PRODUCTION OF β-GALACTOSIDASE USING LACTIC ACID BACTERIA AND OPTIMISATION OF FERMENTATION PARAMETERS

A Thesis Submitted to
the Graduate School of Engineering and Science of
Izmir Institute of Technology
in Partial Fulfilment of Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
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July 2007
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ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my advisor, Assist. Prof. Canan Tarı for her guidance, understanding, motivation and excellent support during this study and preparation of this thesis.

I would like to express my gratitude to my co-advisor, Prof. Dr. Şebnem Harsa for her support and contributions. I feel really fortunate to have worked in with in this project. I would also like to thank Assist. Prof. Fuat Doymaz for his suggestions on my thesis, and Assist. Prof. Figen Tokatlı and Assoc. Prof. Ahmet Yemenicioğlu for their support, guidance and the valuable informations they provided. This project would not have been possible without the funding of TÜBİTAK-TOVAG 104 0 270, AB-COST 2005, and DPT-YUUP 2005K120720.

There are too many people who have helped me during my journey that I need to thank. However, there are some people that definitely deserve a place in my thesis because without them, I would never have been able to get my jobs done and present this thesis, namely Zelal Polat, Ali Emrah Çetin, Fatih Y.G. Yener, Nihan Göğüş, Burcu Çabuk, Şelale Öncü, and Nergiz Doğan.

Last but not least, I would like to thank my family for the love and care that they have showered upon me all these years. My family has been my greatest source of strength and inspiration. I owe all my achievements to them and am really thankful to know that they will support me in anything I do. Nezih Üstok, Nurefşan Üstok, and Fatih Üstok, thank you for always being there for me.

ABSTRACT

PRODUCTION OF β-GALACTOSIDASE USING LACTIC ACID BACTERIA AND OPTIMISATION OF FERMENTATION PARAMETERS

Food grade thermostable β-galactosidase preparations are always in demand for a number of industrial applications. Thermostable β-galactosidases from LAB having a neutral pH-optimum can be safely used to reduce the lactose content of milk for the lactose intolerant people. In this study, β-galactosidase was produced with high productivities by novel *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* strains isolated from traditional Turkish yogurt samples in Toros mountain region.

A full factorial statistical design was used separately for *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* strains in screening experiments. Among the strains, *Lactobacillus delbrueckii subsp. bulgaricus* 77 and *Streptococcus thermophilus* 95/2 were found to have high potential for β-galactosidase and lactic acid production, therefore these were used in the further optimisation studies. The efficiency of different cell disruption methods was investigated on the extraction of β-galactosidase. Among these, lysozyme enzyme treatment was determined as the most effective method. Optimisation studies were carried out using response surface methodology to optimize fermentation conditions for pure strains as well as for mixed ones. Therefore, symbiotic relationship between St 95/2 and Lb 77 were investigated as well. Symbiotic relationship provided 39% and 6.1 % more β-galactosidase activity and 44 % and 9.73 % more lactic acid production when compared to the optimisation results of pure strains Lb 77 and St 95/2, respectively.

Overall, characterization studies showed that enzymes obtained from these strains can be considered as food grade and thermostable since they are obtained from thermophile, food originated novel LAB of local microflora.

ÖZET

LAKTİK ASİT BAKTERİLERİNDEN β-GALAKTOSİDAZ ÜRETİMİ VE FERMENTASYON PARAMETRELERİNİN OPTİMİZASYONU

Gıdalarda kullanılabilir termostabil β-galaktosidaz preparatları, endüstriyel uygulamaların birçoğunda her zaman talep edilmektedir. Laktik asit bakterilerinden üretilen nötral optimum pH'ya sahip termostabil β-galaktosidazlar, laktoz intolerant kişiler için sütün laktoz içeriğini düşürmede güvenle kullanılabilmektedir. Bu çalışmada, β-galaktosidaz, Toros dağları bölgesindeki geleneksel Türk yoğurtlarından izole edilen *Lactobacillus delbrueckii* subsp. *bulgaricus* ve *Streptococcus thermophilus* kültürlerden yüksek verimde üretilmiştir.

Tam faktoriyel istatistiksel tasarım, *Lactobacillus delbrueckii* subsp. *bulgaricus* ve *Streptococcus thermophilus* kültürleri için ayrı ayrı kullanılmıştır. Kültürler arasında *Lactobacillus delbrueckii* subsp. *bulgaricus* 77 ve *Streptococcus thermophilus 95/2* suşlarının yüksek β-galaktosidaz ve laktik asit üretime potensiyeline sahip olduğu bulunmuştur. Bu nedenle, bu suşlar ileriki optimizasyon çalışmalarında kullanılmıştır. β-galaktosidaz enziminin ekstraksiyonunda farklı hücre parçalama yöntemlerinin etkinlikleri de incelenmiştir. Bu yöntemler arasında, lizozim kullanımı en etkili yöntem olarak belirlenmiştir. Optimizasyon çalışmaları, hem saf hem de kültürlerin bir arada kullanımı için fermentasyon parametrelerinin optimizasyonunda cevap yüzey yönteminin kullanılmasıyla gerçekleştirilmiştir. Böylece St 95/2 ve Lb 77 arasındaki simbiyotik ilişki de incelenmiştir. Simbiyotik ilişki, saf kültür Lb 77 ve St 95/2 kullanımı ile yapılan optimizasyon çalışmaları sonuçlarıyla karşılaştırıldığında; sırasıyla Lb 77 ve St 95/2'ye göre % 39 ve % 6 daha fazla β-galaktosidaz aktivitesi ve % 44 ve % 9.73 daha fazla laktik asit üretimi sağlamıştır.

Sonuçta, karakterizasyon çalışmaları; yerel floradan izole edilen, gıda kaynaklı bu termofil laktik asit bakterilerinden elde edilen enzimlerin gıdalarda kullanılabilir ve termostabil olarak ele alınabileceğini göstermiştir.

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CHAPTER 1

INTRODUCTION

Lactase also known as β-galactosidase (E.C 3.2.1.23) is an enzyme that hydrolyzes lactose (abundant disaccharide found in milk) to glucose and galactose, has a potential importance in the dairy industry (Domingues et al. 2005, Montanari et al. 2000, Voget et al. 1994). The nutritional value of lactose is limited due to the fact that a large portion such as 50% of world's inhabitants lacks this enzyme and cannot utilize lactose therefore developing lactose maldigestion or intolerance (Vasiljevic and Jelen 2002, Furlan et al. 2000). This however creates a potential market for the application of β-galactosidase. The current share of food enzymes is 37 % of total enzyme sales corresponding to 720 million dollar in the year of 2004. This value is expected to increase to 863 million dollar by the year 2009, increasing the demand for the discovery of new species, producing enzymes such as β-galactosidase with novel characteristics, which will be of great value to the enzyme industry for different applications (Cortes et al. 2005). Enzymatic hydrolysis of lactose has beneficial effects on assimilation of foods containing lactose for lactose intolerant population, as well as possible technological and environmental advantages for industrial applications (Jurado et al. 2002, Linko et al. 1998). These can be summarized such as improving the technological and sensorial characteristics of foods by increasing the solubility, providing greater sweetening power and formation of monosaccharides (Jurado et al. 2002). Furthermore, the application of β-galactosidase is important in the conversion of cheese whey, a waste from dairy industry into different value added products (Linko et al. 1998).

Although β-galactosidase (lactase) has been found in numerous biological systems, microorganisms such as yeasts, mold and bacteria still remain the only sources for commercial purposes (Vasiljevic and Jelen 2001). In recent years, thermophilic lactic acid bacteria (LAB) have gained great interest because of their GRAS status (generally regarded as safe). Amongst lactic acid bacteria, yogurt bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) are the highest β-galactosidase producers (Shah and Jelen 1991). The β-galactosidase of these cultures has been characterized, showing high stability and activity at high temperatures (Kreft and Jelen 2000, Greenberg and Mahoney 1982). Such conditions, besides enhancing the

rate of lactose hydrolysis prevent the growth of undesirable microorganisms as well. However the β-galactosidase from thermophilic LAB is an intracellular enzyme. Its release from microorganisms is obtained either by mechanical disruption or by chemical permeabilization of the cell membrane. The effectiveness of the various disruption methods differs for different microbial genera and strains. Generally, β-galactosidase activity in the medium can be greatly increased by rupturing cells using different cell disruption methods. The literature tends to focus on the disruption of yeasts with much less information being available on the disruption of lactobacilli. (Bury et al. 2001).

