded by the plasmid Plz15 . It was shown that glucose has a clearly inhibitory effect on the expression of the lactose operon in *L. casei* ATCC 393. It was shown that glucose can enter the cell by two different transport systems; a proton motive-force driven permease and a EII^{Man} –like transporters (Gosalbes et al. 1997).

1.2.5 Industrial Importance of β-Galactosidase

1.2.5.1 Industrial Applications of β-Galactosidase

β-galactosidase has catalytic property to hydrolyse lactose into glucose and galactose. Due to its hydrolyzing property of lactose, it has been used for new milk and fermented milk products. Potential beneficial effects on the assimilation of foods containing lactose, as well as the possible technological and environmental advantages of industrial application are listed in Table5 (Jurado et al. 2002; Gekas and López-Leiva 1985).

Lactose is used for improving sweetness, the solubility of the milk product, broader fermentation possibilities, more ready fermentation of these sugars, and reduced lactose concentration with associated diminished possibility of lactose crystallization. In Figure 9 relative sweetness of 10% aqueous sugar solutions were given (Gekas and López-Leiva 1985).

Low lactose milk, dairy products and yogurt are consumed by lactose malobsorbers whose problem is generally related with lactase insufficiency. Low lactose milk, dairy products and yogurt consumption generally decrease the intolerance symptoms arising due to lactose consumption.

Lactase has been used is cheese industry for a long time. Whey is the relatively clear supernatant that remains after the coagulated casein is separated from the milk for cheese making. Its lactose content is 4.2 to 4.4 %. Because little more than half of the 32 billion lb of whey produced annually in the U.S. is utilized, there appear to be significant environmental and economic incentives for converting this high-BOD waste water by-product into useable products and thereby returning this whey

fraction of milk to the food chain. Modification of whey by lactose hydrolysis could lead to new pathways toward practical and economic use for whey that is presently a waste product (Gekas and López-Leiva 1985).

The sweet syrup prepared from whey by lactose hydrolysis can be used as a source of sugar and, in some cases, of protein in bakery products, in confectionery, in soft drinks, in ice cream, in feedstuffs for cattle instead of molasses, in dairy desserts, or as basis for further fermentation to alcohol. Hyrolysed demineralized lactose syrup was produced by Valio Process in Finland.3 (for references, see Gekas and López-Leiva 1985). In Turkey, Pınar Süt (Yaşar Holding) have produced lactose-hydrolyzed milk.

Table 5. Some applications of β-Galactosidase (Jurado et al. 2002; Gekas and López-Leiva 1985).

1.Elimination of lactose intolerance
2.Formation of galacto-oligosaccharides during lactose hydrolysis for favor the growth of intestinal bacterial microflora.
3.Improvement in the technological and sensorial characteristics of daily foods
4.Greater biodegradability of whey

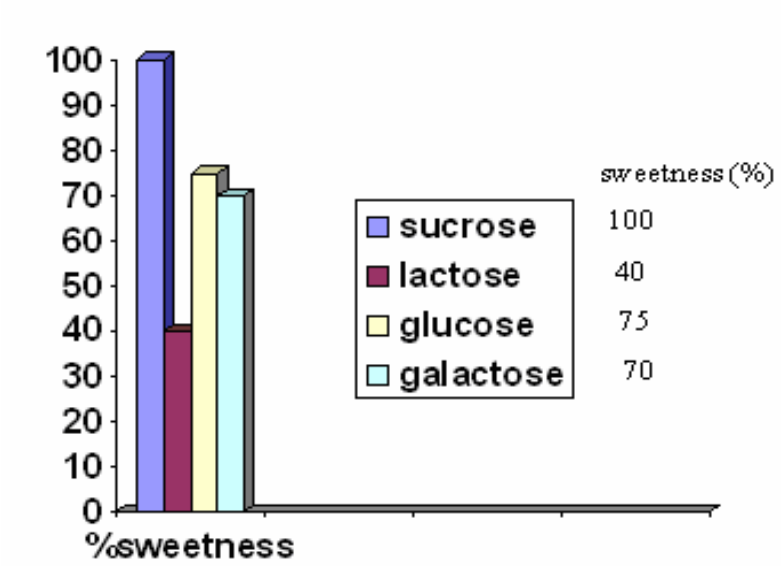

Figure 9. Relative sweetness. Sucrose has the highest sweetness. Therefore it was accepted as % 100. The other sugar's sweetness was calculated relative to sucrose (Gekas and López-Leiva 1985).

1.2.5.2 Sources of β-Galactosidase

Many organisms have been selected due to their high levels of lactase activity for commercial use. Even if yeasts (intracellular enzyme), fungi or molds (extracellular enzyme) are known to produce β-galactosidase (Gekas and Lopez-Leiva 1985), bacterial sources are preferable because of ease of fermentation, high activities of enzyme and good stability. At Table 6, the list of organisms that produce lactase was shown (Godfrey and Stuart 1996).

The commercially exploited sources of β-galactosidase have been of microbiol origin, mainly yeast and moulds. Properties of lactases from different sources are shown at Table 7. Yeast lactase have an optimum pH in the range of 6.0- 7.0. Fungi lactases have pH optima in the acid range (2.5- 4.5) and bacterial lactases have pH

optima in the almost neutral region (6.5-7.5). Thus, fungal lactases are used for acid whey hydrolysis while yeast and bacterial lactases are suited for milk (pH 6.6) and sweet whey (pH 6.1) hydrolysis. Commercial Preparation of Lactase are shown at Table 8 (Gikas and López-Leiva 1985).

Due to secretion abilities, stability and simplicity of purification of the enzyme, still the Bacillus strain is one of the common strains that has been used for lactase production. The most common bacilli used in the industry are *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis*. Regardless of the source organism, long-term stability of the enzyme continues to be a problem (For references, see Sani et al. 1999)

Lactobacilli strains are the commonly used in industry as probiotic. It is well known that β-galactosidase is an intracellular enzyme, and it is not released to the outside of cells under conventional fermentation conditions (Wang et al., 1997). *Lactobacillus delbrueckii* subsp. *bulgaricus* 11842, used in the production of yogurt, is capable of producing relatively high levels of intracellular β-galactosidase in comparison to other dairy cultures (for references see Bury and Jelen 2000).

There is a definite need for β-galactosidase that is stable at high and low temperatures and could be approved as GRAS for hydrolysis of lactose in milk and other dairy products (Kim and Rajagopal 2000)

1.3 Sonication as a Practical Way of Disrupting the Cell to Release β-galactosidase

Since the cost of purified β-galactosidase is prohibitive in most intances, the use of sonicated dairy cultures was proposed to produce a relatively impure source of β-galactosidase for a potentially more economical process of lactose hydrolysis. The

Table 6. The list of organisms that produce lactase (Godfrey and Stuart 1996)

Category	Source	Product Name	Comments
Yeast	Candida pseudotropicalis Kluyveromyces (Sacchoramoyces)fragilis Kluyveromyces lactis	Neutral Lactase Hydrolact Maxilact	Used for hydrolysis of whey Used for hydrolysis of whey Used for hydrolysis of whey
Animal organs	Intestine Brain and skin		
Bacteria	Bacillus megaterium Escherichia coli Lactobacillus acidophilus Lactobacillus bulgaricus Lactobacillus belatericus Lactobacillus crispatus Lactobacillushelveticus Lactobacillus pentosus Streptoccus lactis Streptococcus thermophilus Thermus aquaticus	Acidophilas (Wakunaga Probiotics)	It contains lactase enzyme
Fungi	Aspergillus flavus Aspergillus foetidus Aspergillus niger Aspergillus oryzae Aspergillus phoenicis Curvularia inaqualis Mucor meibei Mucor pusillus Neurospora crassa	Valio Lactase F Amano Enzeco	Used for hydrolysis of whey
Plants	Almands Apricot Coffee berries Kefir grains Peach		

Table 7. Properties of Lactases (Gikas and López-Leiva 1985)

Sources	pH optimum	Temperature optimum	Molecular weight (kD)	Activating ions. Other remarks
A.niger	3,0 -4,0	55-60	124	
A. oryzae	5,0	50-55	90	Mn^{+2}, K^{+}
K. fragilis	6,9-7,3	37	201	Mn^{+2}, Na^{+}
K. lactis	7,2	35	135	K^{+} , Na^{+}
E.coli	6,2-7,1	40	540	
L. thermophilus	3,4-4,3	55-57	530	
C. inaegualis	6,0	30-55		High activity for skim milk
B.circulans	6,8	60-65		
Bacillus sp.	6,5-7,5	65		
L .bulgaricus		42-45		
S. thermophilus		55	500-600	

Table 8. Commercial preparations of Lactase(Gikas and López-Leiva 1985)

Aspergillus niger
Baxter Laboratories, USA
Dairy Food Labs, USA
Kluyveromyces or *Sacchroaromyces lactis*
Gist-Brocades, Holland
Nutritional Biochemical, USA
Kluyveromyces or *Sacchroaromyces fragilis*
Kyowa Hakko Kogyo, Japan
Novo A/S, Denmark (Lactozym)
Escherichia coli
CF Boeringer GmbH, Mannheim, Germany
Worthington Biochemical Corp, USA
Yeast preparations are supplied by:
British Drug House, England
DEBI, Italy
Fungal preparations
Miles Laboratories, USA

following sections will deal with theory of sonication, use of sonication to disrupt the cell for releasing β-galactosidase, and advantages and disadvantages of sonication system in dairy use will be discussed.

1.3.1 Mechanical Disruption Method :Sonication Definition and Description

Among the methods used for cell disruption, sonication (ultrasonics) is one of the most widely used cell disruption technique at laboratory scale. Ultrasound technology has been applied to various fields (Wang et al. 1997). On one hand it is hardly suitable for the industrial purpose, but on the other hand, it requires neither sophisticated devices nor extensive technical training at laboratory (Feliu et al. 1998).

Ultrasound, i.e. sound waves of frequency higher than 15-20 kHz, can cause inactivation of cell; besides ultrasound at higher acustic power inputs, can cause disruption of microbial cells in suspension (Geciova et al. 2002).

1.3.1.1 Mechanisms of Microbial Inactivation

When the power of input is increased, microbubbles begin to form at various nucleation sites in suspension medium. These bubbles grow during the rarefacting phase of the sound wave and then in the compression phase the bubble content is compressed to a minimum radius where the bubble collapses releasing a violent shock wave which propagates in the medium. All these phenomena owing to the action of intense sound waves are termed as cavitation. In the collapse phase of cavitation bubbles, a large quantity of sonic energy is released as mechanical energy in the form of elastic waves. When the elastic waves propagate, in the field, they are

assumed to interact with each other and with the medium of the cavitation field. As a result, the waves disintegrate into eddies which in turn will break-up further to form new sets of eddies. The process of eddy disruption continous till the eddies are so small that they are damped by the viscosity of the medium. During the eddy disruption process the larger eddies will trasmit their energy to the smaller eddies and finally the energy content of the smallest eddies will be dissipated through viscosity as heat. Eddies bigger than the cell will move it from place to place while eddies smaller than the cell form shear stress around the cell wall which is responsible for disintegration of cell (Doulah 1977). As a result of disintegration, protein release is observed.

1.3.1.2 Process Factors

It has been found that release of proteins is effected mainly by the acoustic power and the volume of the sample being sonicated. In the small volumes used (6-7 ml), the concentratation of *L. delbrueckii spp. Bulgaricus* 11842 (12- 46%) wet wt.) did not appear to affect the release of the active β-galactosidase under the sonication conditions employed (Bury et al. 2001). In another study the release kinetics of β-D-galactosidase protein have been determined during small-scale ultrasonication of *E. coli* cells. According to this sudy among several studied parameters, ionic strength and cell concentration have the least influence on the rate of protein recovery, whereas sample volume and acoustic power dramatically affect the final yield of soluble protein in the cell-free fraction (Feliu et al., 1998). The release constant, *k*, of brewers yeast sonicated at powers up to 200 W at 20 kHz has been shown to be independent of cell concentration up to values of 60 g made up to 100 ml. It is inversely proportional to the volume of the treatment vessel in the range 75 to 450 ml, and almost proportional to the input acoustic power from 60 to 195 acoustic

watts (James et al. 1972)

1.3.1.3 Advantages and Disadvantages of Sonication

Disruption of cells by sonication has both advantages and disadvantages. One of the disadvatages of sonication is heat release: During the sonication process larger eddies will tranmit their energy to the smaller eddies, finally the energy content of the smallest eddies will be dissipated through viscosity as heat (Doulah 1977). Therefore, adequate cooling is necessary to decrease the irreversible enzme deactivation. The other disadvantage is that, free radicals are generated in the cavitation bubbles (Visscher et al. 1996). The resistance to disintegration by sonication shows variability among different strains of bacterial species (Feliu *et al*. 1998). Therefore the operational parameters such as time and acoustic power should be optimized for each of the enzyme (Özbek et al. 2000). Sonication is limited to small-scale volumes. Disruption of *Lactobacillus delbrueckii* ssp. *Bulgaricus 11842* cells were studied by Bury et al. (2001) to compare sonication, high-pressure homogenization and bead milling according to release of the β-galactosidase. Sonication was not found as effective as high-pressure homogenization and bead milling.

Although sonication has many disadvantages, it requires neither sophisticated devices nor extensive technical training. Evaluation of technical and economical feasibility of using sonicated dairy cultures to produce a relatively impure source of lactase for lactose hydrolysis has been studied by Bury et al. (2000). They mentioned that if production facility only has to purchase the equipment for cell disruption, the production of partially-lactose-hydrolyzed milk using the whey-based broth appears to be economically beneficial (Bury et al. 2000).

1.4 Scope of This Study

Lactobacillus plantarum is considered as an important organism as a starter culture of fermented products and as probiotic microorganisms. *Lactobacillus plantarum* can be used as a probiotic to reduce the lactase-intolerance symptoms.

For this approach to be commercially successful, strains possessing high levels of β-galactosidase activity need to be identified. Since lactase from *Lactobacillus plantarum* is an intracellular enzyme, sonication method can be used to release enzymes including β-galactosidase to the extracellular environment.

The enzyme released after sonication could be used to hydrolyze a portion of lactose in milk or whey thus reducing the lactose content of the product, which could be an advantage for lactose intolerant consumers.

The objectives of this study are:

(i) To test lactase activity of *L. plantarum* strains from different sources by using X-gal.

(ii) To observe change in β-galactosidase activity at different stages of growth of the microorganisms. It is known that β-galactosidase activity did not show the same pattern with growth. Therefore it is important to find how the growth time and growth conditions affect the β-galactosidase activity.

(iii) To evaluate β-galactosidase specific activity by using different methods to optimize the specific activity measurements. When making strain comparisons, it can be advantageous to evaluate β-galactosidase-specific

activity by using a reproducible and easy method.

(iv) To optimize the sonication parameters to increase the yield of protein release from cell into the extracellular environment.

(v) To investigate the amount of cell lysis caused by sonication. In some cases it is known that sonication causes release of enzyme without decreasing viable cell number. On the one hand viability of the cell is important for being used as probiotic. On the other hand it has been found that viability may not be so important in case lactase was secreted into the environment.

(vi) To characterize β-galactosidase from cell-free crude extract of sonicated *Lactobacillus* strain.

Sonication method has been used to disrupt the cells to release the intracellular enzyme to the extra-cellular environment. β-galactosidase is an intracellular enzyme in lactobacilli. Its application from in-house grown culture will have to include the enzyme release by disruption of the microbial cells. As Vasiljevic and Jelen (2001) suggested, if sonication method as a cell disruption method is optimized, the disrupted cells would be used for many different purposes. For example they can be used as probiotic or they can be simply added into the milk products without extensive purification.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Strains

The bacterial strains that were used through this study were shown in Table 9.

Table 9. Bacterial strains used in this study

Strains	Origin or reference
Lactobacillus plantarum DSM 20246	DSM (Deutsche Sammlung von Microorganismen und Zellkulturen GmbH)
Lactobacillus plantarum NCIMB 1193	NCIMB (National Collection of Industrial and Marine Bacteria)
Lactabacillus plantarum E081	Our laboratory culture (isolated from cheese)
	RSKK (Refik Saydam Kültür Koleksiyonları, Turkey)
Escherichia coli ATCC 25922	ATCC (American Type Culture Collection)

2.1.2 Media

All the required culture media, their composition and preparation were given in Appedix I.

2.1.3 Buffers and Reagents

List of the buffers and reagents, which were used during experiments, their composition and preparation are given in Appendix B.

2.1.4 Chemicals

List of chemicals is given in Appendix C.

2.2 Methods

2.2.1 Cultivation for Activation

Stock cultures were maintained at -80^0 C on Microbank vials (Pro-Lab Diagnostics). Prior to assay, strains were transferred into MRS medium and incubated at 37^0 C for 24 hours. Each culture was subcultured successively three times in MRS broth. For routine daily use, agar plate cultures were prepared. The cultures were stored at 1^0 C to 5^0 C between transfers. All experiments were performed as batch fermentations under anaerobic conditions.

E. coli were maintained in the same way on Nutrient agar slants. Prior to gram staning assay, strains were transferred into Nutrient agar and incubated at 37^0 C for 24 hours

2.2.2 Isolation of *L. plantarum* from Erzurum Cheese

For isolation of the microorganism from Erzurum cheese, the cheese sample (10 g in 90 ml sterile peptone water) was homogenized with stomacher laboratory blender (Seward laboratory blender-stomacher 400), and serially diluted with peptone water (0.1 % g/L). After dilutions, samples were plated on selected agar media. The agar media was incubated in anaerobic jar at 37^0 for 24 hours. Single colonies were selected and inoculated into Eppendorf tubes (1.5 ml) containing MRS broth and then they were further incubated at 37^0 C for 24 hours. The Streak plate method was used to obtain single colony from these cultures. Cultures were maintained in slants (MRS agar) and in glycerol (%20 v/v) at -20^0 C and in microbanks at -80^0 C.

2.2.3 Basic Tests for Identification of Lactobacilli and β-galactosidase Production Test

The microorganisms were checked whether they belong to lactic acid bacteria or not. The tests which were used for this purpose were ; gas production test, catalase test, gram staining test. API 50 CHL kit were used for identification of the genus *Lactobacillus*. Temperature requirements for growth were checked for each strain. X-gal was used for screening of β-galactosidase activity.

2.2.3.1 Gas Production from Glucose

Lactobacillus species are classified according to the type and amount of the products, which are forming during fermentation, as homofermentative or heterofermentative.

Homofermentative lactic acid bacteria produce lactic acid at a yield of % 95-100 as a result of fermentation. Besides lactic acid, acetic acid, ethanol, or formic acid are produced depending on the characteristics of the medium and the strain.

Heterofermenters can not break down fructose bisphosphate to triose phosphate. Instead they oxidize glucose 6-phosphate to 6-phosphogluconate and then decarboxylate this to pentose phosphate, which is broken down to triose phosphate and acetylphosphate by means of the enzyme phosphoketolase. Heterofermentative lactic acid bacteria form %50 lactic acid as a result of fermentation, where they produce acetic acid, ethanol, CO_2, and other compounds in minute amounts.