With this perspective, traditional yogurt samples obtained from Toros mountain region of Turkey with highly bio-diverse environment were used as the source for the isolation of LAB cultures. Almost 136 isolated strains were screened for β-galactosidase activity based on their lactose consumption and lactic acid producing characteristics according to the method described by Bulut et al. and Mora et al. (Bulut et al. 2004, Mora et al. 1998). Among these isolates only three *Lactobacillus delbrueckii* subsp. *bulgaricus* strains and three *Streptococcus thermophilus* strains showed such a high potential. In this study, the goal was to further optimize the fermentation conditions of these isolates whose phenotypic and genotypic identification were completed and determine the strains with highest β-galactosidase activity and lactic acid production that could be potential candidates for industrial use.

As it is well known lactic acid is an economically valuable product with different applications in pharmaceutical, chemical, textile and food industry. The current worldwide production (including the polymer uses) is estimated to be around 120 000 tyr^{-1}. Therefore in the last decade, lactic acid production has increased considerably, due to the development of new applications, and the production technology is currently based on microbial fermentation which emphasizes the demand for the discovery of new microbial sources with novel characteristics (Datta and Henry 2006).

Furthermore, these strains can also be considered as starter cultures with unique characteristics for dairy industry. Therefore, the objectives of this study were;

(i) To determine the effects of strains, cultivation media and agitation speed on biomass, lactic acid and β-galactosidase activity for both *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains using full factorial statistical design.

(ii) To investigate the effectiveness of different cell disruption methods such as, homogenization, glass beads and lysozyme enzyme treatment and compare them in order to select the most effective method for further studies.

(iii) To optimize the fermentation conditions with respect to temperature, inoculum ratio and media composition for the best isolates determined as potential lactic acid and β-galactosidase enzyme producer using response surface methodology.

(iv) To investigate the effect of symbiotic relationship between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* on biomass, lactic acid and β-galactosidase activity.

(v) To scale-up the optimized conditions to bioreactor experiments.

(vi) To characterize the cell-free crude β-galactosidase extracts from *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

CHAPTER 2

LACTOSE INTOLERANCE

Lactose intolerance, in other words inability to hydrolyze lactose (a type of sugar found in milk and other dairy products), is a problem prevalent in more than half of world's population (Vasiljevic and Jelen 2001). It is caused by the deficiency of the β-galactosidase enzyme. The prevalence of β-galactosidase deficiency in different population groups around the world is shown in Table 2.1.

Table 2.1. Prevalence of β-galactosidase deficiency in the world. (Source: Vesa et al. 2000)

Ethnic group	Lactase deficiency (% of population)
Northern European	<10
Central and Southern European	70
East Asia	100
Native American	80-100
Mexican American	53
South Africa	13-90

The absence of β-galactosidase can be described as congenital, primary or secondary deficiency. Congenital deficiency is an extremely rare condition in which detectable levels of β-galactosidase are absent at birth (Pray 2000). Primary β-galactosidase deficiency is genetically inherited, age related decrease in β-galactosidase activity. It occurs in early childhood and progress through the life (Miller et al. 1995). However, secondary β-galactosidase deficiency can occur at any age. It is a transient state of β-galactosidase deficiency due to the damage to the intestinal mucosa where β-galactosidase is produced. This damage can be caused by a severe bout of gastroenteritis, malnutrition, uncontrolled coeliac disease, inflammatory bowel disease (IBS), cancer or toxins (Savaiano and Lewitt 1987).

Mechanism of lactose hydrolysis and absorption and pathogenesis of diarrhea in lactose intolerance is shown in Figure 2.1. The main symptoms of lactose intolerance include flatulence, bloating, diarrhea and abdominal pain. The symptoms are caused by undigested lactose passing from the small intestine into the colon. In the colon, the bacteria normally present ferment unabsorbed lactose producing short chain fatty acids

and gases (carbon dioxide and hydrogen). Gas production might result in flatulence, bloating and distension pain. Unabsorbed lactose also has an osmotic effect in the gastrointestinal tract, drawing fluid into the lumen and causing diarrhea (de Vresa et al. 2001).

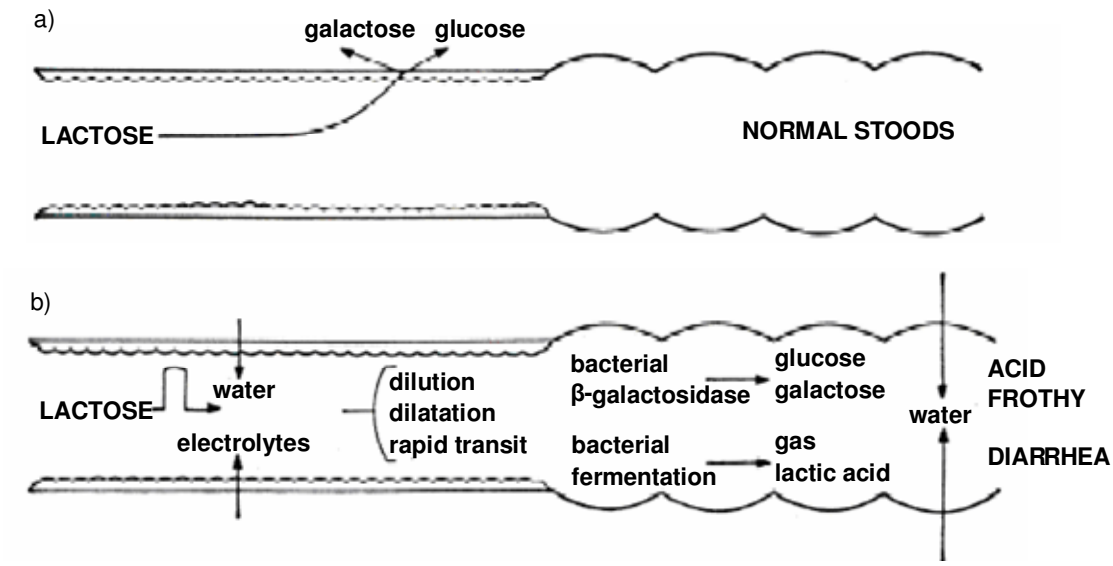


Figure 2.1. a) Mechanism of lactose hydrolysis and absorption b) Pathogenesis of diarrhea in lactose intolerance (Source: Kern and Struthers 1966)

As lactose intolerant people do not have ability to synthesize β-galactosidase enzyme, the treatment of milk and its derivatives with β-galactosidase is required. Therefore, products free of lactose or low lactose content can be consumed without any problems by lactose intolerant people (Furlan et al. 2000). Yogurt is usually better tolerated than fresh milk products by lactose maldigestors. This is because the β-galactosidase in the live yogurt bacteria can aid lactose digestion in the colon. Marteau et al. (2001) also summarized that lactose intolerant people have better digestion and tolerance of the lactose contained in yogurt.

Among β-galactosidase sources, bacterial sources are preferable due to ease of fermentation, high enzyme activities and good stability (Vasiljevic and Jelen 2001). Therefore, lactic acid bacteria (LAB) which constitute a diverse group of lactococci, streptococci and lactobacilli have become a focus of studies for several reasons (Somkuti et al. 1998). These reasons can be listed as;

a) Lactose intolerant people may consume some dairy fermented foods with little or no adverse effects.

b) Lactic acid bacteria are generally regarded as safe (GRAS) so enzyme derived from them might be used without extensive purification (Vasiljevic and Jelen 2002).

c) Some strains have probiotic activity resulting in improved digestion of lactose (Vinderola and Reinheimer 2003)

2.1. Improvement of Lactose Metabolism by Using Lactic Acid Bacteria

2.1.1. Lactic Acid Bacteria

The lactic acid bacteria constitute a various group of microorganisms associated with plants (cabbage, corn, barley, mashes, kale, and silage), meat, and dairy (Carr et al. 2002). In general, lactic acid bacteria may be characterized as Gram-positive, usually non-motile, non-spore forming, catalase-negative, aerobic to facultative anaerobic requiring complex media, cocci, coccobacilli, or rods that produces lactic acid as the major end product from the energy yielding fermentation of sugars (Wood and Holzaphel 1995, Axelsson 1998, Carr et al. 2002).