Lactobacillus plantarum strain is homofermentative bacteria. Therefore when the glucose is the main sugar in culture media, no gas formation is observed.

To determine CO_2 production; MRS broth were prepared and distributed into tubes in 10 mL volumes. Durham tubes were also put into the broth before sterilization. After sterilization, the microorganisms were inoculated into the MRS broth. The inoculated growth medium were then incubated at 37 C for 24 hours (Özcangaz 2000).

2.2.3.2 Gram Staining Procedure

The microorganisms that are stained by the Gram's method are commonly classified as Gram-positive or Gram non-negative and appear purple brown under the microscopic examination. Others that are referred to as Gram negative, and appear red. It is known that lactic acid bacteria are gram-positive bacteria. The method was described in Appendix B

2.2.3.3 Catalase Activity

Most microorganisms growing aerobically posses the enyzme catalase. The lactic acid bacteria do not normally produce a detectable amount of catalase. The isolated strains were checked for the production of the enzyme catalase. Colonies were grown on MRS agar plates. One ml %3 H_2O_2 solution was poured over the surface of agar culture, to test whether they form gas bubbles or not. Formation of free oxygen bubbles indicates the presence of catalase.

2.2.3.4 Temperature Requirements For Growth

It is known that generally *Lactobacillus plantarum* strain do not grow over 37^0 C. Growth of the *Lactobacillus plantarum* DSM 20246, *Lactobacillus plantarum* NCIMB 1193, *Lactabacillus plantarum* E081 at 15^0 C, 37^0 C, 45^0 C, 55^0 C, 60^0 C in MRS broth were observed. The cultures were incubated at these temperatures at least two days.

2.2.3.5 API Fermentation Kit

Ability to ferment different carbohydrates has been used for identification of microorganisms. The API 50 CHL kit allows the study of carbohydrate metabolism of microorganism.The principles of this kit were described in Appendix B

2.2.3.6 β-galactosidase Production Test

X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) is a substrate, which has been used to screen β-galactosidase positive organisms. 50 µl of X-gal (20mg in N-N dimethyl formamide) solution was poured over the MRS agar. This medium was incubated at 37^0 C for one hour. 50 µl from each strain was poured over MRS agar. Mediums were incubated for 24 hours for color formation. X-gal forms blue color if the culture has β-galactosidase activity.

2.2.4 Construction of ONP Standard Curve

ONP standard curve was constructed to find the extinction coefficient (ε) of ONP.

2.2.4.1 Preparation of ONP Solution for Standard Curve Construction

Free ONP is capable of a tautomeric change that gives it a yellow color in alkaline solution, with absorption peak at 420 nm . ONP is a weak acid, pK 7.3, and the acid, benzenoid tautemer is practically colorless. Optical density must therefore be determined either in well- buffered solutions, in which a fixed proportion of the ONP is dissociated (and colored), or in alkaline solutions, pH 10 or higher, in which a negligible fraction remains undissociated (and colorless) (Lederberg 1950).

ONP is an important compound as an indicator for β-galactosidase activity. In this assay, free ONP at different dilutions level was used to construct ONP standard curve. During construction of ONP standard curve, known amount of ONP with

several dilutions were prepared. Each dilution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

ONP (0.033384 g) was mixed with 1 ml 0.05 M phosphate buffer (at pH 7) and 3 ml ethanol. Final volume of the mixture was set to 4 ml. This mixture (4 ml) was agitated until most of the solid particles were become soluble. This mixture was transferred into preheated water bath at 45^0 C. Melting temperature of ONP in water is 44^0 C- 45^0 C. This mixture was labeled as "ONP (60 mM) stock solution". The several dilutions of stock solution were carried out by 0.05 M sodium phosphate buffer at pH 7 (under the assay conditions) (Table 10). Each dilution from the stock solution gave a specific yellow color under the assay conditions with absorption peak at 420 nm.

Blank solution was prepared by mixing 3 ml alcohol and 1ml sodium phosphate buffer (0.05 M at pH 7). For each set of dilutions of stock solution, blank solution was also diluted.

Absorption values were read against the blank solutions in UV-1202 shimatzu spectrophotometer. All readings were carried out as duplicate experiments in tree independent trial. The average for each set of data was plotted against respective concentration.

During the enzymatic assay 1 ml of ONPG solution was mixed with 0.2 ml cell-free crude extract solution. Therefore during standard curve preparation 1 ml of appropriately diluted ONP solution was mixed with 0.2 ml distilled water. Final molarity of ONP was calculated from the equation at below (Table 14).

$M_1 * V_1$ (1ml) = $M_2 * V_2$ (1.2 ml)
M_1: Initial molarity of ONP solution
M_2: Final molarity ONP solution
V_1: Initial volume of appropriately diluted ONP solution
V_2: Final volume of appropriately diluted ONP solution

Table 10. Dilution table: Dilutions were carried out in 20 ml glass tubes

Stock Solution ONP	Buffer (0.05 M sodium phosphate buffer at pH 7 at room temperature)	Final volume of the ONP solution after mixing with buffer for dilutions in glass tubes (ml)	Final concentration of ONP (mM)
			60 mM
0.5 ml (60 mM)	4.5 ml	5 ml	6 mM
0.5 ml (6 mM)	2 ml	2.5 ml	1.2 mM
1 ml (6 mM)	9 ml	10 ml	0.6 mM
3 ml (0.6mM)	3 ml	6 ml	0.3 mM
3 ml (0.3 M)	3 ml	6 ml	0.15 mM
1 ml (0.6 mM)	4 ml	5 ml	0.12 mM
3 ml (0.15 mM)	4.5 ml	7.5 ml	0.06 mM
1 ml (0.06 mM)	4 ml	5 ml	0.012 mM
0.5ml (0.06mM)	4.5 ml	5 ml	0.006 mM

Table 11. Dilution table: Dilution were carried out in reaction medium.

Stock solution ONP (mM)	Initial volume of stock solution	Volume added (ml)	Concentration in reaction medium (mM)
60	1 ml	1.2 ml	50
6	1 ml	1.2 ml	5
1.2	1 ml	1.2 ml	1
0.6	1 ml	1.2 ml	0.5
0.3	1 ml	1.2 ml	0.25
0.15	1 ml	1.2 ml	0.125
0.12	1 ml	1.2 ml	0.1
0.06	1 ml	1.2 ml	0.05
0.012	1 ml	1.2 ml	0.01
0.006	1 ml	1.2 ml	0.005

2.2.4.2 Preparation of O-nitro Phenol (ONP) Solution for ONP Standard Curve by Addition of Sodium Carbonate

The same method was followed for dilutions (Table 12). During the enzymatic assay 0.5 ml of ONPG solution was mixed with 0.1 ml cell-free crude extract solution. Then 0.6 ml sodium carbonate (0.05 M) solution was added into the reaction medium.

During standard curve preparation 0.5 ml of appropriately diluted ONP solution was mixed with 0.1 ml distilled water. Final molarity of ONP was calculated from the equation at above. ONP (0.6 ml) concentration in reaction medium was ranging from 50 mM to 0.005 mM. Sodium carbonate (0.6 ml and 0.05M) was added into reaction medium. Total volume was set to 1.2 ml. So the concentration range of ONP dropped to 25 mM to 0.0025 mM (Table 13).

Blank solution was prepared by mixing equal volumes of 0.6 ml sodium carbonate with 0.6 ml sodium phoshate buffer . Buffer solution was prepared by mixing 3 ml alcohol and 1ml buffer (0.05 M sodium phosphate buffer at pH 7). For each set of dilution buffer solution was also diluted with distilled water.

2.2.5. Protein Determination

2.2.5.1 Bradford Method

The protein concentrations in cell free extract were determined using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein. All dilutions were done from the 0.1 mg/ml stock solution.

Table 12. Dilution table: Dilution were carried out in reaction medium

Stock solution ONP (mM)	Initial volume of stock solution	Volume added (ml)	Concentration of ONP in reaction medium (mM)
60	0.5 ml	0.1 ml	50
6	0.5 ml	0.1 ml	5
1.2	0.5 ml	0.1 ml	1
0.6	0.5 ml	0.1 ml	0.5
0.3	0.5 ml	0.1 ml	0.25
0.15	0.5 ml	0.1 ml	0.125
0.12	0.5 ml	0.1 ml	0.1
0.06	0.5 ml	0.1 ml	0.05
0.012	0.5 ml	0.1 ml	0.01
0.006	0.5 ml	0.1 ml	0.005

Table 13. Dilution table: Dilution were carried out in reaction medium

Stock solution ONP (mM)	Initial volume of stock solution	Volume of sodium carbonate (ml)	Concentration of ONP in reaction medium (mM)
50	0.6 ml	0.6 ml	25
5	0.6 ml	0.6 ml	2.5
1	0.6 ml	0.6 ml	0.5
0.5	0.6 ml	0.6 ml	0.25
0.25	0.6 ml	0.6 ml	0.125
0.125	0.6 ml	0.6 ml	0.0625
0.1	0.6 ml	0.6 ml	0.05
0.05	0.6 ml	0.6 ml	0.025
0.01	0.6 ml	0.6 ml	0.005
0.005	0.6 ml	0.6 ml	0.0025

Distilled water was used to make appropriate dilutions. Standard curve of BSA (mg/ml) was constructed.

2.2.6 Growth Studies

Lactobacillus plantarum DSM 20246, *Lactobacillus plantarum* NCIMB 1193 *Lactabacillus plantarum* E081 were used during growth studies. Modified MRS (MMRS) (Appendix A) medium was used during the assays.

The growth studies were performed in two different volumes of MMRS: 100 ml and 600 ml. The modified MRS medium, which was 600 ml, was used for growth studies which includes calculation of cell dry weight, change in pH, change in turbidity of medium and β-galactosidase activity of *Lactobacillus plantarum* NCIMB 1193. The modified MRS medium, whose total volume was 100 ml, was used for calculation of total β-galactosidase activity and measurement of change in turbidity of medium for construction of growth curve of *Lactobacillus plantarum* DSM 20246, *Lactabacillus plantarum* E081. The modified MRS medium, whose total volume was 100 ml, was used for measurement of change in turbidity of medium for construction of growth curve of *Lactobacillus plantarum* NCIMB 1193

2.2.6.1 Growth Studies for *L. plantarum* NCIMB 1193

For inoculum preparation, *Lactobacillus plantarum* NCIMB 1193 strain was subcultured successively three times in MRS broth and then three times in modified MRS broth (Appendix A) for 14 h at 37^0 C. During the transfers, 200 µL of samples were transferred into 10 ml fresh medium.

Optical density (OD_{600}) of inoculum culture (10 ml) was measured before the inoculation into 600 ml modified MRS medium at pH 6.35. Inoculum culture was diluted with peptone water. Dilutions were carried out by mixing 10 ml of peptone water with 10 ml of inoculum culture. Blank solutions were prepared by mixing 10 ml of peptone water with 10 ml of sterile modified MRS medium (Appendix B). Readings were recorded against the blank solution. Optical density of diluted inoculum was measured at 600 nm. In parallel assays turbidity of inoculum culture was set to same optical density by appropriate dilution. Therefore in each assay the concentration of the culture kept constant.

From "Diluted inoculum culture", 6 ml of inoculum was transferred into 600 ml modified MRS medium for growth curve analysis. Every two hours, 30.4 ml sample was taken from growth medium; 20 ml for pH and β-galactosidase assay, 2.4 ml for OD_{600} readings, 8 ml for cell dry weight (CDW) calculation. During the assay, readings were carried out as parallel readings in triplicate assay. All absorption values were read against the blank solutions in UV-1202 shimatzu spectrophotometer

2.2.6.1.1 Cell Dry Weight Determination for *L. plantarum* NCIMB 1193

Eight mililiter of sample from the growth medium was transferred into the ependorf tupes (1.5 ml) with two hours periods. These samples were harvested by centrifugation at 6000 rpm (RC5C Sorvall Rotor GSA code 10) for 15 min at room temperature. The pellet was washed with 8 ml distilled water one times. The pellet obtained from the second centrifugation was resuspended in 4 ml distilled water. One mililiter of sample was poured on preweighted aluminium papers. These papers were incubated at 100^0 C for 10 hours. Then their weight was calculated and substracted from the initial weight to find the dry weight of 1 ml sample.

2.2.6.1.2 pH and OD_{600} Measurements for *L. plantarum* NCIMB 1193

At two hour periods, OD at 600 nm and pH of the medium were measured. Growth was measured turbidimetrically at 600 nm and cells were diluted with water when the OD_{600} was higher than 0.8. Cell turbidity at OD_{600} was kept between 0.4 and 0.8 by appropriate dilutions. 20 ml of sample was incubated at 5^0 C for 15 minutes to stop further growth. pH of the medium was measured by pH meter (model 3010, Jenway).

2.2.6.1.3 Preparation of Cell Free Crude Extract and β-galactosidase Assay of *L. plantarum* NCIMB 1193

For two hour periods, 20 ml of growth medium was transfered from the growth medium (MMRS) and then, it was centrifuged at 12000 rpm for 15 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10). The pellet was washed twice with 20 ml distilled water. Finally, the pellet was resuspended in 9 ml, 0.05 M sodium phosphate buffer at pH 7; after that cells were chilled on ice until use.

0.2 g lysozyme (sigma- L 6876; 70,000 units/mg) was added into the tube containing 9 ml pelet solution. After gentle mixing, this mixture was incubated at 37^0 C for 15 minutes. After 15 minutes, 1 ml NaCl (4 M) solution was added into the pellet solution. The solution was further incubated at 37^0 C for 50 minutes. This

solution was centrifuged at 11423 g (RC5C Sorvall Rotor GSA code 10) at 9^0 C for 15 minutes. After the centrifugation, the supernatant was immediatly transferred into eppendorf tubes for enzyme assay. Supernatant was stored at 5^0 C until the assay time. This solution was labelled as “cell-free crude extract”.

Continuous enzyme assay protocol as described in section 2.2.8.1 was performed for specific and total β-galactosidase activity measurements of cell-free crude extract of *Lactobacillus plantarum* NCIMB 1193. During the β-galactosidase activity assay, 40 mM ONPG solution at 37^0 C (in 0.05 M sodium phosphate buffer at pH 7) was used as the substrate.

2.2.6.2 Construction of Growth Curve from MMRS Growth Medium (100 ml)

Lactobacillus plantarum DSM 20246, *Lactobacillus plantarum* E081 and *Lactobacillus plantarum* NCIMB 1193 were used for growth studies. 100 ml modified MRS medium was used as growth medium during these experiments.

For inoculum preparation, the cultures from each strain were subcultured successively three times in MRS broth and then three times in 2% modified MRS broth (Appendix A) at 37^0 for 14 h. Optical density (OD_{600}) of inoculum cultures were measured before the inculation into 100 ml modified MRS medium (pH 6.35). By appropriate dilutions turbidity of the inoculum was set up between 0.45 and 0.6 at 600 nm. This solution was labelled as "diluted incolum". During activation transfers 100 µl of each samples were transferred into 1.5 ml eppedorf tubes containing 1 ml fresh MMRS medium.

Dilutions of inoculum were carried out by mixing 1 ml cell suspension with 9 ml peptone water (0.1% g/ml). Blank solution was prepared by mixing 1ml sterile growth medium with 9 ml sterile peptone water. Absorbance at 600nm was recorded against the blank solution in UV-1202 shimatzu spectrophotometer. 0.5 ml of inoculum from "diluted culture" was transferred into 100 ml MMRS medium (Appendix B) for growth curve analysis. The turbidity measurements were carried out at 600 nm each 2 hours of periods against blank solution. Each 2 hour periods,

2.4 ml of sample was transferred from the growth medium for turbidity measurements. At 6th, 8th, 10th, 12th, 14th hr, 10ml culture medium was transferred from the growth medium for β-galactosidase activity test.

Before the turbidity measurement and β-galactosidase assays, samples, taken from the growth sample (MMRS), were stored at 5^0 C for at least 15 minutes to stop the further growth. The optical density of cell suspension was read against the blank solution at 600 nm. For each reading 1.2 ml sample was used. 1.2 ml sterile growth medium was used as a blank solution. All readings were carried out as parallel.

2.2.6.2.1 Preparation of Cell Free Crude Extract from MMRS Growth Medium (100ml) and Measurement of β-galactosidase Activity of *L. plantarum* ATCC 20246 and *L. plantarum* E081

10 ml of culture was taken from the 100 ml modified MRS and harvested by centrifugation at 12000 rpm for 9 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10). After the second centrifugation, the pellet was washed with same amount of distilled water (10ml). The pellet was resuspended in 4.5 ml distilled water followed by vigorous vortexing to disperse the cells homogeneously. This solution was labelled as "pellet solution".

100 mg lysozyme (sigma- L 6876; 70,000 units/mg) was added into the tubes containing 4.5 ml pellet solution. After gentle mixing, this mixture was incubated at 37^0 C for 15 min. After 15 minutes incubation at 37^0 C, 0.5 ml 4 M NaCl solution was added into the pellet solution. The solution was further incubated at 37^0 C for 50 minutes. This solution was centrifuged at 12000 rpm for 9 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10). After the centrifugation, the supernatant was immediatly transferred into eppendorf tubes. Until the experiment, supernatant was stored at 5^0 C. This solution was labelled as cell-free crude extract.

The cell-free crude extract of *Lactobacillus plantarum* DSM 20246 and E081 obtained by lysozyme (sigma- L 6876; 70,000 units/mg) treatment were used for β-galactosidase enzyme assay. Continuous enzyme assay protocol as described in section 2.2.8.1 was performed for total enzyme activity measurements. 40 mM ONPG solution (in 0.05 M sodium phosphate buffer at pH 7) was used as substrate during the β-galactosidase activity assay. The reaction temperature during the assay was 37^0C.

2.2.7 Sonication and Liquid Nitrogen Method for β-galactosidase Release from *Lactobacillus*

At the end of the exponential phase, 80 ml of cultures from modified MRS (100 ml) were harvested by centrifugation at 12000 rpm for 9 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10). After harvesting the cells and washing them at least once with distilled water, the pelleted cells were dissolved in 25 ml distilled water. 5 ml of this suspension was put into a morter for liquid nitrogen assay. The rest of the solution was used for sonication assays. For *Lactobacillus plantarum* NCIMB 1193, total β-galactosidase activity of cell-free crude extract obtained from the liquid nitrogen and sonication methods were compared.

2.2.7.1 Liquid Nitrogen Method

Lactobacillus plantarum NCIMB 1193 was used for this assay. The liquid nitrogen was poured on 5ml cell suspension in the morter**.** The frozen droplets were grounded by a pestel. This procedure was repeated at least twice. Then, the grounded suspension was collected and centrifuged at 12000 rpm for 9 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10)**.** Supernatant was used for assaying enzyme activity

(Rejaee 1993). The β-galactosidase activity was measured by continuous enzyme assay protocol as described in section 2.2.8.1 in Materials and Methods. 40 mM ONPG solution (in 0.05 M sodium phoshate buffer at pH 7) was used as the substrate during the assay. The assay temperature was determined as 37^0 C.

2.2.7.2 Sonication Method

2.2.7.2.1 Optimization of Sonication Method

Throughout this study, the sonication experiments were carried out at 16 kHz on a Soniprep 150 sonifier (Appendix D) equipped with a prop of 9.5 mm. The power was kept constant during the study. The voltage of the sonifier was set to 240 V.

The resistance to disintegration by sonication shows variability among different strains of bacterial species (Feliu et al. 1998). Therefore the operational parameters such as time and acoustic power should be optimized for each of the enzyme (Özbek et al. 2000). For three different bacterial species (*L. plantarum* NCIMB 1193, *L. plantarum* DSM 20246 and *L. plantarum* E081), sonication time was optimized by keeping the acoustic power constant. The same sonicator probe and acoustic power out put were used throughout the study.

The cell-free crude extract of *L. plantarum* NCIMB 1193, *Lactobacillus plantarum* DSM 20246 and E081 obtained by sonication cell disruption method were used for β-galactosidase enzyme assay. Continuous enzyme assay protocol as described in section 2.2.8.1 was performed for total enzyme activity measurements under the assay conditions. ONPG solution (in 0.05 M sodium phosphate buffer at pH 7) was used as substrate during the enzymatic assays.

2.2.7.2.1.1 Sonication Time Optimization

At the end of the exponential phase of the microorganisms, 80 ml culture medium from modified MRS (100 ml) were harvested by centrifugation at 12000 rpm for 9 min at 9^0 C (RC5C Sorvall Rotor GSA code 10). After harvesting the microorganisms and washing them at least ones with distilled water, the pelleted cells were dissolved in 25 ml distilled water. 12 ml of this suspension was used for sonication.

For *L. plantarum* NCIMB 1193, *Lactobacillus plantarum* DSM 20246 and E081, the sonication time was optimized by keeping the acoustic power constant. One ml sample was taken every 30 seconds sonication to determine enzyme activity. To prevent enzyme deactivation due to a rise in temperature during sonication, the cultures were sonicated in an ice bath and after each 30 seconds sonication period, 2 minutes break was given. Meanwhile the solution was incubated in refrigrator to decrease the temperature of the solution. Before sonication, the temperature of the solution was measured as 5^0 C.

After sonication, the samples were centrifuged at 12000 rpm for 8 minutes (Mikro 12-24, Hettich) in 1.5 ml eppendorf tubes. The supernatant was seperated from the pellet and used as cell-free crude extract during the β-galactosidase assay under the assay conditions by following the continous assay protocol as described in section 2.2.8.1

2.2.7.2.1.2 Sonication and Viable Cell Organism

Sonication is a mechanical disruption method; it disrupts the cell wall of the microorganism which leads to cell death.

12 ml cell medium was sonicated for 3.5 minutes by giving breaks for each 30 second sonication periods. After 3.5 minutes sonication, 1 ml of sonicated sample enumerated to determine the residual microbial population after the sonication. Initial counts were obtained from the control samples which was not sonicated (for references, see Mussa and Ramaswamy 1997).

One millilitre aliquots of sonicated *L. plantarum* NCIMB 1193 culture were transferred aseptically from test sachets into sterile dilution tupes containing 9 ml peptone water, from which further dilutions were made. Standard plate count was used for the enumaration of microorganisms employing a pour plate method.

Duplicate plates were incubated anaerobically using anaerobic jar at 37^0 C for 48 hours. Lactobacilli MRS agar was used for the enumeration.

2.2.7.2.1.3 Effect of Sonication on the Release of Cellular Protein

Effect of sonication on cellular protein release from *Lactobacillus plantarum* NCIMB 1193 was determined. After each 30 second sonication period, 1 ml of sample was taken from the sonicated medium. Each 1 ml sample was centrifuged at 12000 rpm for 8 minutes (Mikro 12-24, Hettich) in 1.5 ml eppendorf tubes. After the centrifugation, the supernatant was seperated from the pellet and used for protein anaylsis by using Bradford Protein Determination method. Total sonication time was estimated as 3 minutes.

2.2.8 β-galactosidase Assays

During the experiments, β-galactosidase activity was determined either by using

continuous assay protocol or stopped assay protocol. In continuous assay method, increase in the OD at 420 nm was measured continuously for one minute with 10 sec intervals. The reaction was started with addition of enzyme into the substrate medium (ONPG solution). In stopped assay, the reaction was allowed to proceed one minute. Then, the reaction was stopped by addition of sodium carbonate. Sodium carbonate elevates the pH of the reaction medium up to 10; at this pH the enzyme was deactivated therefore no color formation occurs. Appropriate enzyme and substrate blanks were included

2.2.8.1 Continous Enzyme Assay Protocol

Before the enzyme activity assay, all solutions were incubated in a shaker water bath at 37^0 C for 5 minutes. After that continuous enzyme activity assay reaction was started by addition of appropriately dilluted enzyme solution (0.2 ml) into 1 ml ONPG solution at 37^0 C. The final volume of the reaction medium was 1.2 ml. For appropriate dilutions of the enzyme, distilled water was used. The enzymatic reaction was started by the addition of enzyme to the reaction medium which contains ONPG solution previously incubated at 37^0 C for at least 5 minutes. The total reaction medium becomes 1.2 ml after the addition of 0.2 ml of enzyme solution. The reaction was started by addition of the enzyme solution. The reaction was allowed to proceed for at least 70 seconds. Each ten seconds absorbtion was measured at 420nm. Spectroscopic device allowed continous circulation of 37^0 C water around cuvette during contious enzyme activity assay. The optical density values were read against reagent blank. 1.5 ml dispossible spectroscopic cuvetes were used during the activity assay.

Reagent blank was prepared in 1.5 ml disposable spectroscopic cuvvete. 1ml ONPG solution was mixed with 0.2 ml distilled water. All enzyme activity readings were

performed against the reagent blank.

To eliminate the possible contribution to $0D_{420}$ due to nonenzymatic dissociation of substrates, a control set was prepared in the same way; 1ml ONPG solution was mixed with 0.2 ml of distilled water in 1.2 ml of total volume. For at least 70 seconds with 10 seconds intervals, the change in absorbance at 420 nm under the assay conditions was recorded.

The number of mole of ONP (O-nitrophenol) liberated was determined from the standard curve of ONP. One unit (U) of the enzyme activity was defined as the amount of enzyme required to release 1 μmol of ONP per minute under the assay conditions. The extinction coefficient of dissolved o-nitrophenol at 420 nm was found to be 1.3546 ml μmol^{-1} cm^{-1} (Figure 11). For all continous enzyme activity measurements, this extinction coefficient was used.

Each experiments were done at least twice. Then, means of the results were taken. The composition and preparation of the reagents is given in Appendix B.

Activity was calculated as follows (Baran 1996)

$$U/_{ml} = \frac{\triangle A_{420/min}}{\varepsilon}$$

ε (extinction coefficient): 1.3546 ml μmol^{-1} cm^{-1}

The rate of absorbance change (A420/min) was calculated from the linear portion of the curve by using linear regression. Specific activity was calculated as follows:

$$U/mg\ protein = \frac{\triangle A_{420/min}}{\varepsilon \times mg\ protein/mL\ reaction\ medium}$$

or

$$U/mg\,CDW = \frac{\triangle A_{420/min}}{\varepsilon \times mg\,CDW/mL\,reaction\,medium}$$

ε (extinction coefficient) of ONP: 1.3546 ml μmol^{-1} cm^{-1}

CDW: Cell dry weight

2.2.8.2 Stopped Enzyme Assay Protocol

Another enzyme assay protocol was achieved. Before the assay, the ONPG (30 mM ONPG in 0.05 M sodium phosphate buffer at pH 7.2; Appendix B) solution was preincubated at 37^0 C for at least 5 minutes. The appropriately diluted enzyme solution was stored at 5^0 C until assay time. Reaction was started by addition of 0.1 ml appropriately dilluted enzyme solution into 0.5 ml ONPG solution (30 mM ONPG in 0.05 M sodium phosphate at pH 7.2; Appendix B). Final volume of reaction solution becomes 0.6 ml after the addition of enzyme into the reaction solution to start reaction. The reaction was allowed to proceed for 70 seconds. Reaction was stopped by addition of ice-cold 0.6 ml 0.5 M sodium carbonate (Appendix B) into reaction medium. After the addition of sodium carbonate, total volume of the reaction medium becomes 1.2 ml. The solution was immediatly transferred into the ice.The absorbance was measured at 420 nm against appropriate enzyme and substrate blanks.

Two types of blank solution was used. One of them was zero time blank; enzyme activity was stopped at zero time by addition of 0.6 ml 0.5 M sodium carbonate, then 0.5 ml ONPG (30 mM ONPG in 0.05 M sodium phosphate at pH 7.2) solution which was preinbuted at 37^0 C for 5 minutes was transferred into the reaction

medium. The solution was immediatly transferred into the ice. Reagent blank was also used. Reagent blank was prepared in the same way with enzyme solution except it lacked 0.1 ml enzyme solution, instead of 0.1 ml distilled water was added.

The number of mole of ONP (O-Nitrophenol) liberated was determined from the standard curve of ONP. Units of the enzyme activity were expressed as the amount of enzyme required to release 1 µmoles of ONP per minute under the assay conditions. The extinction coefficient of dissolved o-nitrophenol at 420 nm was found to be 3.1703 ml μmol^{-1} cm^{-1} (Figure 12).

The rate of absorbance change (A_{420}/min) was calculated as follows:

$\Delta(A_{420}/min) = \Delta A_{420}/min$(Enzyme reaction) - $\Delta A_{420}/min$(Blank).

Specific activity was calculated as follows (Baran 1996):

$$U/mg\ protein = \frac{\triangle A_{420}/min}{\varepsilon \times mg\ protein/mL\ reaction\ medium}$$

ε (extinction coefficient) of ONP: 3.1703ml μmol^{-1} cm^{-1} (Figure 12)

2.2.9 Characterization of β-galactosidase from *L. plantarum* NCIMB 1193 Cell-Free Crude Extract From Sonication

Actively growing MMRS cultures was inoculated into 100 ml of fresh MMRS and allowed to grow at 37^0C for 12 hours. Cultures were than cooled in ice water for approximately 15 min and 80 ml of this sample was centrifuged at 12000 rpm for 9 minutes at 9^0 C (RC5C Sorvall Rotor GSA code 10). The pellet was washed with 80 ml distilled water. The pellet was resuspended in 25 ml distilled water. 12 ml of this

suspension was sonicated for 3 minutes on ice water bath in order to disrupts the cells and release the intracellular β-galactosidase. During sonication after each 30 second sonication period, 2 minutes break was given to prevent excessive heat formation. The sonicated suspension was tranferred into 1.5 ml eppendorf tubes and centrifuged at 12000 rpm for 8 minutes (Mikro 12-24, Hettich) in 1.5 ml eppendorf tubes. Supernatant solution was used for characterization of β-galactosidase of *L. plantarum* NCIMB 1193.

2.2.9.1 Effect of Substrate Concentration (ONPG) on β-galactosidase Activity

Cell free crude extract of *Lactobacillus plantarum* NCIMB 1193 was obtained as described in section 2.2.9. This solution was used to observe the effect of substrate concentration on β-galactosidase activity. Kinetic constants, K_m and $V_{max,}$ of the β-galactosidase were determined by changing the ONPG substrate concentration. ONPG substrate concentraion in the assay medium were 0.833 mM, 8.33 mM, 16.6 mM, 33.33 mM (in 0.05 M phosphate buffer at pH 7). Michaelis-Menten plot and Eadie-Hofstee plot were constructed to calculate the K_m and V_{max}.

In this study to find the actual number of ligand binding sites on the β-galactosidase obtained from *Lactobacillus plantarum* NCIMB 1193, Hill plot was constructed by plotting log (ν / ν_{max}- ν) versus log(s) on a linear scale.

2.2.9.2 pH Effect on β-galactosidase Activity

Cell free crude extract of *Lactobacillus plantarum* NCIMB 1193 was obtained as described in section 2.2.9. To determine the optimum pH, the β- galactosidase

activity was studied within the range of pH from 4 to 8. ONPG (30 mM) was used as the substrate during this experiment. Three different buffers were used; between pH 4 and 6.2, 0.05 M citrate buffer, between 6.2 and 8, 0.05 M sodium phosphate buffer and at pH 8, 0.05 M tris-HCl buffer were used. Composition and preparation of buffers were defined in Appendix B. Continuous enzyme assay protocol was used as described in section 2.2.8.1. Specific enzyme activity was calculated and it was defined as μmol ONP/ min/mg protein.

2.2.9.3 Temperature Effect on β-galactosidase Activity

Cell free crude extract of *Lactobacillus plantarum* NCIMB 1193 was obtained as described in section 2.2.9**.** The influence of different temperatures on β-galactosidase activity was observed at pH 7.2 and 30 mM substrate (ONPG) concentration. In order to determine the optimum temperature of β-galactosidase, the activity of enzyme was measured at different temperatures (15^0 C, 20^0 C, 25^0 C, 30^0 C, 35^0 C, 37^0 C, 40^0 C, 45^0 C, 50^0 C, 55^0 C). Stopped enzyme activity method was used as described in section 2.2.8.2. Specific enzyme activity was calculated and it was defined as μmol ONP/ min/mg protein.

CHAPTER 3

RESULTS

During this study *L. plantarum* NCIMB 1193, *L. plantarum* DSM 20246, *L. plantarum* E081 were used for the following assays; (i) β-galactosidase activity measurements, (ii) Growth curve analysis, (iv) Sonication optimization

L. plantarum NCIMB 1193 was used to investigate the effect of sonication on protein release, and viable cell number. Liquid nitrogen method was compared with sonication methods in terms of β-galactosidase release from *L. plantarum* NCIMB 1193. Sonication time optimization was carried out for *L. plantarum* NCIMB 1193, *L. plantarum* DSM 20246, *L. plantarum* E081. The cell-free extract of sonicated *L. plantarum* NCIMB 1193 was used to characterize the β-galactosidase. Temperature optimum and pH optimum were found. Km and V_{max} values were calculated. Hill plot was constructed to find the number of ligand binding sites on the enzyme.

3.1 Isolation of the *L. plantarum* from Erzurum Cheese

The isolate from Erzurum Cheese was identified as *Lactobacillus plantarum* by API identification software (Table 15). In Table 14, the results of gas formation, gram staining, catalase test were given. According to these results it could be concluded that, the isolate belongs to lactic acid bacteria group. In Table 16, the results of the identification tests applied for grouping the isolates were shown.

3.2 Basic Tests for Identification of Lactobacilli and β-galactosidase Production Test

All microorganism were checked for their purity. For this purpose we decided to re-idenitify the microorganisms. Erzurum cheese isolate also used throughout this study. It was found that it produced β-galactosidase. Therefore we also included this strain into the experiments.

L. plantarum NCIMB 1193, *L. plantarum* DSM 20246, *L. plantarum* E081 were found as gram-positive, catalase negative rods. They were not producing gas and they were able to grow at 15^0 C and 37^0 C (Table 14). For all strains, X-gal containing MMRS agar plates became blue in color. It means that all strains produced β-galactosidase (Figure 10).

Table 14. Results of the identification tests, temperature requirement for growth and β-galactosidase test

Strains	15^0 C	37^0 C	45^0C /55^0C /60^0C	Gram staining	Catalase activity	Gas Formation	X-gal
L. plantarum NCIMB 1193	+	+	-	+	-	-	+
L. plantarum DSM 20246	+	+	-	+	-	-	+
L. plantarum E081	+	+	-	+	-	-	+
E. coli				-			
- .....negative results,+.....positive results							

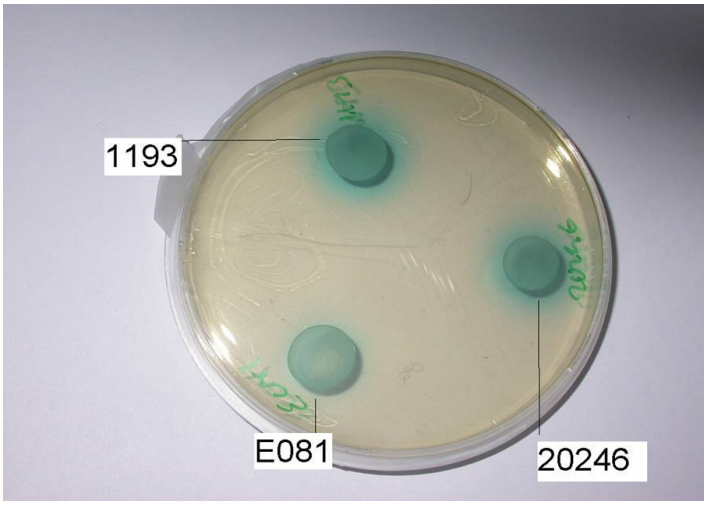

Figure 10. β-galactosidase activity on X-gal containing MMRS agar plates. Blue color indicates the β-galactosidase activity. 1193- *Lactobacillus plantarum* NCIMB 1193, 20246-*Lactobacillus plantarum* DSM 20246, E081-*Lactobacillus plantarum* E081

3.2.1 API Fermentation Kit

According to the results obtained from mini API computer program, all of the microorganisms were identified *as Lactobacillus plantarum* (Table 15).

Table 15. Results of the API kits which were analysed with API identification software

Strain	Results taken from by mini API computer program
L. plantarum DSM 20246	Excellent Identification %99.9 T= 0.96 *L. plantarum* 1
L. plantarum E081	Very Good Identification % 99.3 T=0.78 *L. plantarum* 1
L. plantarum NCIMB 1193	Excellent Identification %99.9 T=0.97 *L. plantarum* 1

Table 16: Identification tests applied for grouping the isolates

		E081	20246	1193
0	Control	—-	—	—
1	Glycerol	—	—	—
2	Erythritol	—	—	—
3	D-Arabinose	—	—	—
4	L-Arabinose	—	+	+
5	Ribose	+	+	+
6	D-Xylose	—	—	—
7	L-Xylose	—	—	—
8	Adonitol	—	—	—
9	ß Methyl-xyloside	—	—	—
10	Galactose	+	+	+
11	D-Glucose	+	+	+
12	D-Fructose	+	+	+
13	D-Mannose	+	+	+
14	L-Sorbose	—	—	—
15	Rhamnose	—	—	+
16	Dulcitol	—	—	—
17	Inositol	—	—	—
18	Mannitol	+	+	+
19	Sorbitol	+	+	+
20	α Methyl-D-mannoside	+	+	+
21	α Methyl-D-glucoside	—	—	—
22	N Acetyl glucosamine	+	+	+
23	Amygdaline	+	+	+
24	Arbutine	+	+	+
25	Esculine	+	+	+
26	Salicine	+	+	+
27	Cellobiose	+	+	+
28	Maltose	+	+	+
29	Lactose	+	+	+
30	Melibiose	—	+	+
31	Saccharose	+	+	+
32	Trehalose	+	+	+
33	Inuline	—	—	—
34	Melezitose	+	+	+
35	D-Raffinose	—	—	+
36	Amidon	—	—	—
37	Glycogene	—	—	—
38	Xylitol	—	—	—
39	β Gentiobiose	+	+	+
40	D-Turanose	—	+	+
41	D-Lyxose	—	—	—
42	D-Tagatose	—	—	—
43	D-Fucose	—	—	—
44	L-Fucose	—	—	—
45	D-Arabitol	—	—	—
46	L-Arabitol	—	—	—
47	Gluconate	+	+	+
48	2 ceto-gluconate	—	—	—
49	5 ceto-gluconate	—	—	—

3.3 ONP Standard Curve

For continuous β-galactosidase assay, the liberated ONP amount was calculated by using standard curve of ONP. The extinction coefficient (ε) of o-nitrophenol was found as 1.3546 ml μmol^{-1} cm^{-1} (Figure 11).