The lactic acid bacteria are grouped as either homofermentative or heterofermentative based on the end product of their fermentation. The homofermentative bacteria produce lactic acid as the major product of fermentation of glucose. However, the heterofermenters produce a number of products besides lactic acid, including carbon dioxide, acetic acid, and ethanol from the fermentation of glucose. The homofermenters possess the enzyme aldolase and they are able to ferment glucose more directly to lactic acid than the heterofermenters. The heterofermenters use the alternate pentose monophosphate pathway, converting six carbon sugars (hexoses) to five carbon sugars (pentoses) by the enzyme phosphoketolase, producing in the process both aldehyde and diacetyl-highly desirable aromatic and flavor-enhancing substances. The heterofermenters are often used in dairy industry because of these flavor-enhancing substances (Wood and Holzaphel 1995, Carr et al. 2002). Figure 2.2 shows the three main pathways associated with hexose metabolism in LAB.

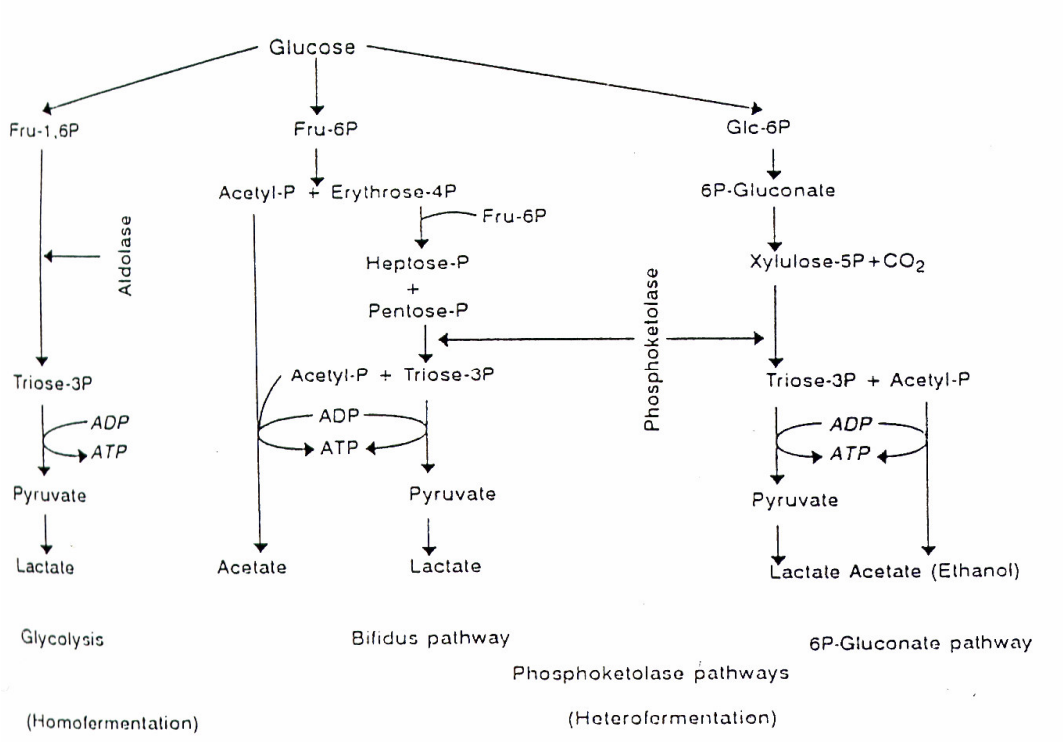


Figure 2.2. Schematic presentation of the main pathways of hexose fermentation in lactic acid bacteria (Source: Kandler 1983)

As it is well known, lactic acid bacteria (LAB) play a significant role in the food fermentation processes (Wood 1997). They are very useful in the food industry owing to their availability to acidify and therefore preserve foods from spoilage. They also improve texture, flavor and aroma of the fermented food products.

Yogurt is the fermented milk product which is produced with thermophilic lactic acid bacteria including *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. It has become a major food item where its consumption is still increasing due to its healthy properties arising from the action of variable yogurt bacteria and their metabolites (Kristo et al. 2003a, 2003b).

2.1.1.1. *Lactobacillus delbrueckii* subsp. *bulgaricus*

Lactobacillus delbrueckii are Gram-positive, facultative anaerobic, non-motile and non-spore-forming, rod-shaped members of the industrially important lactic acid bacteria. Like other lactic acid bacteria, they are acid tolerant, cannot synthesize porphyrins, and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product (Kandler and Weiss 1986, Hammes and Vogel 1995, Axelsson

1998). Within the genus *Lactobacillus, L. delbrueckii* are part of the obligately homofermentative, which produce D-lactic acid from hexose sugars via the Embden-Meyerhof Parnas (EMP) pathway and are incapable of fermenting pentoses (Axelsson 1998). The *L. delbrueckii* species contains three subspecies, *L. delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *lactis*, and *L. delbrueckii* subsp. *bulgaricus*.

L. delbrueckii subsp. *bulgaricus* (Lb) grows on a relatively restricted number of carbohydrates and typically requires pantothenic acid and niacin (Hammes and Vogel 1995). Phylogenetically, *L. delbrueckii* subsp. *bulgaricus* is closely related (<10% sequence divergence) to *L. amylovorus*, *L. acidophilus, L. helveticus, L. acetotolerans, L. gasseri,* and *L. amylophilus* (Schleifer and Ludwig 1995).

L. delbrueckii subsp. *bulgaricus* is a component of "thermophillic" starter cultures used in the manufacture of a number of fermented dairy products (Hassan and Frank 2001). These cultures have an optimum growth temperature of approximately 42 °C and contain *Streptococcus thermophilus* with *L. delbrueckii* subsp. *bulgaricus* and/or *L. helveticus*. These cultures are utilized in the yogurt, Swiss-type and Italian-type cheese varieties.

2.1.1.2. *Streptococcus thermophilus*

Streptococcus thermophilus (St) is in the group of thermophilic lactic acid bacteria. It is traditionally used in association with one or several lactobacillus strains as a starter culture in the production of several fermented dairy products.

Like most lactic acid bacteria, *Streptococcus thermophilus* is also non-spore forming, catalase negative, facultatively anaerobic and metabolically homofermentative microorganism. *Streptococcus thermophilus* appears as non-motile, spherical or ovoid cells (0.7-0.9 µm in diameter) in chains or pairs when grown in liquid media (Wood and Holzaphel 1995).

As it is well known, lactic acid bacteria are fastidious microorganisms requiring simple carbohydrates as an energy source and amino acids as nitrogen source. *Streptococcus thermophilus* is one of those microorganism has an optimum growth temperature of 40-45 °C. It ferments limited number of sugar including lactose, fructose, sucrose and glucose. In addition to the source of fermentable carbohydrate, it also requires hydrolysed proteins as the source of amino acids. These include glutamic

acid, histidine, methianine, cysteine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine. Most of bacteriological media used to support growth of *Streptococcus thermophilus*, therefore contain hydrolysed casein, tryptone or beef extract. Most of the vitamin and nutrient requirements are satisfied by addition of yeast extract (Wood and Holzaphel 1995).

Due to its moderate thermophilic nature, *Streptococcus thermophilus* survives and produces acid at temperatures higher than can be tolerated by the mesophilic lactic acid bacteria. This characteristic makes *Streptococcus thermophilus* useful in the fermentation of dairy products such as yoghurt and Swiss and Italian cheeses that are ordinary manufactured or incubated at elevated temperatures (Wood and Holzaphel 1995).

CHAPTER 3

β-GALACTOSIDASE

β-galactosidase (β-D-galactoside galactohydrolyse; E.C. 3.2.1.23), in other words lactase, is a commercially important enzyme that catalyzes the hydrolysis of lactose into its constituent monosaccharides glucose and galactose (Figure 3.1).