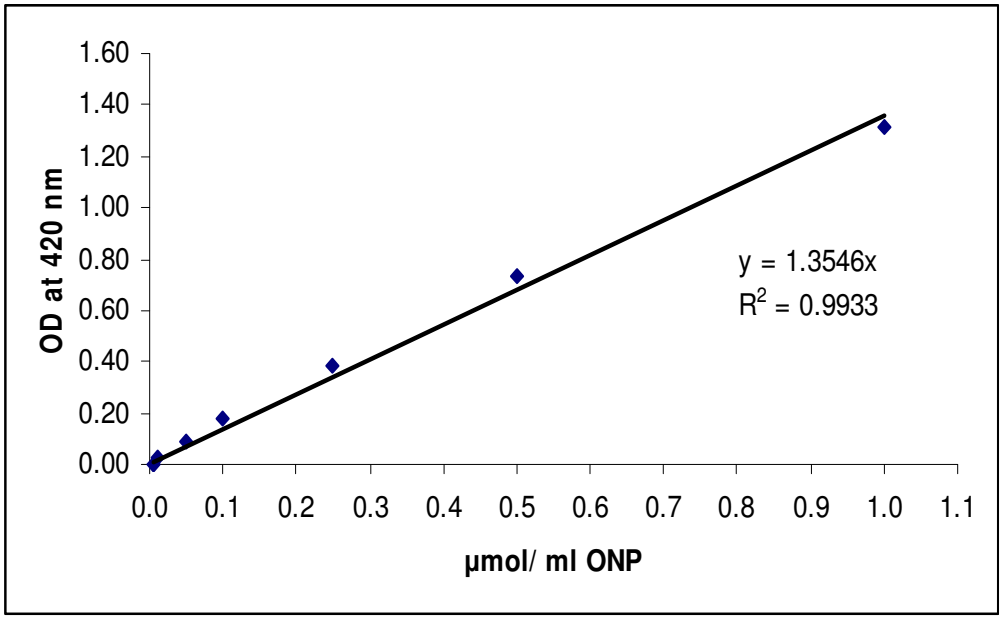

Figure 11. ONP (o-nitrophenol) Standard Curve for continous enzyme assay.

For stopped enzyme activity method, the extinction coefficient of o-nitrophenol was found as 3.1703 ml μmol^{-1} cm^{-1}.

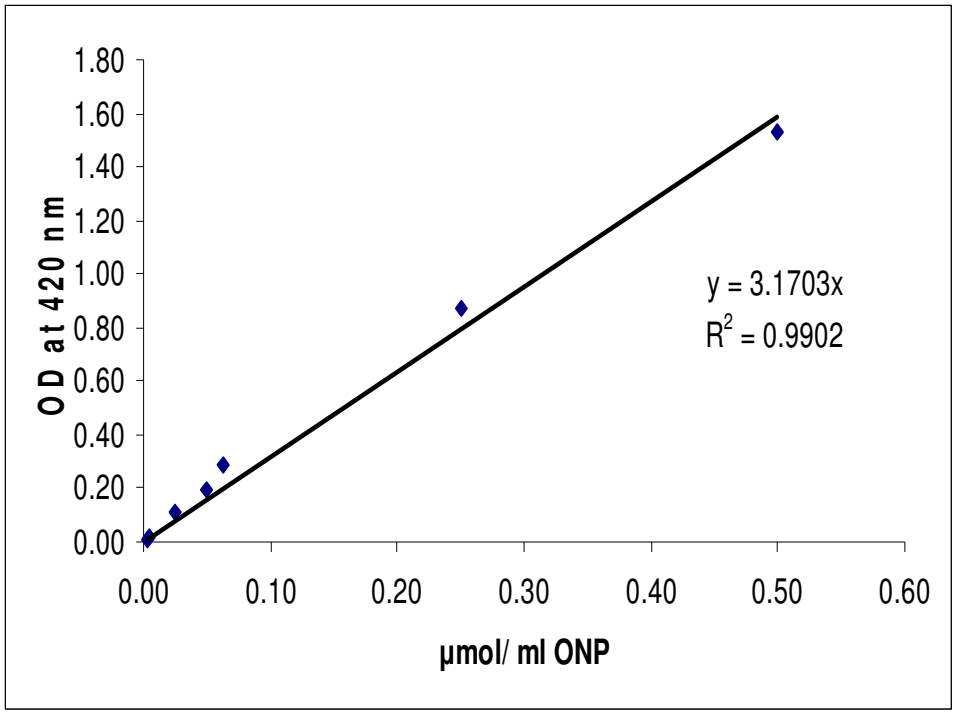

Figure 12. ONP (o-nitrophenol) Standard Curve for stopped enzyme assay.

3.4 Protein Determination

3.4.1 Bradford Method

Standard curve of Bovine Serum Albumin (BSA) was constructed tor determine the protein concentration of cell-free crude extract.

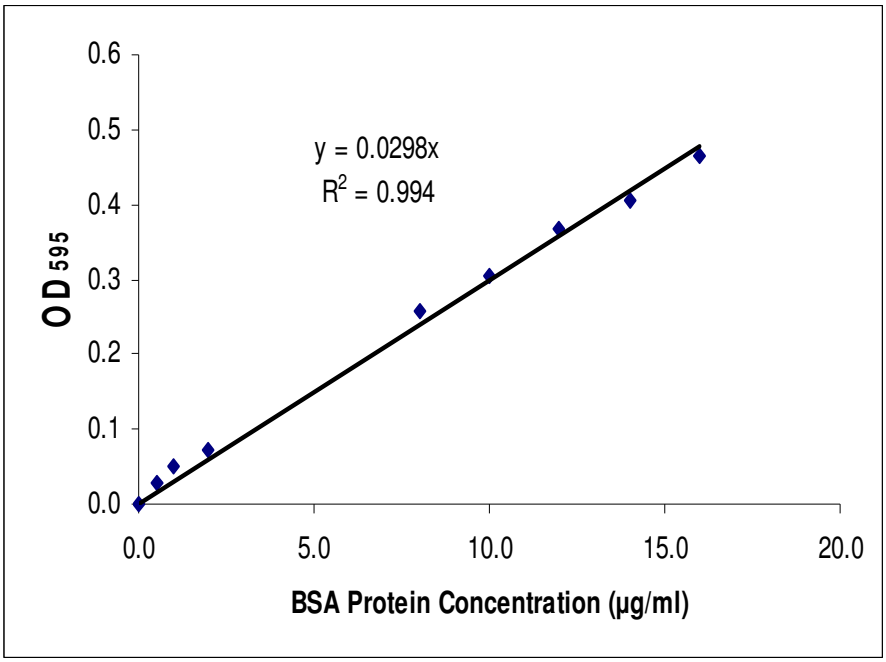

Figure 13**.** Standard curve for protein determination

3.5 Growth Studies and β-Galactosidase Production

3.5.1 Growth Studies for *Lactobacillus plantarum* NCIMB 1193

Lactobacillus plantarum NCIMB 1193 inoculated in 600 ml MMRS broth were used for growth studies. Total β-galactosidase and specific β-galactosidase activities were assayed in parallel to pH and growth (Figure 14, Figure 15). Relation pattern of cell dry weight and β-galactosidase production (Figure 16), differential rates of β-galactosidase production (Figure 17), and comparison of two types of specific

activities (Figure 18) (μmol min^{-1} mg^{-1} CDW and μmol min^{-1} mg^{-1} protein) were carried out under assay conditions.

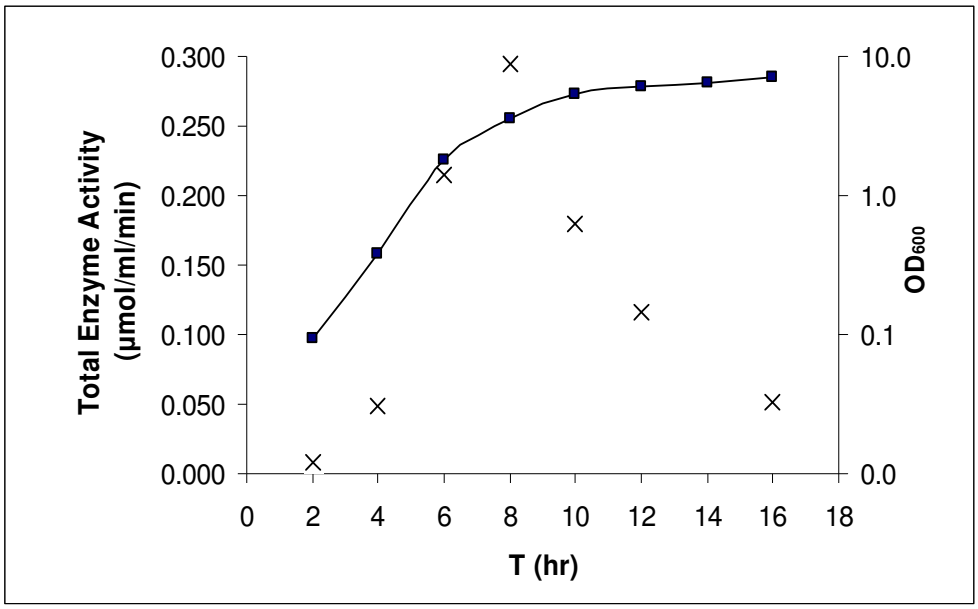

Figure 14: Cell growth of *Lactobacillus plantarum* NCIMB 1193 in batch cultures on MMRS (600ml) at 37^0 C. The following parameters were evaluated: optical density (■), total β-galactosidase activity (×).

Figure 14 shows the evolution of total β-galactosidase activity and cell growth of *Lactobacillus plantarum* NCIMB 1193 in batch cultures on MMRS (600ml) at 37^0 C. Cultivation were done in triplicate as described in section 2.2.6.1 in Material and Methods. It can be observed that the total β-galactosidase activity initially increased and attained a maximum of 0.295 μmol/ml/min after 8 h of incubation. This initial part where the increase in activity is observed comprised the exponential phase and early stationary phase. However, as the incubation continued, total β-galactosidase activity decreased.

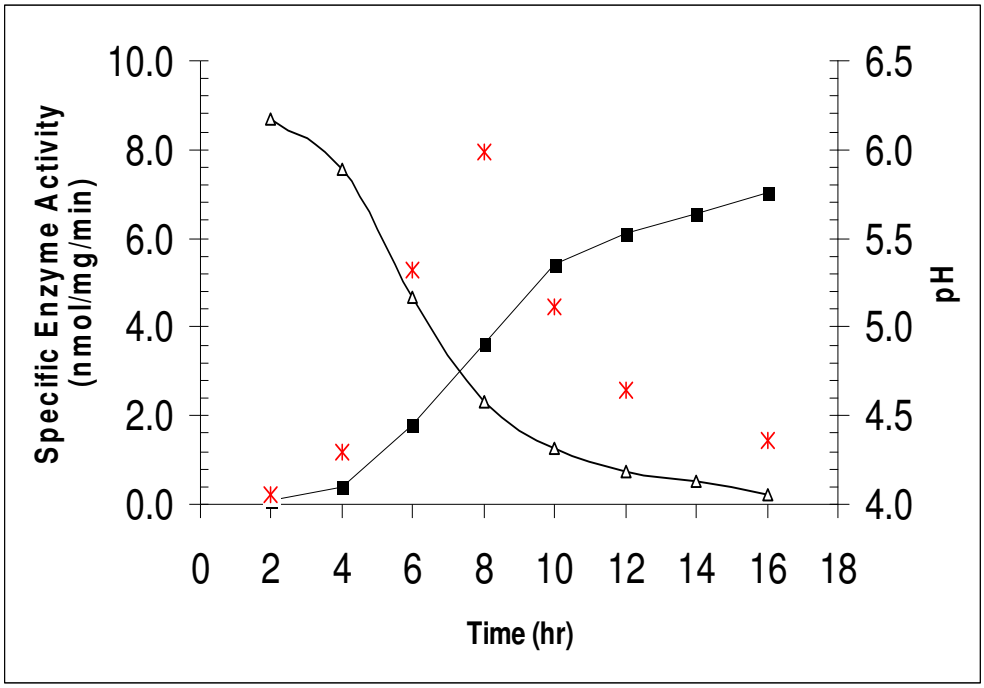

Figure 15. Growth analysis of *Lactobacillus plantarum* NCIMB 1193 in batch cultures on MMRS (600ml) at 37^0 C. The following parameters were evaluated: OD_{600} (■), specific β-galactosidase activity (ж), pH (Δ)

In Figure 15, specific β-galactosidase of *L. plantarum* NCIMB 1193, which was calculated from the total β-galactosidase activity, was shown. It can be observed that specific enzyme activity initially increased and attained a maximum of 7.949 nmol/mg/min after 8 h of incubation. As the incubation continued, the pH of the medium increased. The rate of change in the pH is high between pH 5.9 and pH 4.6 values. However in the ranges of pH above 5.9 and below 4.6, the change in the pH is slow. The slow change in pH in these regions can be explained by the slower growth rate of the cell.

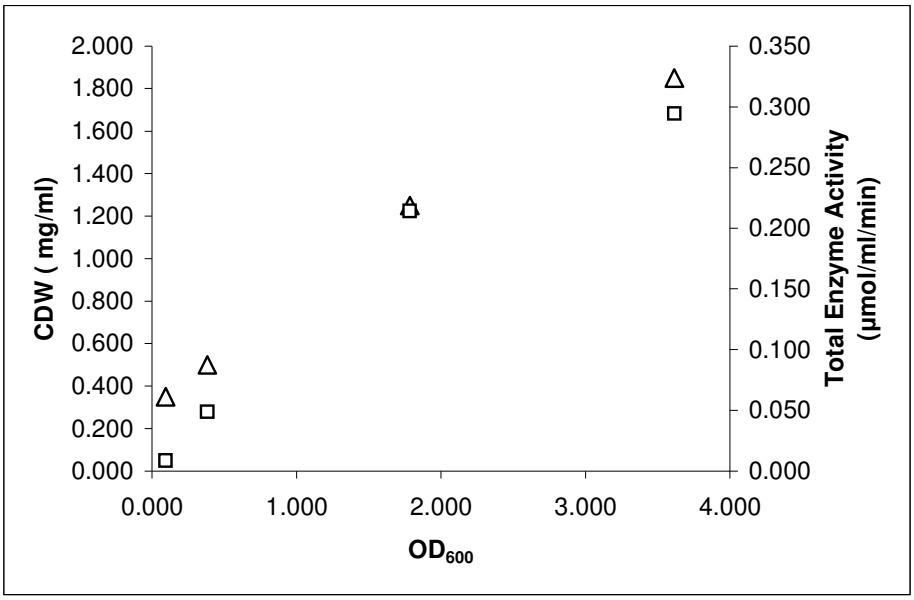

Figure 16. Cell dry weight (CDW) and total β-galactosidase activity of *Lactobacillus plantarum* NCIMB 1193. The following parameters were evaluated: cell dry weight (CDW) (Δ), total β-galactosidase activity (□)

In Figure 16, total β-galactosidase activity (µmol/ml/min) and cell biomass as miligram of cell dry weight (CDW) per ml of culture were shown. The values were obtained as described in section 2.2.6.1.1 Materials and Methods. A positive corellation was found between cell dry weight (CDW) and total β-galactosidase acitivity (µmol/ml/min). In other words both biomass and total β-galactosidase activity increased while the turbidity increased.

When enzyme activity values were plotted against the corresponding CDW values, a linear graph appeared (Fig 17), the slope of this graph was called as "differential rate of enzyme production" which not only relates the specific activity values to population concept, in other words is the average value of population dependent specific activity but also is a standardized parameter which provides comparison among strains.

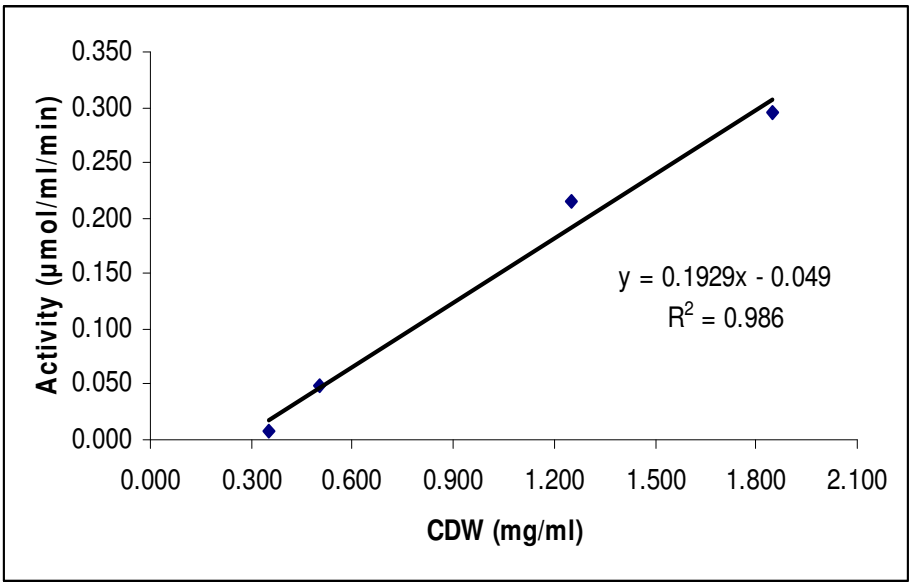

Figure 17. Differential rates of β-galactosidase production by *Lactobacillus plantarum* NCIMB 1193.

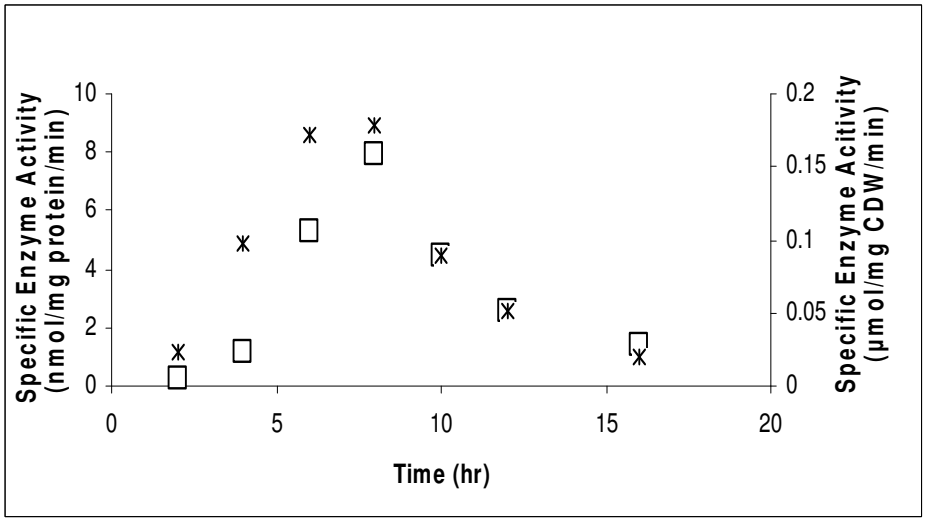

Figure 18. Comparison of two types of specific activities by using β-galactosidase from *Lactobacillus plantarum* NCIMB 1193. The following parameters were evaluated:specific β-galactosidase activity (nmol/mg protein/min) (ж), specific β-galactosidase activity (µmol/mg CDW/min) (□)

In Figure 18, two different expression of specific activities of β-galactosidase from *L.plantarum* NCIMB 1193 was compared. In this assay, cell dry weights and protein amounts were used to measure specific activies. It was found that there is a correlation between two specific activities. Thus either the protein amount or the CDW (cell dry weight) could be used to calculate specific activity.

3.5.2 Construction of Growth Curve from MMRS Growth Medium (100ml)

3.5.2.1 Growth Analysis of *Lactobacillus plantarum* NCIMB 1193

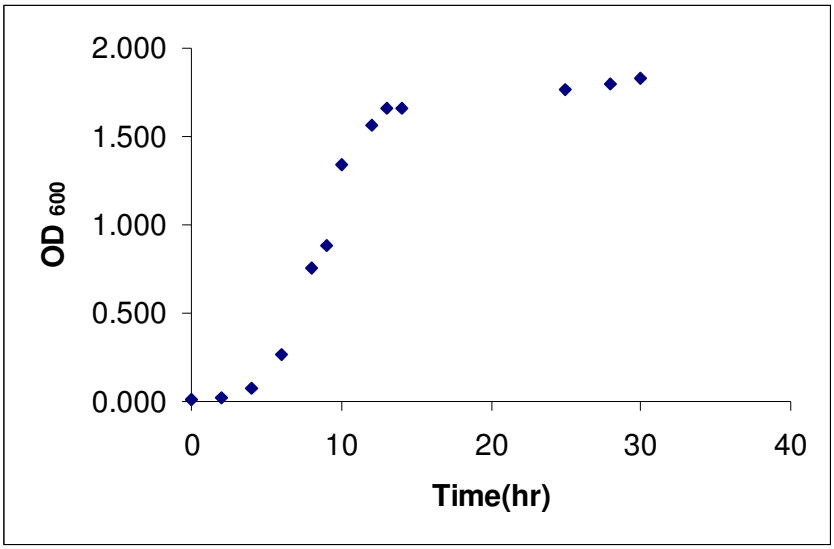

Figure 19. Growth curve of *Lactobacillus plantarum* NCIMB 1193 at 37^0 C.

It was found that the *Lactobacillus plantarum* NCIMB 1193 strain entered to the late logaritmic phase (early stationary phase) at around 12 hours of incubation at 37^0 C in 100 ml MMRS (Figure 19). Cultivation were done in triplicate as described in section 2.2.6.2.in Materials and Methods.

3.5.2.2 Growth Analysis of *Lactobacillus plantarum* E081

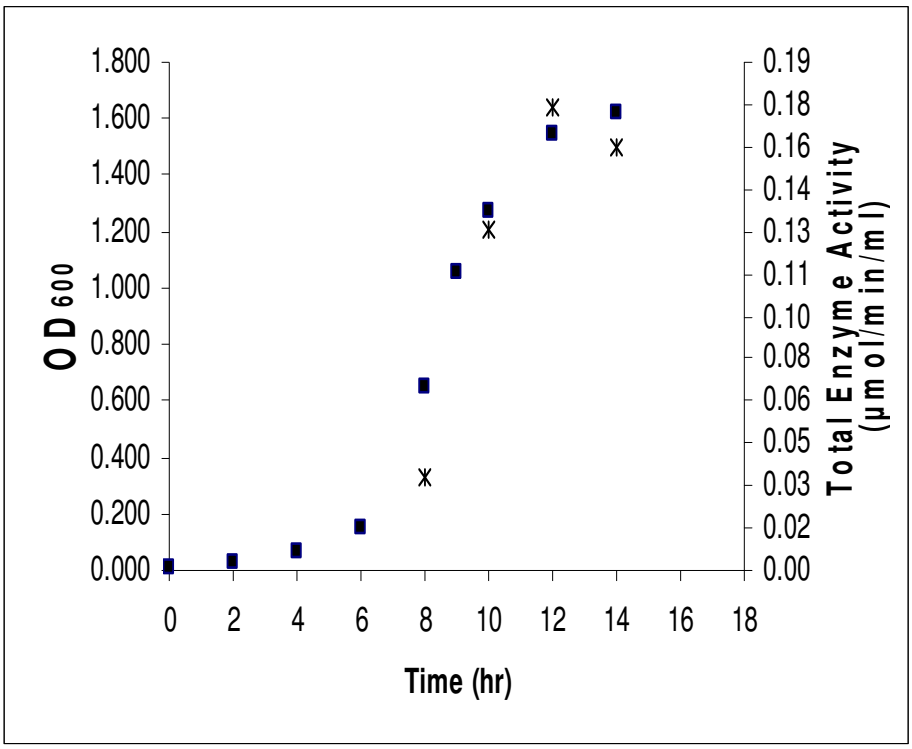

Figure 20. Growth Analysis of *Lactobacillus plantarum* E081 at 37^0 C . The following parameters were evaluated:OD_{600} (■), total enzyme activity (ж).

It was found that the *Lactobacillus plantarum* E081 strain entered to the late logarithmic phase or early stationary phase at around 12 hours of incubation at 37^0 C in 100 ml MMRS (Figure 20). It can be observed that the total β-galactosidase activity initially increased and attained a maximum of 0.175 µmol/ml/min after 12 h of incubation. This initial part where the increase in activity is observed comprised the exponential phase and early stationary phase. However, as the incubation continued, total β-galactosidase activity decreased. Cultivation was done in duplicate as described in section 2.2.6.2.1 in Materials and Methods.

3.5.2.3 Growth Analysis of *Lactobacillus plantarum* DSM 20246

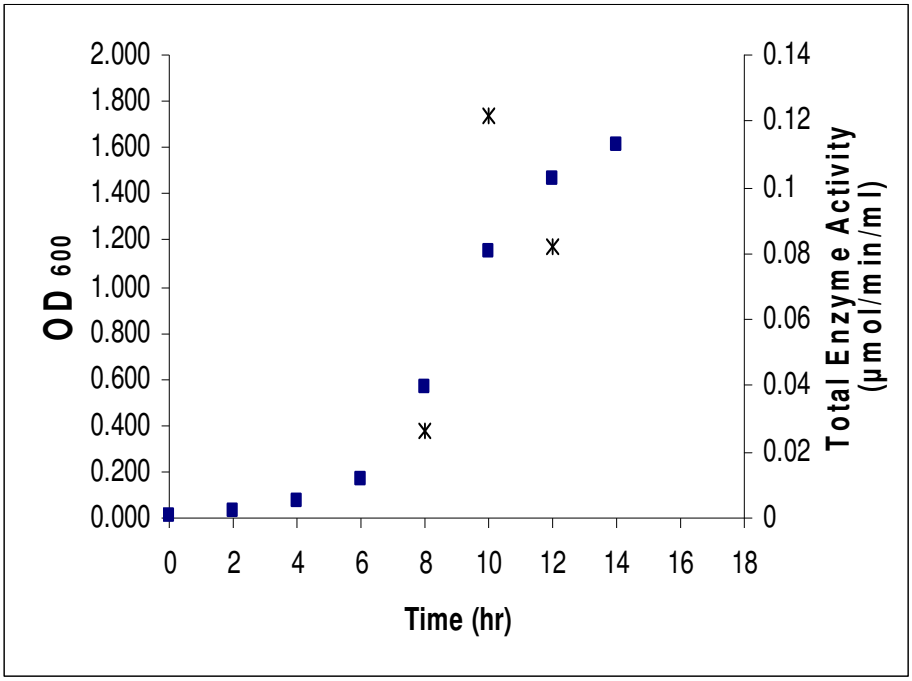

Figure 21. Growth analysis of *Lactobacillus plantarum* DSM 20246 at 37^0 C . The following parameters were evaluated:OD_{600} (■), total β-galactosidase activity (ж).

It was found that the *Lactobacillus plantarum* DSM 20246 strain entered to the late logarithmic phase or early stationary phase at around 10 hours of incubation at 37^0 C in 100 ml MMRS (Fig 21). It can be observed that the total β-galactosidase activity initially increased and attained a maximum of 0.122 µmol/ml/min after 10 h of incubation. This initial part where the increase in activity is observed comprised the exponential phase and early stationary phase. However, as the incubation continued, total β-galactosidase activity decreased. Cultivation was done in duplicate as described in section 2.2.6.2.1 in Materials and Methods.

3.6 Sonication and Liquid Nitrogen Methods for β-galactosidase Release from *Lactobacillus*

3.6.1 Liquid Nitrogen Method

Total β-galactosidase activity of cell-free extract of *Lactobacillus plantarum* NCIMB 1193 was found by using continous enzyme activity method as described in section 2.2.8.1 in Materials and Methods. From the results in Table 17, it could be concluded that liquid nitrogen method is not as effective as sonication method interms of β-galactosidase release from the cell.

Table 17. Total β-galactosidase activity results of *Lactobacillus plantarum* NCIMB 1193.

Source Microorganism: *Lactobacillus plantarum* NCIMB 1193		
	Liquid Nitrogen Method	Sonication
Total β-galactosidase Activity	0.076.(μmol /min/ml)	1.061 (μmol /min/ml)
Substrate Concentration and Assay Conditions	40 mM ONPG (in 0.05 M sodium phosphate buffer) at 37^0C	40 mM ONPG (in 0.05 M sodium phosphate buffer) at 37^0C

3.6.2 Sonication Method

3.6.2.1 Optimization of Sonication Method

Sonication method was used as a mechanical disruption method for protein release from the cell. Time is an important parameter of sonication. This parameter was optimized for *Lactobacillus plantarum* NCIMB 1193, *Lactobacillus plantarum* DSM 20246, *Lactobacillus plantarum* E081 for β-galactosidase release from the cell. The sonicated cell were checked for total β-galactosidase activity as decribed in section 2.2.6.2.1 in Materials and Methods.

3.6.2.1.1 Effect of Sonication Time on the Release of Cellular β-Galactosidase from *L.plantarum* NCIMB 1193

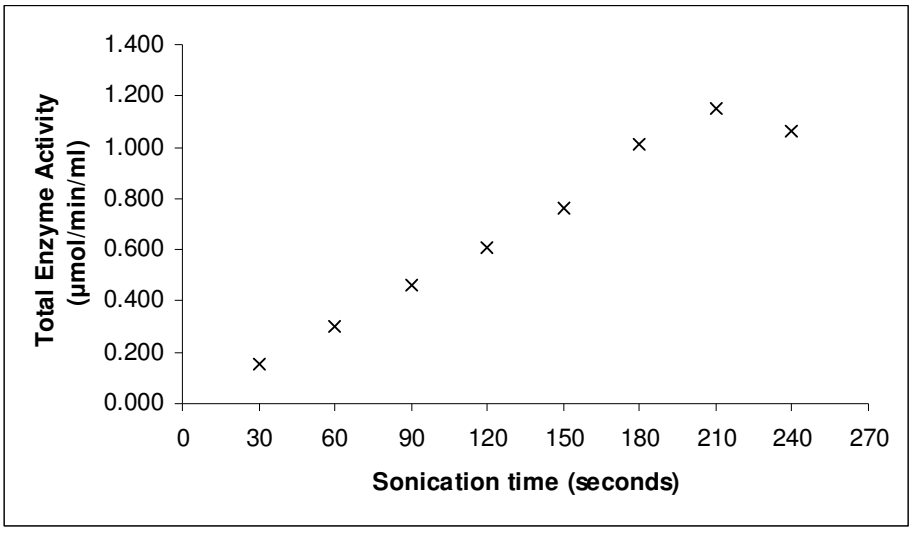

Figure 22. Effect of sonication time on release of cellular β-galactosidase from *Lactobacillus plantarum* NCIMB 1193. Following parameter was evaluated: total β-galactosidase activity (×)

In Figure 22, total β-galactosidase activity of *L. plantarum* NCIMB 1193 was shown. It can be observed that total enzyme activity initially increased and attained a maximum of 1.150 μmol/min/ml after 210 second sonication. As the sonication continued, the total β-galactosidase activity decreased. The results are the average of tree trials.

3.6.2.1.2 Effect of Sonication on the Release of Cellular β-Galactosidase from *L. plantarum* DSM20246

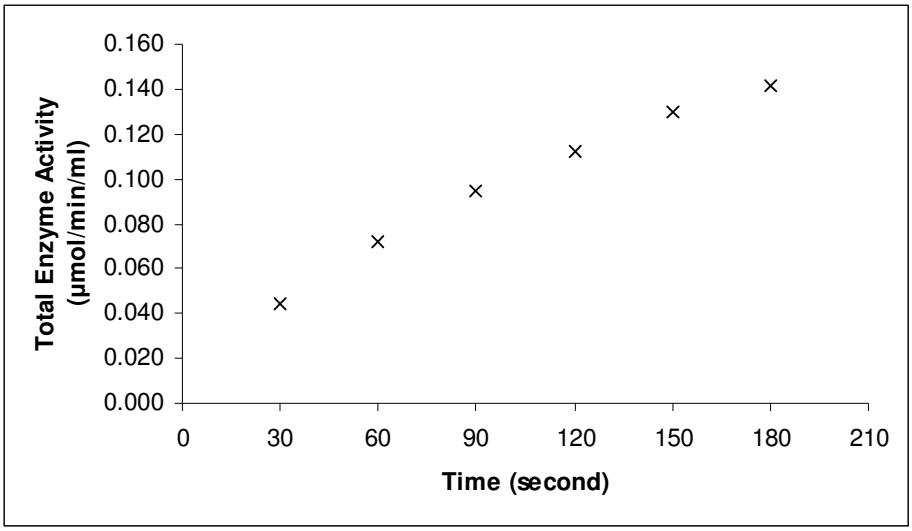

Figure 23. The effect of sonication time on release of cellular β-galactosidase from *L. plantarum* DSM 20246. Following parameter was evaluated: total β-galactosidase activity (×)

In Figure 23, total β-galactosidase activity of *L. plantarum* DSM 20246 was shown. It can be observed that total enzyme increased and attained a maximum of 0.142 μmol/min/ml after 180 second sonication. The results are the average of tree trials.

3.6.2.1.3 Effect of Sonication on the Release of Cellular β-Galactosidase Activity of *L. plantarum* E081

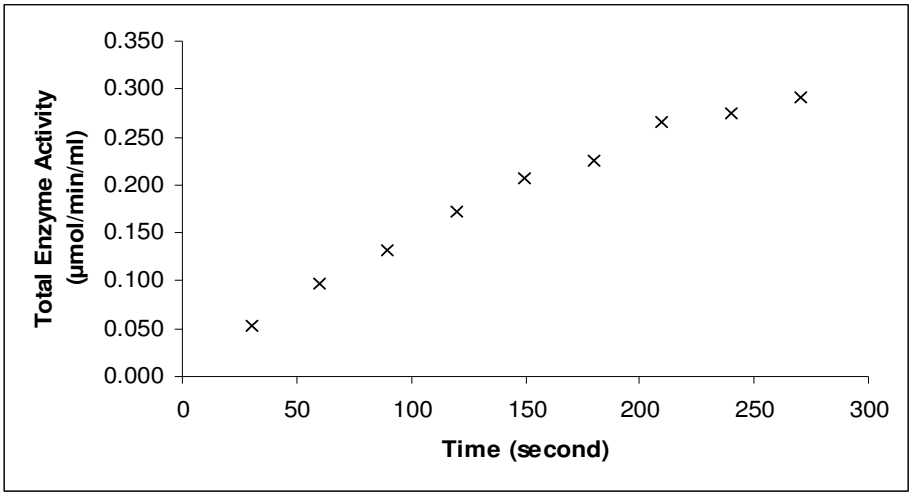

Figure 24. The effect of sonication time on release of cellular β-galactosidase from *L. plantarum* E081. Following parameter was evaluated: total β-galactosidase activity (×).

In Figure 24, total β-galactosidase activity of *L. plantarum* E081 was shown. It can be observed that total enzyme activity increased and attained a maximum of 0.292 µmol/min/ml after 270 second sonication. After the 210 second sonication, the increase in total β-galactosidase activity is low. The results are the average of two trials.

3.6.2.2 Viable Cell Count of *Lactobacillus plantarum* NCIMB 1193 Before and After Sonication

The viability of the *Lactobacillus plantarum* NCIMB 1193 culture before and after sonication was determined as described in 2.2.7.2.1.2 Materials and Methods.

According to results in Table 18, it can be concluded that the decrease in the cell viability is very low after sonication.

Table 18. The effect of sonication on cell viability of cultures of *Lactobacillus plantarum* NCIMB 1193.

log (plate count)(cfu.mL^{-1})	
Before sonication	After sonication
9.769	9.125
cfu:colony forming unit	

3.6.2.3 Effect of Sonication on Protein Release from Cell

Effect of sonication on the release of cellular protein from *Lactobacillus plantarum* NCIMB 1193 was determined as decribed in section 2.2.7.1.3 in Materials and Methods. Figure 25 shows the evalutation of protein release from the sonicated *Lactobacillus plantarum* NCIMB 1193 strain. It can be observed that the concentration of protein released from the sonicated cell increase after each 30 second sonication period.