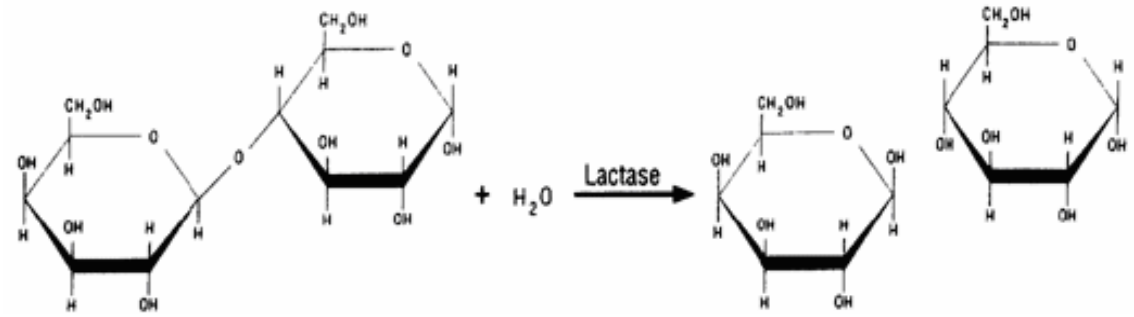


Figure 3.1. Schematic presentation of lactose hydrolysis by β-galactosidase (lactase) enzyme. (Source: Prescott, et al. 1990)

This enzyme is widely distributed in nature, found in numerous plants, animals and microorganisms including yeast, fungi and bacteria.

The enzyme from several sources have been well characterized, especially the enzyme from *Escherichia coli* serves a model for the understanding of the action of the enzyme (Jacobson 1994).

3.1. Hydrolysis Mechanism

Lactose hydrolysis can be performed in two ways including acid hydrolysis and enzymatic hydrolysis.

3.1.1. Acid Hydrolysis

First way of lactose hydrolysis is acid hydrolysis. It is carried out by a homogenous reaction in acid solution or in a heterogeneous phase with ion exchange resins. Acid hydrolysis can be performed under harsh conditions. For example; 80 %

hydrolysis may be achieved in three minutes at pH 1.2 and 150 °C (Gekas and Lopez-Leiva 1985). Although this procedure seems to be simple, it has several disadvantages. Most important one is protein denaturation due to low pH and high temperature. It causes reduction in the function of the proteins. Thus, it prevents their uses in many products (Bury et al. 2001). Moreover, the presence of salts in whey causes deactivation of acid and requires a demineralization step. Other disadvantages are off-colour and off-flavour formation. Due to many drawbacks of acid hydrolysis, enzymatic hydrolysis by β-galactosidase is the preferred method of lactose hydrolysis (Gekas and Lopez-Leiva 1985).

3.1.2. Enzymatic Hydrolysis

3.1.2.1. Reaction Mechanism

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry.

Lactose hydrolysis mechanism was explained by using the enzyme β-galactosidase obtained from *Escherichia coli* (Jacobson 1994).

A double-displacement reaction mechanism was proposed in which β-galactosidase formed and hydrolyzed a glycosyl-enzyme intermediate via carbonium ion galactosyl transition state (Wallfels and Malhrotra 1961). In literature, it is proposed that the active site of β-galactosidase contains cysteine and histidine amino acids which function as proton donor and proton acceptor, respectively. Cysteine amino acid contains the sulphydryl group acted as proton donor and histidine residues contains imidazole group acted as nucleophile site to facilitate cleavage of the glycosidic bond, respectively, during the enzymatic hydrolysis procedure (Figure 3.2) (Mahoney 1998, Zhou and Chen 2001).

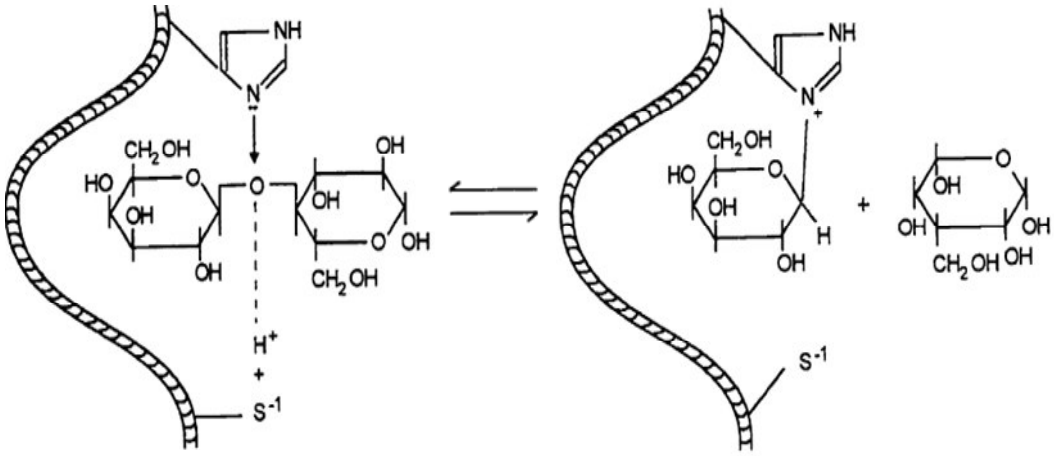


Figure 3.2. Proposed mechanism of lactose hydrolysis by β-galactosidase.
(Source: Richmond et al. 1981)

The second reaction occurred is called galactosyl (transgalactosyl) reaction. In this reaction, β-galactosidase transferred the galactosyl moiety from the intermediate to an acceptor containing a hydroxyl group (Figure 3.3) (Mahoney 1998).

When this acceptor is water, free galactose is formed by hydrolysis. However, under certain conditions, other sugars are able to act as acceptors and give rise to oligosaccharide formation (Mahoney 1998).

3.1.2.2. Oligosaccharide Formation

Enzymatic hydrolysis of lactose is accompanied by galactosyl transfer to other sugars, thereby producing oligosaccharides. The amount and nature of the oligosaccharide formation by transgalactosyl reaction depends mainly on the enzyme source and the nature and concentration of the substrate. The yield of oligosaccharides can be increased by using higher substrate and/or by decreasing the water content (Mahoney 1998). Quantitatively, the disaccharide allolactose is one of the major oligosaccharides produced by neutral pH β-galactosidases (Mahoney 1998). It is also primary transferase product (Huber et al. 1976). At high initial lactose concentrations, maximum oligosaccharide production can be reached between 30-40% of the total sugars present. However, at lower initial lactose levels, such as those found in milk and whey, transferase activity is reduced, where maximum oligosaccharide levels can reach 22 to 25% (Huber et al. 1976).

Oligosaccharides are hydrolyzed slowly, both *in vitro* and *in vivo.* They can be considered as low molecular weight, non-viscous, water-soluble, dietary fibre. They are considered to be physiologically functional foods which promote the growth of bifidobacteria in the colon and a wide variety of health benefits has been claimed in connection with this effect. The appearance and disappearance of oligosaccharides is investigated through consideration of the kinetics of transferase activity (Mahoney 1998).

Table 3.1. Structures of some oligosaccharides formed during β-galactosidase action on lactose. (Source: Mahoney 1998)

Disaccharides	β-D-Gal (1→6)-D-Glc	allolactose
	β-D-Gal (1→6)-D-Gal	galactobiose
	β-D-Gal (1→3)-D-Glc	
	β-D-Gal (1→2)-D-Glc	
	β-D-Gal (1→3)-D-Gal	
Trisaccharides	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Glc	6' digalactosyl-glucose
	β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	6' galactosyl-lactose
	β-D-Gal (1→6)-β-D-Gal (1→6)-D-Gal	6' galactotriose
	β-D-Gal (1→3)-β-D-Gal (1→4)-D-Glc	3' galactosyl-lactose
	β-D-Gal (1→4)-β-D-Gal (1→4)-D-Glc	4' galactosyl-lactose
Tetrasaccharides	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	6' digalactosyl-lactose
	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	
	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	
Pentasaccharide	β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→6)-β-D-Gal (1→4)-D-Glc	6' trigalactosyl-lactose

*Gal, galactose; Glc, glucose.