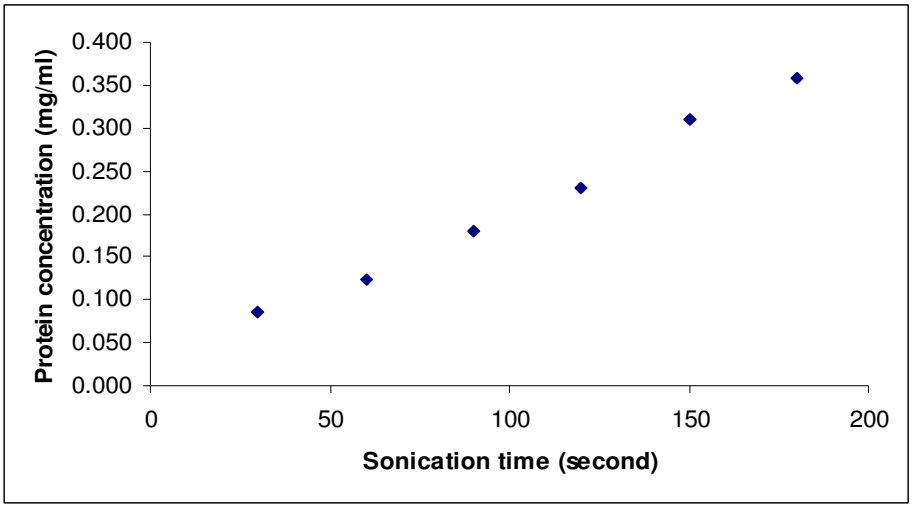

Figure 25. Effect of sonication on release of cellular protein from *Lactobacillus plantarum* NCIMB 1193. Protein concentration (mg/ml) of cell free extract was evaluated.

3.6.3 Characterization of β-Galactosidase from Cell-Free Crude Extract of Sonicated *Lactobacillus plantarum* NCIMB 1193 Culture Medium

3.6.3.1 Effect of Substrate Concentration on β-Galactosidase Activity

The enzyme activity was measured at different concentrations of the substrate ONPG. The amount of the substrate used in the reaction medium was varied between 33.333 and 0.833 mM within the assay medium. As seen in Figure 26, β-galactosidase follows a parabolic Michaelis-Menten kinetics. The reaction rate increased while the substrate (ONPG) concentration increased. K_m and V_{max} values of β-galactosidase were calculated from the reciprocal plots of substrate concentration versus reaction velocity (Figure 27). The Lineweaver-Burk plot was linear, suggesting a simple Michealis-Menten kinetics. The V_{max} was found as 1.721 (μmol/ min mg) and K_m was found as 3.47 mM.

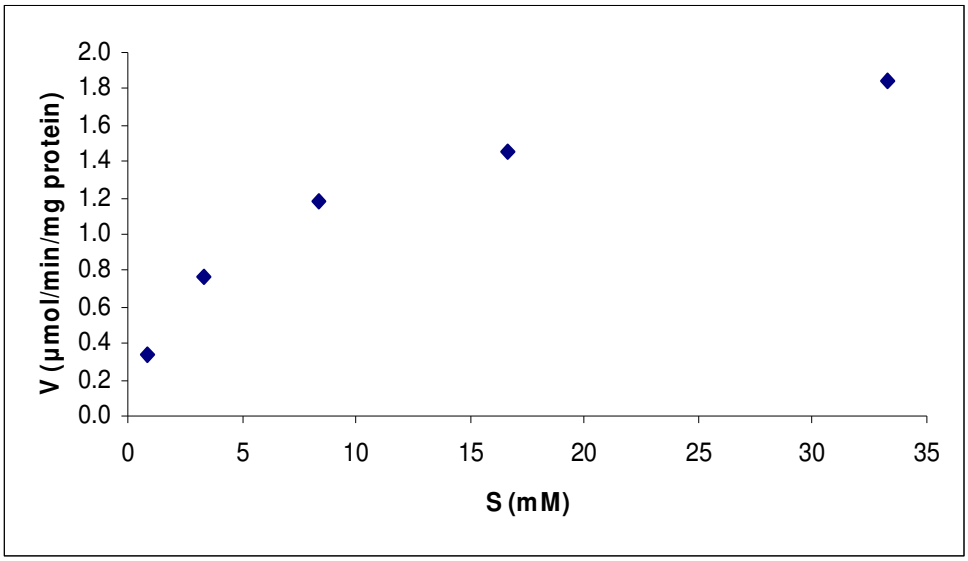

Figure 26. Michaelis-Menten plot for β-galactosidase of *Lactobacillus plantarum* NCIMB 1193. The following parameters were evaluated: rate of the β-galactosidase from cell free crude extract (µmole.min^{-1} per mg protein) (V), substrate concentration (mM) (S).

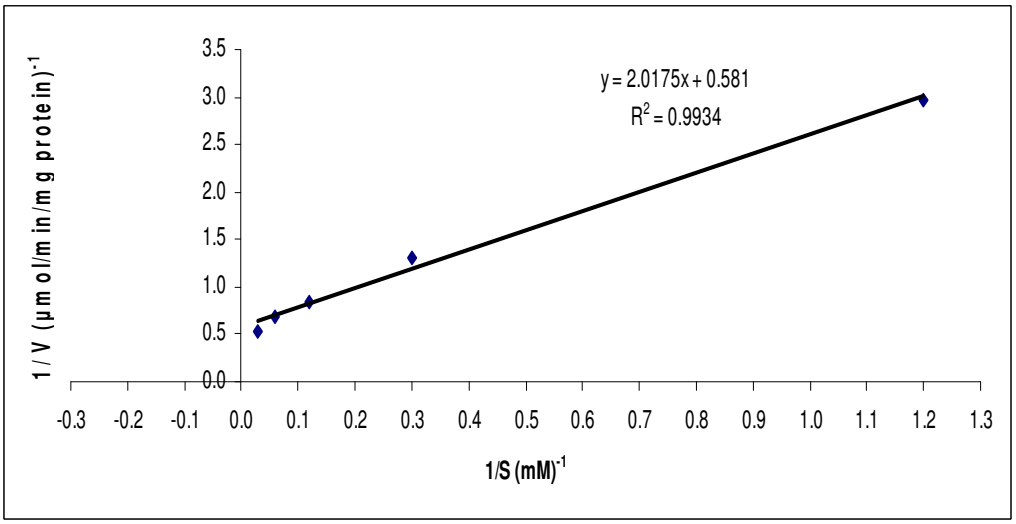

Figure 27. Lineweaver-Burk double reciprocal plot of β-galactosidase.

The Eadie-Hofstee plot of β-galactosidase of *Lactobacillus plantarum* NCIMB 1193 was constructed as decribed in section 2.2.9.1 in Material and Methods (Figure 28). The Eadie- Hofstee plot is preferred for discovering deviations from linearity. In Table 19, V_{max} and K_m values calculated from Eadie-Hofstee plot and Lineweaver-Burk double reciprocal plot were given. V_{max} and K_m values calculated from different graphs were found to be close to the each other (Table 19).

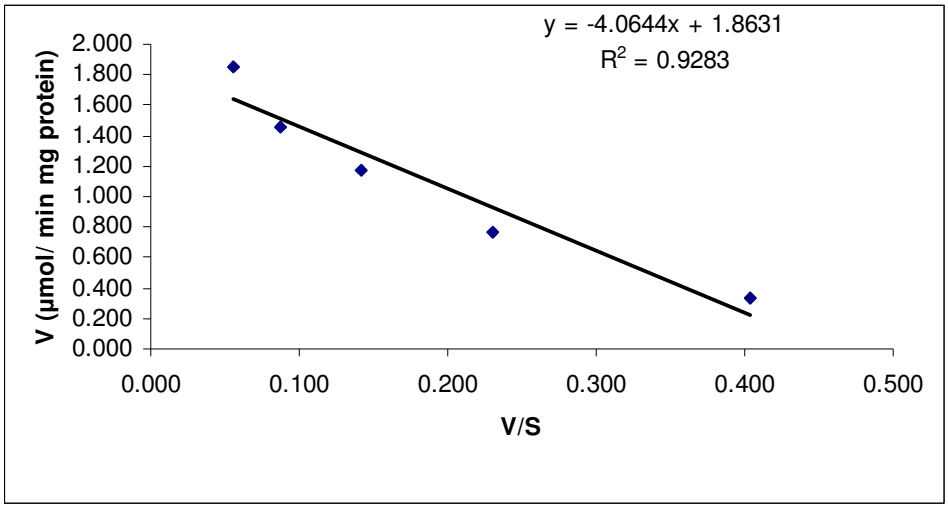

Figure 28. Eadie-Hofstee plot for β-galactosidase. The following parameters were evaluated: rate of the β-galactosidase (µmole min^{-1} per mg protein)(V), substrate concentration(mM) over rate (V/S).

Table 19. V_{max} and K_m values

	Eadie-Hofstee plot	Lineweaver-Burk double reciprocal plot
V_{max}	1.863 µmol/ min mg protein	1.721 µmol/ min mg protein
K_m	4.064 Mm	3.47 mM

The slope of the Hill plot was used to find the "n_{app}" value. "n_{app}" refers to the actual number of ligand binding sites on a molecule of enzyme. It was found as 1.03 (Figure 29). In Figure 29, the rate (V) was defined as μmole.min^{-1} per mg protein and S was defined as mM substrate concentration in the reaction medium. V_{max} was obtained from the Lineweaver-Burk double reciprocal plot of β-galactosidase.

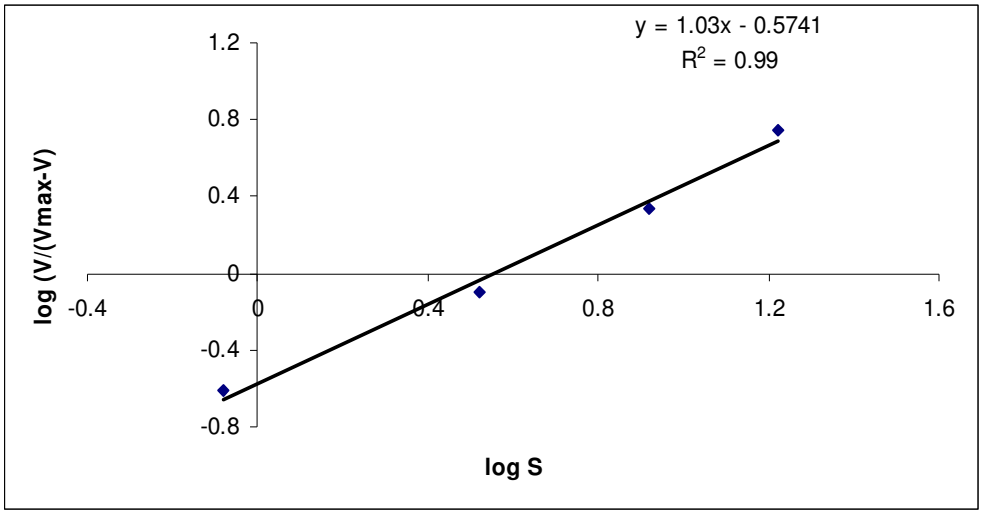

Figure 29. Hill plot. The following parameters were used: specific β-galactosidase activity (V), maximum β-galactosidase activity (V_{max}), substrate concentration (S).

3.6.3.2 Effect of pH on β –galactosidase Activity

In Figure 30, it can be observed that specific β-galactosidase activity increase up to pH 7.2. The maximum specific activity value was calculated as 1.626 μmol /min/mg protein under the assay conditions as described in section 2.2.9.2 in Materials and Methods. After pH 7.2, decrease in specific activity value was observed.

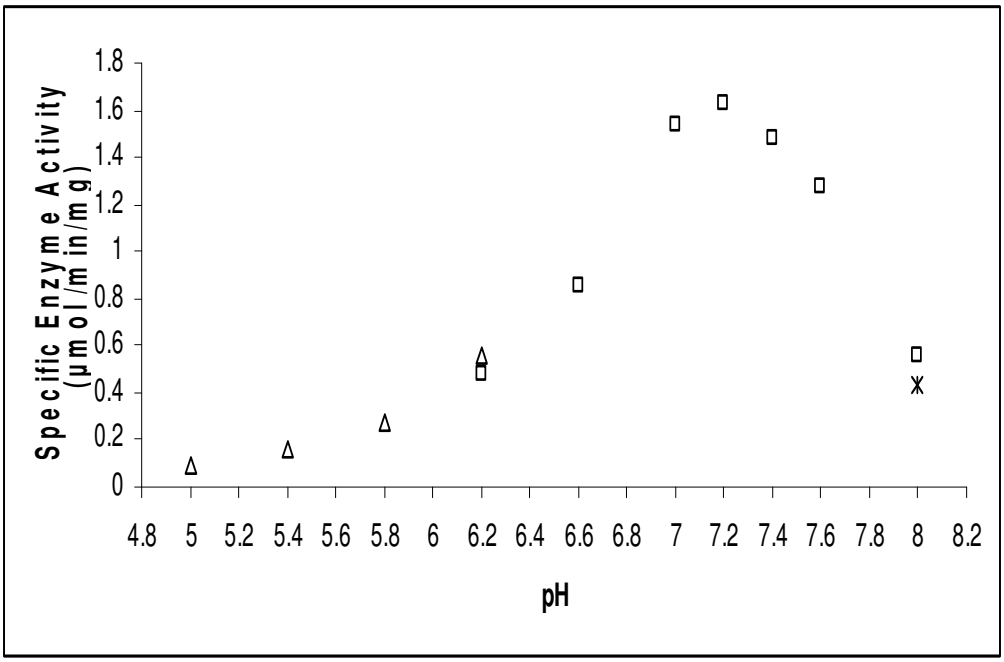

Figure 30**.** Effect of pH on β–galactosidase activity. *Lactobacillus plantarum* NCIMB 1193 was used. The following parameters were evaluated: specific β-galactosidase activity in citrate buffer (Δ), specific β-galactosidase activity in phosphate buffer (□), specific β-galactosidase activity in tris-HCl buffer (ж).

3.6.3.3 Effect of Reaction Temperature on β-galactosidase Activity

In Figure 31, it can be observed that specific β-galactosidase activity increase up to 37^0 C. The maximum specific activity was calculated as 3.805 µmol /min/mg protein under the assay conditions as described in section 2.2.9.3 in Materials and Methods. After 37^0 C, decrease in specific activity was observed.

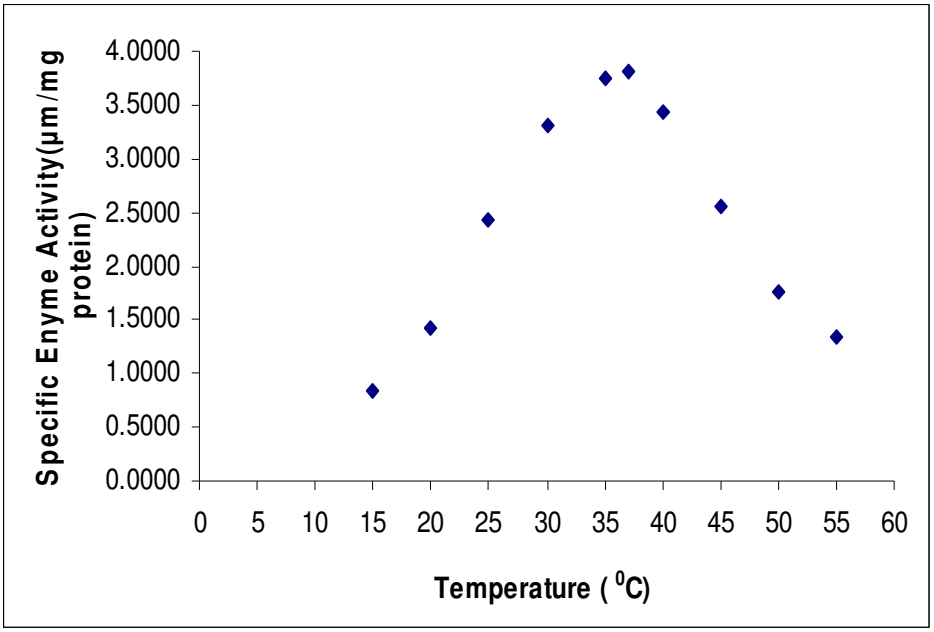

Figure 31. Effect of temperature on β–galactosidase activity (µmole min^{-1} per mg protein). *Lactobacillus plantarum* NCIMB 1193 was used

CHAPTER 4

DISCUSSION

Lactose, the main sugar in milk and whey, and its corresponding hydrolase, β-galactosidase, have been the subject of extensive research during the past decade. Partly, this is because of the interesting possibilities of using low lactose or lactose free products.

In this study, β-galactosidase production was measured and sonication parameters were optimized for three *Lactobacillus plantarum* strains. *Lactobacillus* strains were selected for three particular reasons (Somkuti et al. 1998): a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects b) LAB are generally regarded as safe (GRAS) so the enzyme derived from them might be used without extensive purification (Vasiljevic et al. 2002) c) some strains have probiotic activity such as improved digestion of lactose by releasing β-galactosidase into the environment (Vinderola and Reinheimer 2003).

In this study for all strains, positive correlation between growth and β-galactosidase activity was investigated. It was observed that, maximum total β-galactosidase activity corresponds to the early stationary phase for three of the strain (Figure 14, Figure 20, Figure 21). A decline in total enzyme activity was observed after the early stationary phase during the growth experiments. The decline in total enzyme activity could be considered as a result of inhibition of cellular functions due to high pH, depletion of a nutritional factor from the growth medium,deactivation of the enzyme due to low pH catabolite repression, or/and inducer exclusion. Lactic acid bacteria maintain a cytoplasm that is more alkaline than the medium, but the medium is acidified during growth by secretion of lactic acid. However, if the cytoplasmic pH decreases below a threshold pH, cellular functions are inhibited and

the intracellular enzymes can be deactivated (Kashket 1987). The amount of glucose and galactose could regulate the enzyme activity by catabolite repression or inducer exclusion. Hickey et al. (1986) proposed that addition of glucose into growth medium containing lactose decreases the β-galactosidase activity. Other studies suggested that the glucose must be present in the external medium for it to exert an repressive effect (Rephali and Saier 1980). In this experiment, lactose was used as a main carbohydrate source by the bacteria during the growth. During cell growth in MMRS medium, the amount of lactose decreased while the amount of glucose, galactose, and oligosachharide concentrations increased. When the cytoplasmic glucose increased over a threshold, β-galactosidase activity could be inhibited in gene level.

In the literature, definition of β-galactosidase activity differs considerably. Throughout this study, a unit activity was defined as the amount of the enzyme required to release one μmol of o-nitrophenol in one minute under the assay conditions. It was found that both CDW and protein amount could be used to calculate the specific enzyme activities (Figure 18).

In this study three different cell disruption methods were used and two of them were compared with each other (Table 17). Lysozyme method, sonication method, and liquid nitrogen method were used for protein release from the cell. Sonication and liquid nitrogen method were compared with each other. Sonication was found to be the most effective method in terms of β-galactosidase release from the cell (Table 17). The amount of protein release from the cell during application of disruption method is directly related with the composition of the cell surface of the bacteria. In Gram-positive bacteria a thick layer of mucopeptide (peptidoglycan network) confers a considerable rigidity to the cell wall. This polymeric compound is composed of N-acetyl-D-glucosamine and N-acetyl-muramic acid in β-(1—>4) linkage as alternating units in polysaccharide (glycan) chains. The major resistance to disruption of bacterial cells appears to be the peptidoglycan network.

β-galactosidase is an intracellular enzyme in the lactobacilli strain. Therefore, the amount of protein release is directly related with the efficiency of the disruption method.

During growth studies, enzymatic lysis method was used to release the β-galactosidase from the cell. On one hand, enzymatic lysis has the advantage of being specific and gentle, but on the other hand it is an expensive method. Lysozyme is often used for lysis of peptidoglycan layers as it catalyses hydrolysis of β-1.4-glycosidic bonds. During lysozyme lysis, a different technique was used to increase the efficiency of the method. The customary procedure was to wash and suspend the cells in a phosphate buffer containing sodium chloride, and then to add lysozyme. However, it was proposed that to add sodium chloride after addition of lysozyme could be more effective in terms of protein release from the cell. The addition of sodium chloride after addition of lysozyme into the cell solution decreases the effect of growth phase of culture on protein release from the cell. During the transition from exponential to stationary phase, considerable changes in peptidoglycan structure occur. The degree of cross linkage increases significantly (Geciova et al. 2002). As a result, β-galactosidase release is significantly affected from the growth phase of the culture. However, when lysozyme was added firstly into the cell suspension and then sodium chloride, it was found that the growth phase of the culture did not significantly affect the reaction rate of lysozyme (Metcalf and Deibel 1969). Therefore during this study, lysozyme was added to cell suspension before sodium chloride. This solution was incubated in 37^0 C water bath for 15 minutes. After this incubation period, sodium chloride was added into the solution. It is known that sodium chloride increases the ionic strength of the solution; therefore the rate of lysozyme-catalysed reaction would increase.

The second cell disruption method used in this study was liquid nitrogen method. Liquid nitrogen method is generally used to disrupt yeast and fungi. In this study it was not found as effective (in terms of β-galactosidase release into the medium) as

sonication method (Table 17). The efficiency of this method would be increased by the grinding the sample several times. But this application is time consuming.

In Table 17, it can be seen that total β-galactosidase activity of sonicated cell suspension is higher than the liquid nitrogen method. Sonication method has many advantages. In several studies cell lyses by sonication method was used to increase the lactase activity several folds in comparison to substrates containing intact cells (Shah and Jelen 1991). As Geciova and others (2002) summarized, many researchers studied sonication as a method of accelerating dairy fermentation process. After sonication of high lactase producing strains during yogurt fermentation, 71-74% depletion of the initial lactose content was reported. In the same study, 39-51% lactose hydrolysis was obtained in non-sonicated milk. In another study about 55% lactose hydrolysis was reported after sonication-enhanced fermentation. On the other hand, about 36% lactose hydrolysis was reported in non-sonicated milk (for references see Geciova et al. 2002).

Feliu et al (1998) reported that the resistance to disintegration by sonication shows variability among different strains of bacterial species. Özbek et al. (2000) reported that optimizing operational parameters, such as time and acoustic power for each of the organisms, was necessary. Feliu et al (1998) summarized the sonication parameters that affect protein release; among these parameters, cell concentration have the least influence on the rate of protein recovery, whereas sample volume and acoustic power dramatically affect the final yield of soluble protein in the cell-free fraction (Feliu et al. 1998).

As Özbek and Ülgen (2000) summarized, the enzyme release process during sonication is mostly reported to follow first-order kinetics. In this study the effect of sonication on the rate of protein release and sonication time were found to be correlated with each other (Figure 25). In Figure 25, it can be seen that the concentration of protein released from the cell increases with increasing sonication

time.

The enzyme activity and the sonication time of each strain were found to have positive correlation (Figure 22, Figure 23, and Figure 24). It was found that the peak of the enzyme activity correspond to different sonication time (seconds) for each strain; 210 seconds for *Lactobacillus plantarum* NCIMB 1193, 180 seconds for *L. plantarum* DSM20246 and 270 seconds for *L. plantarum* E081. When the sonication continued after maximum total activity was obtained, decrease in the total enzyme activity was observed (Figure 22). The decrease of the enzyme activity could be explained by excessive heat release during sonication. After each sonication period, 1 ml of sample was transferred into 1.5 ml eppendorf tubes for enzyme activity assay. Therefore after each sonication period, the total volume of the cell suspension was decreased. When the volume was decreased under a threshold level, the heat released from the sonication became higher. In this study it was found that when the volume of medium decreased, more heat was released.

Before and after sonication, the viable cell count of the *Lactobacillus plantarum* NCIMB 1193 culture was determined by standard enumeration techniques. It was found that when the volume of the cell medium and the acoustic power was kept constant, the decrease in the cell viability was very low (Table 18). After 210 seconds sonication, viable cell concentration was found as $2.554\ 10^9$ cfu/ml. Viable count of unsonicated culture was found as $5.834\ 10^9$ cfu/ml. According to these results, it could be suggested that sonicated cultures would be used to hydrolyse lactose in milk and as probiotic. In an early study *Lactobacillus plantarum* NCIMB 1193 was found to be promising as probiotics (Cebeci and Gürakan 2003).

As Wallenfels and Weil (1972) summarized, enzymes from different sources have widely different pH optimum, temperature optimum and ionic requirements, no general procedure for assaying enzymatic activities can be found in literature. But, for specific strains there are general procedures for assaying activities. In this study

modified enzyme activity method of Cesca et al. (1984) was used for enzyme activity.

Wallenfels and Weil (1972) summarized standardization methods of the beta-galactosidase assay. The hydrolysis of lactose by β-galactosidase can be followed by measurement of the liberation of either the glycon or the aglycon. During hydrolysis of lactose, galactose and glucose are formed. Zhou et al. (2001) reported that in high lactose content solution, lactose molecule has more changes to act as the acceptor, binding with the enzyme-galactose complex to form oligosaccharides. Therefore determination of liberated galactose can not be used for measurement of activity of β-galactosidase. Most procedures for assaying beta-galactosidase are based on the determination of the liberated aglycon. Wallenfels and Weil (1972) were summarized the methods to determine the liberated aglycon: glucose (aglycon) liberated from lactose can be estimated enzymically using glucose oxidase or hexokinase coupled with glucose 6-phospate dehydrohenase. There exist non enzymatic methods for measurement of activity of β-galactosidase: High Pressure Liquid Chromatography (HPLC) method have been used to estimate the liberation of glucose.

As Wallenfels and Weil (1972) summarized, the most common substrates for assaying beta-galactosidase are chromogenic galactosidase. The first of these substrates was p-nitro phenyl -β -D-galactoside. After the use of o-nitrophenyl-β-D-galactoside (ONPG) was described as a sensitive and convenient assay, ONPG was started to be used extensively. 6-bromo-2 –naphtyl β-D-galactoside was introduced as another chromogenic galactosidases which gives rise to insoluble 6-bromo 2-napthol after enzyme hydrolysis. The amount of liberated aglycon is measured after coupling with a diazonium salt. The low solubility of 6-bromo-2 –naphtol makes the galactoside a valuable tool for histochemical use. Sensitivity of the galactosidase assay was increased considerably by using fluorogenic substrates. Thus, methylumbelliferyl β-D-galactoside was introduced for estimating low enzymic

activities. X-gal was also introduced as a good screening chromogenic galactosidases. It is generally used to screen β-galactosidase activity on solid mediums or for detecting enzymically active zones after electrophoresis of enzyme preparations on polyacrylamide gels.

In this study ONPG and X-gal were used as substrate for detecting β-galactosidase activity. X-gal was used to test the stains β-galactosidase activity. Greenish blue color was accepted as positive β-galactosidase activity (Figure 10). It was observed that the cultures, which were incubated in modified MRS broth (Appendix A), could still produce β-galactosidase when they were transferred on the MRS agar. Greenish-blue color was formed on the MRS agar (Figure 10). It is interesting to note that glucose has a clearly inhibitory effect on the expression of the lactose operon in *L.casei* ATCC 393 (Gosalbes et al. 1997). Other studies also suggest that the sugar must be present in the external medium for it to exert an repressive or inductive effect (Rehaeli and Saier 1980). However it was reported that the regulation of the *Lactobacillus bulgaricus* lac operon differs from that of *E.coli* and *S. thermophilus* since high levels of expression are seen during growth in media containing glucose (Gasson and Vos 1994). In this study the expression of beta-galactosidase on glucose containing MRS medium could be explained in two ways: *Lactobacillus plantarum* strain may have similar lac operon which shows high level of expression during growth in media containing glucose or it could be explained by low amouth of the glucose taken into the cell by tranporters.

In this study Km and V_{max} values were calculated (Table 19). pH and the temperature optimum values of β-galactosidase enzyme from *Lactobacillus plantarum* NCIMB 1193 strain by using ONPG as the substrate were found (Figure 30, Figure 31).

In Table 19, K_m and V_{max} values were given. Segel (1975) summarized the importance of K_m: K_m is numerically equivalent to the substrate concentration that

yields half-maximal velocity. The numerical value of K_m is of interest for several reasons. If S (substrate in the reaction medium) is too low than Km, the rate of the enzyme would be very sensitive to changes in substrate amount. (b) Since K_m is a constant for a given enzyme, its numerical value provides a means of comparing enzymes from different organisms or from different tissues or the same organisms, or from the same tissue at different stages of development. In this way, it might be determined whether enzyme A is identical to enzyme b, or whether they are different proteins that catalyze the same reaction. (c) By measuring the effects of different compounds on Km, important activators and inhibitors might be identified.

In Figure 27, the linearity of Lineweaver-Burk double reciprocal plot was suggesting a simple Michealis- Menten kinetics. To discover deviations from linearity, Eadie-Hofstee plot was also constructed (Figure 28). The Eadie-Hofstee plot is a way of plotting kinetic enzyme data so as to yield a straight line for reactions obeying Michaelis-Menten kinetics. This is done by plotting reaction velocity (V) versus velocity/substrate concentration (V/[S]). The slope of the line is equal to $-K_M$ and the x-intercept is V_{max}. An advantage of an Eadie-Hofstee plot over a Lineweaver Burk plot (Figure 27, Figure 28) is that the Eadie-Hofstee plot does not require a long extrapolation to calculate K_M.

Hill plot was constructed and “n” was found as 1.03 (Figure 29), which means that the number of ligand binding sites on the enzyme is one.

It is known that pH influences the velocity of an enzyme-catalyzed reaction. Therefore it is important to know effect of pH on enzyme activity. The optimum pH differs when the substrate of the enzyme changes. Therefore if the enzyme is used in milk system for lactose hydrolization, the optimum pH should be found by using lactose as the main substrate. In this study the pH optimum of β-galactosidase was found by using ONPG as substrate.

As Wallenfels and Weil (1972) summarized, early studies on the influence of pH on the enzymatic hydrolysis of ONPG had give data which were fitted to a bell-shaped curve with maximal enzymatic activity between pH 7.2 and 7.4. In Figure 30, it can be observed that a bell-shaped curve was formed between pH 6.6 and pH 7.6. The optimum pH of β-galactosidase from *Lactobacillus plantarum* NCIMB 1193 was found as pH 7.2 under the assay conditions. In Figure 30, it can be observed that there is decline in activity at above and below the optimum pH. Wallenfels and Weil (1972) suggested some reasons for the decline in activity at above and below of optimum pH: a) the decline could result from the formation of an improper ionic form of the substrate or enzyme (or both), or from inactivation of the enzyme, or from a combination of these effects. In this study the effect of pH on enzyme stability was not studied. Therefore there was no indication why the velocity declines above and below pH 7.2. In Figure 30, it can be observed that two different buffers at same pH were used during activity assays to oberve the specific buffer effect; (a) at pH 6.2 Citrate and sodium phosphate buffers were used to examine specific buffer effect. (b) at pH 8 Tris-HCl and sodium phosphate buffer were used to observe specific buffer effect. The term, specific buffer effect, is used to define the effect of different anions and cations on enzyme activity at the same pH value. In this study it was found that the β-galactosidase is more active in phosphate buffer (at pH 8) than Tris-HCl (at pH 8) and it was less active in phosphate buffer (at pH 6.2) than Citrate buffer (at pH 6.2) (Figure 30). In a study, which was performed by Cesca et al. (1983), none of the β-galactosidase obtained from different lactobacilli strains showed a maximal activity at 7.2 under the assay conditions. Cesca et al. found that the optimum pH of β-galactosidase of lactobacilli varied between pH values of 5.2-6.8 in 15 different strains of lactobacilli strain. Our results were suggested that *L. plantarum* ATCC 1193 has an optimum pH around pH 7.2.

Optimum temperature was found under the assay conditions as described in section 2.2.9.3 in Materials and Methods (Figure 30). Segel (1975) summarized the effect of temperature on enzyme activity: Most chemical reactions proceed at a faster velocity

as the temperature, T, is raised. The tertiary structure of an enzyme is maintained primarily by a large number of weak noncovalent bonds. If the molecule absorbs too much energy, the tertiary structure will disrupt and the enzyme will be denatured, that is, lose catalytic activity. Thus as the temperature increases, the expected increase in rate of enzyme resulting from increased E (enzyme) +S (substrate) collisions is offset by the increasing rate of denaturation. Consequently, a plot of enzyme rate versus temperature usually shows a peak. Influence of temperature on the enzymatic hydrolysis of ONPG had give data, which were fitted to a bell-shaped curve with maximal enzymatic activity between 35^0 C and 40^0 C under the assay conditions (Figure 31).

CHAPTER 5

CONCLUSION

Lactobacillus plantarum is considered as an important organism for dairy industry. In dairy industry, β-galactosidase enzyme is rather used as a crude extract to prevent high cost of purification. Thus it is important to characterize the enzyme in crude extract of β-galactosidase producers. For this approach to be commercially successful, strains possessing high levels of β-galactosidase activity need to be identified.

In this study, we investigated the effect of growth phase on total β-galactosidase activity. It is important to observe the growth phase of the organisms to find the phase of growth where the maximum enzyme activity is observed. It was found that maximum total β-galactosidase activity was obtained at early stationary phase of growth. For purication or other studies, the enzyme can be obtained at early stationary phase of the growth.

Since lactase from *Lactobacillus plantarum* is an intracellular enzyme, protein release by sonication method can be used to increase the β-galactosidase activity. In this study β-galactosidase enzyme from *L. plantarum* NCIMB 1193 obtained as a crude extract was characterized. The methods of obtaining crude extract and/or releasing β-galactosidase were carried out. Sonication was found as an efficient method to release protein from the cell. . It was found that liquid nitrogen method is not as effective as sonication method in terms of protein release.

It was found that sonication method did not cause any dramatic decrease in cell number. Thus it was concluded that sonication could be used as a method of choice for the release of the enzyme from *L. plantarum* during probiotic use.

The enzyme in crude extract was also further characterized. The optimum pH, temperature was determined to analyze the assay conditions. It was found that the enzyme is active in a wide range of temperature and pH values.

REFERENCES

Baran T. E. Comparison of Different Methods of Beta-Galactosidase Immobilization on/in Poly (2-Hydroxyethyl Methacrylate) Supports Master Thesis Biochemistry Department Middle East Technical University 1996

Becker VE, Evans HJ. The influence of monovalent cations and hydrostatic pressure on β-galactosidase activity Biochim Biophys Acta 191:95-104, 1969

Bradford M.M Anal. Biochem. 72: 248-254 1976

Brückner R., Titgemeyer F. Carbon catobolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization FEMS Microbiology Letters 209: 141-148, 2002

Bury D. and Jelen P. Lactose hydrolysis using a disrupted dairy culture: Evaluation of technical and economical feasibility Canadian Agricultural Engineering 42: 75-80, 2000

Bury D., Jelen P., Kalab M. Disruption of *Lactobacillus delbrueckii* ssp. *Bulgaricus* 11842 cells for lactose hydrolysis in dairy products: a comparison of sonication, high-pressure homogenization and bead milling Innovative Food Science & Emerging Technologies 2: 23-29, 2001

Cebeci A., Gürakan C. Properties of potential probiotic *Lactobacillus plantarum* strains , Food Microbiology 20: 511-518, 2003

Cesca B., Manca De Nadra M. C., Strasser De Saad A.M., Pesce De Ruiz Holgado A., Oliver G. β- Galactosidase of *Lactobacillus* Species Folia Microbiol. 29: 288-294, 1984

Chang Y.-H., Kim J.-K., Kim H.-J., Kim W.-Y., Kim Y.-B., Park Y.-H. Selection of a potential probiotic *Lactobacillus* strain and subsequent in vivo studies Antonie van Leeuwenhock 80: 193-199, 2001

Doulah M. S.: Mechanism of Disintegration of Biological Cells in Ultrasonic Cavitation Biotechnology and Bioengineering Vol. XIX: 649-660, 1977

Davidson B. E., Llanos R. M., Cancilla M. R., Redman N. C., and Hillier A. J. Current research on the genetics of lactic acid production in lactic acid bacteria Int. Dairy Journal 5: 763-784, 1995

de Vrese M., Stegelmann A., Richter B., Fenselau S., Laue C., Schrezenmeir J. Probiotics- compensation for lactase insufficiency Am J Clin Nutr; 73 (suppl): 421S-9S, 2001

Delkour J., Ferain T., Hols P. Advances in the genetics of thermophilic lactic acid bacteria 11 497-504 2000

Feliu J. X., Cubarsi R., Villarde A. Optimized release of recombinant proteins by ultrasonication of *E.coli* Biotechnol.Bioeng, 58 (5): June 5, 1998

Fernandes C. F., Shahani K. M., Amer M. A. Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products FEMS Microbiology Reviews 46: 343-356, 1987

Garman J., Coolbear T., Smart J. The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β-galactosidase from six strains of lactic acid bacteria Appl Microbiol Biotechnol 46**:** 22-37, 1996

Gasson M.J., de Vos W.M. Genetics and Biotechnology of Lactic Acid Bacteria 1994

Geciova J., Bury D., Jelen P. Methods for disruption of microbial cells for potential use in the dairy industry – review International Dairy Journal 12: 541–553, 2002

Gekas V., López-Leiva M. Hydrolysis of lactose: A literature review Process Biochemistry, February: 2-12, 1985

Godfrey T., West S. Industrial Enzymology 2th. Ed. 1996

Gosalbes M. J., Monedero V., Alfred C., Pérez-Martínez G. Establishing a model to study the regulation of the lactose operon in *Lactobacillus casei* FEMS Microbiology Letters 148: 83-89, 1997

Greenberg N.A., Mahoney R.R. Production and Characterization of β-galactosidase from *Streptococcus thermophilus* Journal of Food Science 47: 1824-1828, 1982

Gurr M.I. Nutritional aspects of fermented milk products FEMS Microbiolgy Reviews 46 337-342, 1987

Hickey M. W., Hillier A. J., Jago G.R. Transport and metabolism of lactose, glucose, and galactose in homofermentative *Lactobacilli* Applied and Environmental Microbiology 51 (4): 825-831, 1986

Irwin H. Segel A Enzyme Kinetics Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, Wiley-Interscience Puplication John Wiley & Sons, New York, 1975

James C. J., Coakley W. T., Hughes D. E. Kinetics of protein release from yeast sonicated in batch and flow systems at 20 kHz Biotechnology and Bioengineering 14 33-42, 1972

Jurado E., Camacho F., Luzon G., Vicaria J.M. A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from *Kluyveromyces fragilis* Enzyme and Microbiol Technology 31: 300-309, 2002