The mechanism for the transferase reaction is still not known but it is thought to involve a 1, 4-linked galactose being transferred to a 1, 6-linkage (Huber et al. 1976). The results of oligosaccharide formation may be favourable. Recently, researches suggested that oligosaccharide production is beneficial to human health. They have been added to infant formula as potential “bifidus factors” to promote the growth and the establishment of bifidobacteria in the intestine (Hsu et al. 2005). Moreover, other reported therapeutic benefits of oligosaccharide consumption include decreased serum cholesterol levels, enhanced absorption of dietary calcium and enhanced synthesis of B-complex vitamins (Onishi et al. 1995).

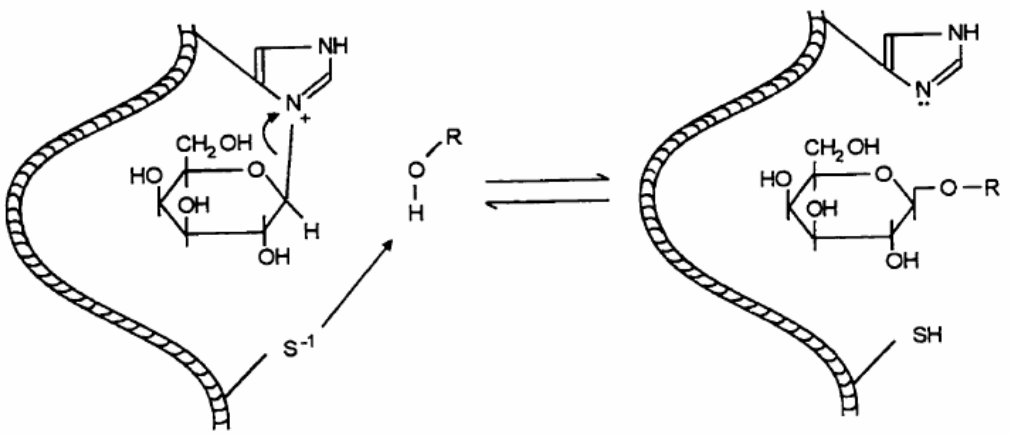


Figure 3.3. Proposed mechanism of galactosyl transfer reaction by β-galactosidase. (Source: Richmond et al. 1981)

3.2. Applications of β-galactosidase

β-galactosidase enzyme used in the hydrolysis of lactose is one of the most important biotechnological processes in the food industry. It has potentially beneficial effects on the assimilation of foods containing lactose, as well as the possible technological and environmental advantages of industrial applications. These applications are listed above; (Gekas and Lopez-Leiva 1985, Jurado et al. 2002)

1. Elimination of lactose intolerance by encouraging the utilization of lactose as an energy source.
2. Formation of galacto-oligosaccharides during lactose hydrolysis to favour the growth of intestinal bacterial micro flora.
3. Improvement in the technological and sensorial characteristics of foods containing hydrolyzed lactose from milk or whey: increased solubility, greater sweetening power, formation of monosaccharides which are easier to ferment in certain products such as yoghurt and reduction of the Maillard reaction.
4. Greater biodegradability of whey.

3.2.1. Industrially Important Strains

Many organisms have been known as high β-galactosidase producer for commercial use. Although yeast (intracellular enzyme), fungi or molds (extracellular

enzyme) are known to produce β-galactosidase (Gekas and Lopez-Leiva 1985), bacterial sources have gained more importance and they are preferable due to ease of fermentation, high activities of enzyme and good stability.

The commercially exploited sources of β-galactosidase have been of microbial origin (mainly yeast and molds). The characteristics of the β-galactosidase from different microbial sources are listed in Table 3.2

Table 3.2. Properties of β-galactosidases from microbial sources.
(Sources: Gekas and Lopez-Leiva 1985, Mahoney 1985)

Sources	pH optimum	Temperature optimum (°C)	Activators	Inhibitors
A. niger	3.0-4.0	55-60	none needed	none
A. oryzae	5.0	50-55	none needed	none
K. lactis	6.5-7.3	35	K^+, Mg^{+2}	Ca^{+2}, Na^+
K. fragilis	6.6	37	K^+, Mn^{+2}, Mg^{+2}	$Ca^{+'}$, Na^+
E. coli	7.2	40	Na^+, K^+	
B. subtilis	6.5	50	none needed	
B. stearothermophilus	5.8-6.4	65	Mg^{+2}	
S. thermophilus	7.1	55	K^+, Mg^{+2}	Ca^{+2}
L. bulgaricus		42-45		
C. inaegualis	6.0	30-55		

Although properties of the microbial β-galactosidase vary with the organism, suitable enzymes must be selected according to their application. Application of an enzyme can be determined by its operational pH range. According to this characteristic, enzymes may be divided into two groups including the acid-pH enzymes from fungi and neutral-pH enzymes from yeast and bacteria. Acid pH enzymes from fungi are suitable for processing acid whey and permeate whereas; neutral-pH enzymes from yeast and bacteria are generally suitable for milk and sweet whey process (Mahoney 1985).

Fungal enzymes have pH optima in the range of 3 to 5. Therefore fungal enzymes are suitable for processing acid whey and permeate. They have also relatively high optimum temperature which is between 55-60 °C. At it is known, combination of low pH and high temperature discourages microbial growth. However, fungal β-galactosidase is not as pure as a yeast source and it may contain other enzymes such as protease, lipase or amylase. As a consequence of these limitations, fungal applications of β-galactosidase have been limited to high acid products and pharmaceutical preparations (Mahoney 1985).

However, yeast β-galactosidase is characterized by their neutral pH optima. Thus, they are widely used in the hydrolysis of lactose in milk (pH 6.6) and sweet whey (pH 6.2). Milk also supplies the potassium and magnesium ions needed for activity. Yeast β-galactosidase can be produced in high yields at relatively low prices and are viewed as safe for use in foods. However, the most important characteristic of this enzyme is its low heat stability. If the temperature increases above 55 °C, the enzyme is inactivated rapidly. To achieve high conversions at these temperatures, with minimal oligosaccharide production, high levels of enzyme are required. This would increase the processing costs. In order to avoid these problems, hydrolysis is often carried out at 4-6 °C for 16 to 24 hours where microbial spoilage is minimized (Mahoney 1985).

Bacterial β-galactosidases are characterized by neutral pH optima as well. They are diverse in their optimum temperature with variation between bacteria and even between strains of same bacteria. A number of bacteria have been considered as potential β-galactosidase sources such *as L. lactis, L. acidophilus, L. bulgaricus* (Bury and Jelen 2000, Vasiljevic and Jelen, 2001, Gueimonde et al. 2002, Akolkar et al. 2005). Thermophilic sources have been found to produce thermo stable β-galactosidase. *S. thermophilus* β-galactosidase has an optimum temperature of 55 °C (Greenberg and Mahoney 1982).

Lactobacilli strains are commonly used in the industry as probiotic. It is well known that β-galactosidase from lactic acid bacteria is an intracellular enzyme, and it is not released to the outside of cells under conventional fermentation conditions (Bury et al. 2001). *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 (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 Rajogopal 2000).

However, the obvious disadvantage of using these enzymes is the low production yield; about 10 times lower than that attainable with yeast. This may be improved through optimisation of growth conditions or recombinant DNA technology.

3.2.2. Commercial Procedures

Enzymatic hydrolysis of lactose by β-galactosidase is one of the most popular technologies to produce lactose-reduced milk for consumption by lactose-intolerant people. Evolution of lactose-reduced products began when the enzyme β-galactosidase became accessible to the consumer. The consumer could add this enzyme to a glass of fresh milk to hydrolyze the lactose. After the overnight incubation at refrigerator, lactose content is reduced by 70% or more depending on the enzyme amount added (Kligerman 1981). Most lactose intolerant people could then consume the "lactose-reduced" milk without adverse effects (Kligerman 1981).

With the developments in processing techniques, hydrolyzing lactose before packaging certain dairy products has become more prevalent. Through the advances in research, the procedure has become more cost effective due to increased understanding of the process and its effects on characteristics of the product (Holsinger and Kligerman 1991). With these new advancements, various types of lactose-reduced foods such as milk, American cheese, cottage cheese and ice cream are made their way into this growing market (Reiter 1991). Many research efforts have been focused on ways to reduce or remove the lactose in dairy products (Kocak and Zadow 1989). One of the more common ways to accomplish this is through the use of enzymes. Three techniques may be utilized to accomplish enzymatic lactose hydrolysis:

1. “Single-use” or “throw-away” batch systems

2. Recovery systems (β-galactosidase reuse systems)

3. Immobilized enzymes which are systems where the enzyme is chemically bound to an inert matrix (Holsinger and Kligerman 1991).