Kashket E. R. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance FEMS Microbiology Reviews 46: 233-244, 1987

Kathy F., Johnson M.C., Ray B. Lactose hydrolyzing enzymes in *Lactobacillus acidophilus* strains Food Microbiology, 2: 23-29, 1985

Kim J.W., Rajagopal Isolation and Characterization of β-galactosidase from *Lactobacillus crispatus* Folia Microbiol 45(1) 29-34 2000

Kreft M.E., Jelen P. Stability and activity of β-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* ssp.b*Bulgaricus* 11842 as affected by temperature and ionic enviroments Journal of Food Science 65 (8), 2000

Ladero M., Santos A., Garcia J.L., Carrascosa A.V., Pessela B.C.C., Garcia-Ochoa F. Studies on the activity and the stability of β-galactosidases from *Thermus* sp strain T2 and from *Kluyveromyces fragilis* Enzyme and Microbiol Technology 30: 392-405, 2002

Lederberg J. The Beta-D-Galactosidase of Escherichia Coli, Strain K-12 J.Bacteriol vol 60: 381-392, 1950

Madigan M.T, Martinko J. M., Parker J. Biology of Microorganisms, 8th Ed.,, Prentice Hall International, Inc., 1997

Mahoney R.R Galactosyl- oligasaccharide formation during lactose hydrolysis: a review Food Chemistry 63 (2): 147-154, 1998

Marteau P. R, de Vrese M., Cellier C. J., and Schrezenmeir J. Protection from gastrointestinal diseases with the use of probiotics 73(suppl): 430S-6S, 2001

Mattila–Sandholm T., Myllärinen P., Crittenden R., Mogensen G., Fondén R., Saarela M. Technological challenges for future probiotic foods International Dairy Journal 12: 173-182, 2002

Metcalf R.H. Deibel R.H. Differential Lytic response of *Enterococci* associated with addition order of lysoyzme and anions Journal of Bacteriolgy 674-680, 1969

Molin G. Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v Am J Clin Nutr;73 (suppl): 380S-5S, 2001

Mussa D.M., Ramaswamy H. S. Ultra High Pressure Pasteurization of Milk:Kinetics of Microbial Destruction and Changes in Physico-chemical Characteristics Lebensm.-Wiss. u.-Technol., 30: 551-557, 1997

Naidu A. S., Bidlack W. R., Clemens R. A. Probiotic spectra of lactic acid bacteria (LB) Critical Reviews in Food Science and Nutrition, 38 (1): 13-126, 1999

Özbek B., Ülgen Ö. K. The stability of enzymes after sonication Process Biochemistry, 35: 1037-1043, 2000

Özcangaz Ç. Characterization of Lactic Acid Isolates From Turkish Sourdough and Interaction with Yeast Master Thesis Biotechnology Department Middle East Technical University 2000

Pivarnik, L., Rand A. G. Assay conditions effect on β-galactosidase activity from *Kluyveromyces lactis*. Journal of Food Science 57(4): 1020-1021, 1992

Pomeranz Y. Lactase (beta-D-Galactosidase) I. Occurrence and Properties Food Technology May 1964

Poolmen B. Biochemistry and molecular biology of galactoside transport and metabolism in lactic acid bacteria Lait 73. 87-96, 1993

Poolmen B. Transporters and their roles in LAB cell physiology Antonie van Leeuwenhock 82: 147-164, 2002

Postma, P., W., Lengeler, J., W., Jacopson, G., R. Phosphoenolpyruvate: carbohydrate phoshotransferase systems of bacteria Microbiology Reviews 57 (3): 543- 594, 1993

Premi L., Sandine W. E., Elliker P. R. Lactose hydrolyzing enzymes of Lactobacillus species Appl Microbiol 24: 51-57, 1972

Prenosil J.E., Stuker E., Bourne J.R. Formation of oligosaccharides during enzymatic lactose hydrolysis. 1. State of art. Biotechnol Bioeng 30:1019-1025, 1987

Reinheimer J. A., Vinderola C.G. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance Food Research International 23: 895-904, 2003

Rephali A.W., Saier M. H., J.R Regulation of genes coding for enzyme constituents of the Bacterial phosphotransferase system Journal of Bacteriology 658-663, 1980

Richmond, M.L. β-galactosidase: Review of recent research related to technological application, nutritional concerns and immobilization J. Dairy Sci. 64: 1759-1771, 1981

Rouwenhorst R.J, Pronk J.T., Van Dijken J.P. The discovery of β-galactosidase. *TIBS* 14: 416-418,1989

Sani R.K., Chakraborti S., Sobti R.C., Patnaik P.R., Banerjee U.C. Chracterization and some reaction –engineering aspects of thermostable extracellular β-galactosidase from a new *Bacillus* species *Folia Microbiol.*44 (4): 367-371, 1999

Sanul A. R. Laktaz yetmezliği tanısında değişik miktarda laktoz içeren test yemekleri ile alınan sonuçlar (Gatroentroloji Uzmanlık Tezi) Ege Tıp Fakültesi, Namık Kemal Menteş Gastroentoloji Kliniği, 1990

Shah N., Jelen P. Lactase activity and properties of sonicated dairy cultures: Milchwissenschaft 46 (9), 1991

Somkuti G. A., Dominiecki M. E., Steınberg D.H. Permeabilization of *Streptoccus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with ethanol Current Microbiology 36: 202-206, 1998

Stiles M. E., Holzapfel W. H. Lactic acid bacteria of foods and their current taxonomy International Journal of Food Microbiology 36: 1-29, 1997

Thompson J. Lactic acid bacteria: model systems for in vivo studies of sugar transport and metabolism in Gram-positive organisms Biochimie 70: 325-356, 1988

Thomson J. Regulation of sugar transport and metabolism in lactic acid bacteria FEMS Microbiology Reviews 46: 221-231, 1987

Titgemeyer F., Hillen W. Global control of sugar metabolism: a Gram-positive solution Antonie von Leeuwenhock 82: 59-71, 2002

Toba T., Hayasaka I., Taguchi S., Adachi S. A new method for manufacture of lactose-hydrolysed fermented milk J. Sci. Food Agric, 52: 403-407, 1990

Vasiljevic T., Jelen P. Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β-galactosidase extracts from *Lactobacillus bulgaricus* 11842 Innovative Food Science & Emerging Technologies 3 :175-184, 2002

Vinderola C. G., Reinheimer J.A. Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance Food Research International 36: 895-904, 2003

Visscher A. D., Eenoo P. V., Drijvers D., Langenhove H. V. Kinetic Model for the Sonochemical Degradation of Monocyclic Aromatic Compounds in Aqueous Solution Journal of Physical Chemistry 100: 11636-11642, 1996

Wallenfels K., Malhotra O.P. Beta-galactosidase in: The Enzymes, Boyer P.D. (Ed.), Academic Press, New York, 1972

Wallenfels K., Malhotra O.P. Galactosidases. Advances in Carbohydrate Chemistry 16: 239-298, 1961

Wang D., Sakakibara M. Lactose hydrolysis and β-galactosidase activity in sonicated fermentation with *Lactobacillus* strains Ultrasonics Sonochemistry 4: 255-261, 1997

Wang D., Sakakibara M., Kondoh N., Kimihiro S. Ultrasound –enhanced lactose hydrolysis in milk fermentation with *Lactobacillus bulgaricus* J. Chem Tech Biotechnol 65: 86-92, 1996

Zhou Q. Z. K., Chen X. D. Effects of temperature and pH on the catalytic activity of the immobilized β-galactosidase from *Klyveromyces lactic* Biochemical Engineering Journal 9: 33-40, 2001

APPENDIX A

LIST OF CULTURAL MEDIA

MRS broth:

This medium was used for the cultivation of lactic acid bacteria.

Peptone from casein 10 g

Meat extracts 8 g

Yeast extracts 4 g

K_2HPO_4 2 g

D (+) Glucose 20 g

Tween ® 80 1 g

Sodium acetate. 5 g

Di-ammonium citrate 2 g

$MgSO_4.7H_2O$ 0.2 g

$MnSO_4.4H_2O$ 0.04 g

Water 1000 ml

pH was measured to before autoclaving.

52.2 g medium was dissolved in 1 litter of demin water and autoclaved

Modified MRS broth

All components are same with MRS broth except that 2% lactose was replaced with 2 % glucose.

pH was measured to before autoclaving.

52.2 g medium was dissolved in 1 litter of demin water and autoclaved

MRS agar

All components are same with MRS except that 15 g Agar per liter of solution was added to MRS broth solution. It was boiled then autoclaved.

Nutrient agar

bacto beef extract.....3g
bacto peptone.....5g
bacto agar.....15g

suspend 23 grams in 1 liter distilled or deionized water and boil to dissolve completely. Sterilize et 121-124 for 15 minutes
pH 6.8 +-0.2 at 25 C

Nutient broth

Peptone from meat..... 5.0 g
meat extract..... 3g

Suspend 8 g in 1 litre of demineralized water; if required, dispense into smaller containers; autoclave (15 min at 121C)
Ph 7.0 +-0.2 at 25C

APPENDIX B

PREPERATION OF BUFFER AND REAGENTS

Preperation of Buffers

0.05 M buffers were prepared by titration of 0.05 M conjugate base with 0.05 M its respective acid until desired pH value. The types of buffers used in different pH ranges are:

Citrate buffer (0.05 M): pH 5.0, pH 5.5, pH 5.8, pH 6.2

Sodium phosphate buffer (0.05 M): pH 6.2, pH 6.6, pH 7.0, pH 7.2, pH 7.4, pH 7.6, pH 8

Tris (hydroxmethyl) aminomethane buffer (0.05 M): pH 8

.

Preparation of chemicals:

O-nitrophenol-B-D-galactopyranoside (ONPG) Solution:

ONPG solutions were prepared by addition of solid ONPG in appropriate buffer solution (0.05 M) under the assay conditions. ONPG has low solubility therefore it should be well mixed. This solution should be prepared freshly.

Mw ONPG=301.26 g/L

ApiFermentation Medium for Lactobacilli

(a): API 50 CHL Medium 10 ml

Polypeptone	10g
Yeast Extract	5g
Dipotassium phosphate	1 ml
Sodium acetate 3 H_20	2g
Diammonium citrate	5g
Magnessium sulphate 7 H_20	2g
Manganse sulphate 4 H_20	0.20g
Bromocresol Purple	0.05g
Tween 80	0.17ml
dwater	1000 ml

(b): Suspension Mediums

2 mL and 5 mL sterile dH_20 were used as suspension medium

(c) Princible

The Api 50 CH strip allows the study of carbahydrate metabolism of microorganisms. It contains of 50 microtubes each containing an anaerobic zone (the tube portion) for the study of oxidation and assimilation. The first tube contain no substrate and is used as a negative control. The remaining tubes contain a defined amount of dehydrated substrate, belonging to the carbohydrate family and its

derivatives. The fermantation is shown by a color change in the tube portion, and is due to the anarobic production of acid detected by a pH indicator included in the chosen medium. Also the substrates may be metabolized by various other pathways like; assimilation, oxidation. The API 50 CH (BioMerieux, France) strip may be used to test any of these three pathways. The medium used for inoculating the strips should be chosen according to the pathway to be studied and the nutritional requirements of the group of microorganisms to be tested.

API 50 CHL medium, intended for the identification of the genus Lactobacillus and related organisms. It is a ready use medium that enables the fermentation of 49 carbohydrates on the API CH strip to be studied. A suspension is made in the medium with the microorganism to be tested and each tube of the strip is inoculated.. During incubation, carbahydrates are fermented to acids which produce a decrease in pH, detected by the colour change of the indicator. The results make up the biochemical profile of the strain and are used in its identification or typing. Composition of the media are given in Appendix B.

(d) Instruction for use

(d 1) Selection of the colonies preparation of inoculum

In the instruction manual of API kit, it is recomended to check the purity of the colonies. Thus before the preparation of inoculum, gram staining, catalayse and gas formation tests were performed. Stock cultures which were maintained at -80^0 C were used. For full activation, the cultures were transferred into a fresh medium (MRS broth) at least three days. Then 2 % inoculum from culture medium (MRS broth) was taken and inoculated on MRS agar for isolation. The culture ,which were inoculated on MRSagar, incubated at 37C for 24 hours in the anaerobic jar.

(d2) Preparation of Inocolum

All the colonies on MRS agar were picked up by a swap and then they were transferred into 10 ml glass tubes containing 2 ml suspension medium (sterile demineralized water) to make a heavy suspension. This tube was labelled as "2 ml heavy suspension medium". From the "2 ml heavy suspension medium", a certain number of drops were transferred into a glass tube containing 5 ml suspension medium (sterile demineralized water) to obtain a turbidity equivalent to 2 McFarland. The tube containing 5 ml suspension medium was labelled as "5ml suspension medium". The numbers of drops to obtain a turbidity equivalent to 2 Mc Farland in "5 ml suspension medium" were recorded as "n".

An ampule of API 50 CHL medium was opened under aseptic conditions. From the "2 ml heavy suspension medium", "2n" (twice the number of drops to obtain a turbidity equivalent to 2 McFarland in "5 ml suspension medium") drops transferred into the API 50CHL medium. Homogenization of API 50CHL was performed by 1 ml micropipete.

The bacterial suspensions were distributed into 50 tubes by using 1 ml micropippette. Minerale oil was used to provide strickly anorobic environment. Each carbohydrate tube was covered with 1 ml of mineral oil. The strips were incubated at the optimum temperature 37^0 C for 48 hrs. Analysis were done by mini API computer program (for furher information see, api 50 CHL medium instruction manual).

Sodium Carbonate

0.05 M sodium carbonate is prepared by dissolving 5.3 g in 1000 ml deionized water.
MNa2CO3=106 g/L

0.1% water peptone

1 g of peptone was dissolved in 1000 ml of distilled water. The solution was then autoclaved at 121^0 C for 15 minutes.

Gram Staining Reagents

(a): Crystal violet

2 g crystal violet
1% ammonium oxalate solution
2 g of crystal violet was dissolved in 20 ml 95% ethanol. This solution was added to 80 ml of 1% ammonium oxalate solution. After 24 hours, it was filtered

(b): Gram iodine

1 g iodine and 3 g potassium iodide was added to 300 ml distilled water .It was stored in a bottle. This solution is light sensitive

(c): Safranin

2.5 g safranin was added to 100 ml 95% ethanol. This solution was added to 100 ml distilled water.

A gram stain is a differential stain requiring a primary stain (crystal violet), which is followed by an iodine solution. Then washed by alcohol (%95 ethanol) after decolorizing step safranin which is the counter strain was applied to the smear. Gram reactions are only reliable for cultures of 24 hours or less. The staining solutions are provided in Appendix.B. From an overnight fresh culture, a loopful culture was spread in a thin film over a microscope slide, air dried and fixed by passing the slide through the flame. Then it was stained with primary stain for one minute, after that the slides were washed with distilled water and iodine solution is let to act for 60-180 seconds. The color resulted after the addition of iodine was then removed by the alcohol wash. After rinsing with water, it was stained with safranine solution; the counterstain for 30 seconds. The slides were then washed, dried, and visuilized under the microscope. The control was *E.coli,* a gram negative species.

The cultures incubated more than 24 hour may give false negative results. Therefore non of the cultures incubated more than 24 hours.

Catalase Test

3% H_2O_2 solution was used. Bubble formation was accepted as positive result.

APPENDIX C

LIST OF CHEMICALS

Table 17. List of the chemicals

Chemical	Suppliers
Agar-agar	Merck
Tween 80	Merck
Nutrient agar	DIFCO
MRS broth	Merck
MRS agar	Merck
Nutrient broth	DIFCO
α-lactose	Sigma
2-Nitrophenyl-β-D-galacto pyranoside (ONPG)	Sigma
Sodium Chloride (NaCl)	Merck
Tris(hydroxymethyl- aminomethan)	Merck
Sodium acetate	Merck
ONP	Sigma
Sodium carbonate (Na_2 CO_3)	Sigma
Meat Extract	AppliChem
Pepton from casein	Merck
Yeast Extract	Merck
X-gal	Sigma
Citric acid	Sigma
Sodium citrate	Merck
Hydrochloric acid (HCl) (%36 g/ml)	Merck
Magnesium Sulfate	Merck
Manganase Sulfate	Merck
Hydrogenperoxide (H_2O_2)	Merck
di-potasium hydrogen phosphate	Merck
di-Ammonium hydrogen citrate	Kindly provided by Chemistry Dept. Middle East TechnicalUniversity,Turkey
Sodium Hydroxide	Merck

Table 17. (continued)

Bovine serum albumin	Sigma
Lysozyme	Sigma (L-6876)
$Na_2 HPO_4$	AppliChem
$NaH_2 PO_4$	AppliChem

APPENDIX D

EQUIPMENTS

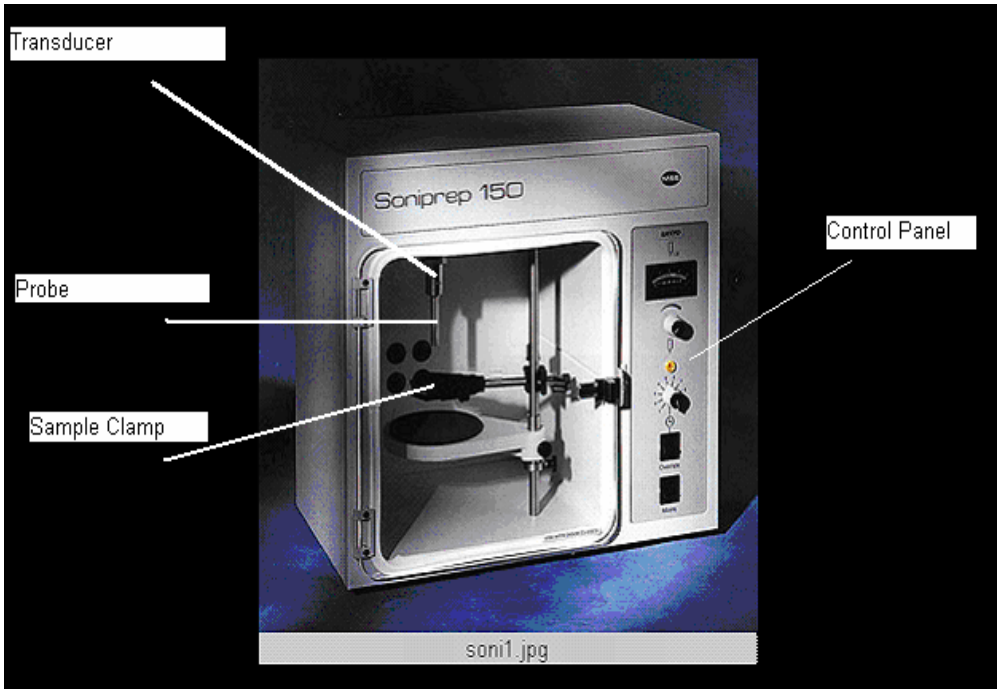

Figure 28. Soniprep 150 Ultrasonic Disintefrator