The advantages of immobilized enzyme are clear: ease in enzyme recovery and reuse, ease in product recovery and continuous operation. The behaviours of immobilized enzyme reactors have been extensively studied as a critical point in the industrial process production (Li et al. 2007).

It has been proposed that the "single-use" enzyme batch system could be used conveniently during the development of new lactose reduced products, whereas the latter systems are better for large-scale operations (Holsinger and Kligerman 1991). The method ultimately used is mainly dependent on a number of factors including pH of the

product, maximum temperature the product will reach and how long it will remain there, contact time, activity of the enzyme, substrate, and cost (Zadow 1986).

3.3. Physicochemical Properties of β-galactosidase

The enzymes derived from various microbial sources have different properties, such as protein chain length, and the position of the active site. However, 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 3.3

Table 3.3. Active sites and other properties of β-galactosidase from various microbial origins. (Source: Zhou and Chen 2001)

Enzyme Origin	*K. lactis*	*E.coli*	*A.niger*
Length (AA)	1025	1023	1006
Proton donor	Glu^{482}	Glu^{461}	Glu^{200}
Nucleophile/base	Glu^{551}	Glu^{537}	Glu^{298}

Molecular weight of β-galactosidase varies between organisms as well. *E. coli* β-galactosidase has a molecular weight of 116,353 kDa per monomer (approximately 540 kDa per molecule). Molecular weights of other bacterial β-galactosidases are presented in Table 3.4.

Table 3.4. Molecular weights of β-galactosidases from bacterial sources

Organism	**Molecular Weight (kDa)**	**Reference**
S. solfataricus	240	Grogan (1991)
S. thermophilus	530	Greenberg and Mahoney (1982)
L. acidophilus	570	Nielsen and Gilliland (1992)
L. lactis	500	McFeters et al., (1969)

The effects mono- and divalent cations have been well documented (Greenberg and Mahoney 1982, Garman et al. 1996, Kreft and Jelen 2000, Vasiljevic and Jelen

2002). Divalent cations such as magnesium and manganese may enhance the β-galactosidase activity, whereas monovalent cations may have a positive or negative effect (Garman et al. 1996, Kreft and Jelen 2000).

In a study performed by Garman et al. six species of lactic acid bacteria were used (Garman et al. 1996). The rate of lactose hydrolysis by β-galactosidase from each species was enhanced by Mg^{+2} however the effect of K^{+} and Na^{+} differed from strain to strain. In another study, manganese was found to be the most effective cation followed by magnesium for the maximum β-galactosidase activity of *Streptococcus thermophilus* (Greenberg and Mahoney 1982).

Ca^{+2} is known as an inhibitor of β-galactosidase (Greenberg and Mahoney 1982). However, all of the calcium in milk is bound to casein. As it is not free in solution, it does not inhibit β-galactosidase activity (Garman et al. 1996).

CHAPTER 4

CELL DISRUPTION

Although most important large-scale products are extracellular, many potentially useful products are retained in the cell. In recent years, intracellular products have gained more importance due to the developments in genetic engineering. Therefore, genetic engineering and strain improvement are mainly focused on developing organisms that excrete important intracellular products (Clementi 1995). As a consequence, majority of intracellular products have been successfully produced on an industrial scale in recent years.

Cell disruption is the first stage in the isolation of intracellular materials and significantly effects the downstream separation and purification operations. As it is well known, microorganisms are protected through cell wall structures ranging from very fragile to tough (Stansbury and Whitaker 1987). In order to release cellular contents, several cell disruption methods have been developed. The cell wall determines the resistance to the cell disruption process. Therefore, knowledge of composition and structure of cell wall is essential for a better understanding of the disintegration mechanism (Clementi 1995).

In bacterial cells, the resistance of cell wall to disruption mainly depends on the presence of peptidoglycan that is composed of glycan chains consisting of N-acetylglucoseamine and N-acetylmuramic acid residues (Clementi 1995). Although nearly all bacterial cell walls contain peptidoglycan, their structural characteristics change considerably due to the degree of cross linking and thickness. In Gram positive bacteria, glycan chains form a thick layer where peptidoglycan represents up to 90% of the wall. However, this is much thinner in Gram negative bacterial cell. Therefore, it is proposed that the resistance of bacterial cells to disruption process increases as the thickness of the peptidoglycan layer, tetra peptide unit content and the degree of cross linking increase (Clementi 1995).

In order to isolate intracellular products, it is required to select suitable methods to breakdown cellular structures. Therefore, one should consider several factors during selection of suitable process. These factors depend on the characteristics of the microorganism, location of the desired product and sensitivity of the product to

denaturation. Although many techniques available that are satisfactory in lab-scale, only a limited number of methods are suitable for large-scale applications, particularly for intracellular enzyme extraction (Clementi 1995).

Cell disruption methods can be classified into two main groups including mechanical and non mechanical disruption methods.

4.1. Mechanical Methods

Mechanical methods for cell disruption based on either solid or liquid shear. Bead mills, homogenizer and sonicator are the main examples of mechanical disruption methods.

4.1.1. Bead Mills

The bead mill has been successfully adapted for cell disruption both in the laboratory and in the industry. At the lowest levels of the technology, the beads are added to the cell suspension in a tube and the sample is mixed on a common laboratory vortex mixer. This process works for easily disrupted cells, is inexpensive and multiple samples can be conveniently processed.

In the more sophisticated level, different designs have been used including vertical or horizontal cylindric grinding chamber filled with small beads (Christi and Moo-Young 1986). As the impeller spins, the beads are "activated" and the cells are torn apart by the shear forces generated between the beads. Laboratory models are capable of milling smaller volumes (100 ml) in batch configuration or larger volumes in a continuous operation. Industrial models operate continuously and are readily available with 275 L stainless steel grinding chambers.

The degree of disruption depends on several factors such as bead loading, bead size, impeller tip speed and mean residence time (Kula and Schütte 1987). Increased bead loading enhances cell disruption up to a certain value of bead density (Schutte and Kula 1993). Above this value a decreased efficiency occurs. Cell disruption can also be enhanced by increasing the agitation speed up to certain point. If this speed is exceeded, no further increase is observed in the disruption rate due to the higher dispersion or backflow effects (Clementi 1995). Bead size also affects the degree of disruption. Beads used for this purpose may be glass, steel or ceramic. A smaller bead size tends to be more

effective because of the increased number of contact points between the grinding media and the material (cell) to be milled. However, the location of the desired protein can also influence the bead size chosen for the disruption. For example, disrupting with larger beads will tend to release periplasmic enzymes in comparison to cytoplasmic proteins (Kula and Schütte 1987) thus facilitating the purification of periplasmic enzymes.

Residence times of the cell suspension in the mixing chamber can be varied to provide the desired level of disruption for a given system. The maximum residence time is limited due to the product inactivation as a result of thermal degradation.

As a consequence of cell disruption in bead mills, thermal or shear denaturation of proteins take place when operation conditions are chosen to give the highest amount of disruption (Clementi 1995).

4.1.2. Homogenization

The homogenizer has been adapted for liquid shear disruptions of microbial cells. It is a vital unit for dairy processing as well. High pressure homogenizers arde used for the treatment of milk or other products in the food industry. Homogenization appears to be favoured and effective technique for cell disruption and also suitable for large scale applications (Stansbury and Whitaker 1987).

A homogenizer is composed of a piston pump or pumps and a valve assembly. The pressure generated by the pump is controlled by the spring loaded valve. The fluid flows through the narrow gap between the valve and valve seat only to change direction abruptly at the impact ring. Considerable amounts of heat are generated during operation, therefore efficient cooling is required between passages to prevent damage to heat sensitive proteins. If the temperature is properly controlled, there is no problem with product degradation or inactivation (Clementi 1995).

4.1.3. Ultrasonic Disruption

Ultrasonic disruption is most commonly used method in laboratory scale. Cell disruption with sonicator occurs when sound waves having a frequency of 20 kHz are converted to a very rapid vibration in a liquid. This phenomenon is known as cavitation (Clementi 1995).

In ultrasonic disruption, the ultrasonic energy is transmitted to the liquid through a sonicator tip or horn. The tip or horn is placed into the sample. The vibration of ultrasonic device causes local low pressure areas within the liquid. The low pressure converts the liquid to gas in the form of very small bubbles. As the local pressure changes and begins to rise, these bubbles collapse. The collapse of these bubbles causes a shock wave travel through the liquid, resulting in a shear force that will disrupt cells. However, the rapid vibration of the tip generates heat during the process. Therefore, sonicator should be turned off every few minutes during operation (Clementi 1995).

4.2. Non-Mechanical Methods

Non-mechanical methods such as enzymatic treatment, chemical treatment and freeze thawing are used in the isolation of cellular contents. In each case, the cell membrane may be totally disrupted or made partially permeable to allow the product release.

4.2.1. Enzyme Treatment

There are number of enzymes which hydrolyze specific bounds in cell walls of a limited number of microorganisms. These enzymes include lysozyme, enzyme extracts from leucocytes, *Streptomyces ssp., Trichoderma ssp, Penicillium ssp*. This method appears very attractive in terms of specificity and is especially promising for the harvesting of particulate (Clementi 1995). Although this method is probably one of the gentlest methods, using enzymes is an expensive process and may complicate purification processes (Stansbury and Whitaker 1987).

In the enzymatic lysis, cell wall is the substrate for the enzyme. Commercially available enzymes such as lysozyme made from egg white are applicable to only few bacteria. An advantage of enzymatic lysis compared to other disruption methods, is better control of pH and temperature, lower capital investment and potential specificity, however enzymes are expensive. When taking all these into consideration, enzymes may be used to release only specific proteins, thus simplefying the downstream operation and reducing the total recovery cost compared with non-specific homogenization methods (Stansbury and Whitaker 1987).

4.2.2. Chemical Treatment

Several chemicals (acids, alkalis, organic solvents and surfactants) are used for cell lysis. Treatment with these chemicals presents several drawbacks such as contamination of the products with potential toxicity and risk of damage to sensitive products (Clementi 1995). Cells may be made permeable by the use of detergents (such as sodium dodecyl sulphate, SDS) to solubilise the lipid bilayer and in some cases disrupt cells. A number of detergents damage the lipoproteins of the microbial cell membrane and lead to release of intracellular components. Unfortunately the detergents may cause some protein denaturation and may need to be removed before further purification stages (Stansbury and Whitaker 1987).

4.2.3. Freeze Thawing

In freeze-thawing, slow freezing rates which promote large crystal growth may disrupt cells. Freezing and thawing of a microbial cell paste will inevitably cause ice crystals to form and melt with some subsequent disruption of cells. Even though, it is slow with limited release of cellular materials, it has been used in combination with other techniques (Stansbury and Whitaker 1987).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

5.1.1. Microorganisms

Lactobacillus delbrueckii subsp. *bulgaricus* species (*Lactobacillus delbrueckii subsp. bulgaricus* 77 (Lb 77), *Lactobacillus delbrueckii* subsp. *bulgaricus* 16 (Lb 16), *Lactobacillus delbrueckii* subsp. *bulgaricus* 22b (Lb 22b) and *Streptococcus thermophilus* species *Streptococcus thermophilus* 77a (St 77a), *Streptococcus thermophilus* 74 (St 74), *Streptococcus thermophilus* 95/2 (St 95/2), isolated from traditional yogurt samples of Toros mountain region of Turkey were used. Phenotypic and genotypic characterizations of the cultures were conducted by the Molecular Food Biotechnology research group at Izmir Institute of Technology (Erkus et al. 2006).

5.1.2. Chemicals

List of chemicals used in this study is given in Table A.1.

5.2. Methods

5.2.1. Culture Propagation

Stock cultures of these strains were prepared by mixing 0.5 ml of activated culture with 0.5 ml of glycerol-broth solution (40%) and stored at - 80 °C in sterile cryovials. Culture propagation was done by transferring 1 loop of stock culture into 10 ml of sterile M17 and MRS broth for *Streptococcus thermophilus* species and *Lactobacillus delbrueckii subsp. bulgaricus* species, respectively, followed by incubation at 43 °C for 16 hours (overnight incubation).

5.2.2. Construction of Growth Curves

Growth curves were constructed in order to determine the specific growth rates and the right incubation time to achieve maximum yield for β-galactosidase production and an easy cell disruption. Therefore, strains St 77a, St 74 and St 95/2 were subjected to fermentation in 100 ml of M17 broth and strains Lb 77, Lb 22b and Lb 16 were subjected to fermentation in 100 ml of MRS broth with an inoculation ratio of 2.5 % (v/v) (determined in preliminary studies) under static conditions at 43 °C. Fermentation samples were taken and analyzed for enzyme activity and cell counts.

5.2.3. Fermentation

5.2.3.1. Fermentation in Shake Flasks

Screening and optimisation studies were carried out in 250 ml Erlenmeyer flasks containing 100 ml of either one of the sterilized broths (113 ºC /10 min) described in statistical designs. In the screening experiments, media were inoculated with 2.5 % activated culture as described in the previous section 5.2.1 and incubated for 8 hours with or without agitation (Table B.1, Table C.1). At the end of fermentation, enzyme extraction procedures were performed for each flask.

Table 5.1. Fermentation media formulations used in screening experiments

Media Components and Concentrations (%) (w/v)
Lactobacillus delbrueckii subsp. bulgaricus species
12 % Skim Milk (S)
6 % Whey (W)
6 % Whey+ 0.2 % Yeast Extract (WYE)
6 % Whey + 1.2 % MRS (WMRS)
Streptococcus thermophilus species
2 % Whey+ 3 % Corn Step Liquor + 2 % Peptone (M1)
2 % Whey+ 3 % Corn Step Liquor + 2 % Peptone + 2% Potassium Phosphate (M2)
7 % Whey+ 3 % Corn Step Liquor + 2 % Peptone (M3)
7 % Whey+ 3 % Corn Step Liquor + 2 % Peptone + 2% Potassium Phosphate (M4)

Experimental conditions used in optimisation studies were shown in Table D.1, Table E.2, and Table F.3. Enzyme extraction was performed by lysozyme enzyme treatment method in all optimisation studies.

5.2.3.2. Fermentation in Bioreactor

After the determination of optimum conditions by central composite design for each strain, scale up from shake flasks (100 ml) to bioreactor (2.5 L) was carried out using Infors-Minifors Bioreactor (5 L). The conditions used in the bioreactor studies were given in Table 5.2.

Table 5.2. Fermentation conditions used in bioreactor studies

Strain	Inoculum Ratio (%)	Media	Temperature	pH adjustment	Fermentation Mode
Lb 77	4	8% Skim Milk	43 ± 0.1	-	Batch
St 95/2	3.9	5% Whey + 4% CSL+2% Potassium Phosphate + 2% Peptone	43 ± 0.1	-	Batch
Lb77 & St 95/2	Lb: 3 St : 2.6	5% Whey + 4% CSL+2% Potassium Phosphate + 2% Peptone	43 ± 0.1	-	Batch
Lb 77	4	8% Skim Milk	43 ± 0.1	6.2 ± 0.1	Batch
St 95/2	3.9	5% Whey + 4% CSL+2% Potassium Phosphate + 2% Peptone	43 ± 0.1	6.2 ± 0.1	Batch
Lb77 & St 95/2	Lb: 3 St : 2.6	5% Whey + 4% CSL+2% Potassium Phosphate + 2% Peptone	43 ± 0.1	6.2 ± 0.1	Batch
Lb77 & St 95/2	Lb: 3 St : 2.6	5% Whey + 4% CSL+2% Potassium Phosphate + 2% Peptone	43 ± 0.1	-	Fed-Batch (addition of 500 ml 5% whey at the end of 4h)

5.2.4. Disruption of Microbial Biomass

Since β-galactosidase from, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* is an intracellular enzyme, three different disruption methods were applied for each run in order to compare and evaluate the efficiency of these methods.

5.2.4.1. Enzyme Extraction by Lysozyme

For the enzyme extraction procedure, 10 ml of fermentation broth was harvested by centrifugation at 6000 rpm for 15 min at 4 ºC followed by washing the pellet with 10 ml of 0.05M Na- phosphate buffer (pH 7) and centrifugating it at 6000 rpm for another 15 min After the washing step the pellet was resuspended in 4.5 ml of the same buffer followed by vigorous vortexing. To this solution, 100 mg lysozyme (Sigma L 6876) was added and incubated at 37 ºC for 15 min. After this period, 0.5 ml of 4 M NaCl solution was added and incubated further at 37 ºC for another 50 min. followed by centrifugation at 6000 rpm for 15 min. After the centrifugation, the supernatant was used for the enzyme assay and protein determination.

5.2.4.2. Enzyme Extraction by Glass Beads

For the glass bead extraction, 10 ml of fermentation broth was harvested by centrifugation at 6000 rpm for 15 min at 4 ºC. The pellet obtained was washed with 10 ml of 0.05M Na- phosphate buffer (pH 7) and centrifuged again at 6000 rpm for 15 min at 4 ºC. The pellet was resuspended in 5 ml of the same buffer followed by vigorous vortexing to disperse the cells homogeneously. To the pellet solution obtained, 5 g of glass beads (Marienfeld glass beads, 1 mm in diameter) was added and vortexed at 2700 rpm using 8 operating cycles (1 operating cycle = 1 minute operation + 30 sec cooling on ice). At the end of 8 cycles, the solution was centrifuged at 6000 rpm for 15 min at 4 ºC and the supernatant was used for the enzyme assay and protein determination.

5.2.4.3. Enzyme Extraction by Homogenizer

For the extraction using the homogenizer, 30 ml of the fermentation broth was harvested by centrifugation at 6000 rpm for 15 min at 4ºC. The pellet obtained was washed with 30 ml of 0.05M Na-phosphate buffer (pH 7) and centrifuged at 6000 rpm for 15 min. After the washing step the pellet was resuspended in 15 ml of the same buffer followed by vigorous vortexing. Cell disruption was performed on this solution using the homogenizer (Yellow Line DI 18 Basic Homogenizer) by applying 4 operating cycles (1 operating cycle = 45 sec operating + 30 sec pause) at 14000 rpm. Afterwards the solution was centrifuged at 6000 rpm for 15 min at 4 ° C; the supernatant was used for the enzyme assay.

5.2.5. Enzyme Assay

β-galactosidase activity was assayed according to the procedure described in Food Chemicals Codex-National Academy of Sciences. The chromogenic substrate o-nitrophenol-beta-D-galactopyranoside (ONPG) (8.3×10^{-3} M) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) was used. The amount of substrate and enzymes used were 2 ml and 0.5 ml, respectively. At time zero, 0.5 ml of enzyme solution was added to the ONPG solution and incubated for 15 minutes. The assay was stopped by the addition of 0.5 ml of 10% sodium carbonate followed by reading the absorbance at 420 nm. One unit was defined as that quantity of enzyme that would liberate 1 mM of o-nitrophenol per minute under the assay conditions. Units were calculated using the equation 5.1.

$$\text{Unit/ml} = A \times \text{dilution factor} / (\varepsilon \times \text{time} \times \text{enzyme suspension}) \quad (5.1)$$

Where A denotes the absorbance at 420 nm, dilution factor was the fold of the enzyme solution, ε equaled the extinction coefficient (determined from the o-nitrophenol standard curve) and time was the incubation time (15 minutes).

5.2.6. Protein Assay

The method of Bradford with BSA as a standard was used to measure protein amount (Roe 2000).

5.2.7. Biomass Determination

The biomass (g/l) was determined gravimetrically by drying the pellet at 43 ºC until constant weight was reached.

5.2.8. Lactose and Lactic Acid Determination

Lactose and lactic acid determination was performed by using HPLC system. The equipment used was a Perkin Elmer HPLC system (Perkin Elmer, Boston, MA, USA) equipped with a pump (PE Series 200), refractive index detector (PE Series 200) and Interface (PE Series 900). Samples were injected with a 20 µl loop. The separation was performed with an Aminex HPX-87H ion exclusion column (300 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA). Column was thermostated at 45 °C in column oven (Metatherm, Lake Forest, CA, USA). The conditions applied were: 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. Data acquisition and peak processing were performed with Total Chrom Workstation Ver.6.2.1 software. Peak identification was based on retention times and peak quantification was based on the external standard method (Altıok et al. 2006).

5.2.9. Experimental Design and Statistical Analysis.

5.2.9.1. Full Factorial Design

Full factorial design was used in order to identify important parameters in the screening analysis. Therefore, effects of strain, media and agitation speed were investigated on the responses of biomass, lactic acid and β-galactosidase activity. The effects of these factors were examined separately for *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* strains. The level of the factors was such as

three types of strains, four types of media formulation mentioned in section 5.2.3.1 and two types of agitation speed (static and 150 rpm). Analysis of data and generation of graphics were done by the software Design Expert 7.0.0 Trial Version, Stat-Ease Inc. The analysis of variance (ANOVA) tables were generated and the effect of factors and interaction terms were determined. The significances of all terms were judged statistically according to their p-values.

5.2.9.2. Central Composite Design

Three different optimisation studies were performed by using statistical software called Design Expert 7.0 Trial Version.

In the first optimisation study, effects of independent variables, temperature (X1), inoculum (X2) and skim milk concentration (X3) were investigated on the responses of lactic acid, biomass and β-galactosidase activity by using Lb 77. A Face centered composite design (FCCD) with three factors were used. Total of 20 treatment combinations were generated and performed.

In the second optimisation study, effects of independent variables temperature (X1), inoculum (X2), whey concentration (X3) and corn steep liquor (X4) were investigated on the responses of lactic acid, biomass and β-galactosidase activity using St 95/2. A Face centered composite design (FCCD) with four factors were used. A total of 30 run in face centered CCD were generated and performed.

According to the results obtained from the optimisation studies described above, third optimisation study was performed to investigate the symbiotic relationship between Lb 77 and St 95/2. Effects of independent variables inoculum amount of St 95/2 (X1), inoculum amount of Lb 77 (X2) and media formulation (X3) were investigated on the responses of lactic acid, biomass and β-galactosidase activity.

In all optimisation studies, analysis of data and generation of response surface graphics were done by the software Design Expert 7 Trial Version, Stat-Ease Inc. The response variables in three set of experiments were lactic acid, biomass and β-galactosidase activity. After running the experiments and measuring the response variables, a second order polynomial equation including the interactions was fitted to the response data by multiple linear regression approach.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_j X_i X_j + \boldsymbol{\varepsilon} \qquad (5.2)$$

where Y is the predicted response, k is the number of factor variables, $ß_o$ is the model constant, $ß_i$ is linear coefficient, X_i is the factor variable in its coded form, $ß_{ii}$, is the quadratic coefficient, $ß_{ij}$, is the interaction coefficient and ε is the error factor. The following equation (5.3) is used for coding the actual experimental values of the factors in a range of [-1, +1]:

$$x = [\text{actual}–(\text{low level} + \text{high level})/2] / (\text{high level}–\text{low level})/2 \qquad (5.3)$$

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically according to their p-values.

5.2.10. Characterization of β-galactosidase

5.2.10.1. Effect of pH on Activity and Stability of β-galactosidase

The effect of pH on the activity of β-galactosidase was determined by assaying the enzyme activity at different pH values ranging from 4.5 to 9.0 using 0.05 M of the following buffer systems: sodium acetate (4.5, 5.0, 5.5), sodium phosphate (pH 6.0, 6.5, 7.0, 7.5) and tris-HCl (pH 8.0, 8.5, 9.0). The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage. The pH stability of β-galactosidase was investigated in the pH range of 4.5-9.0 using 0.05 M of the following buffer systems: sodium acetate (4.5, 5.0, and 5.5), sodium phosphate (pH 6.0, 6.5, 7.0, 7.5) and tris-HCl (pH 8.0, 8.5, 9.0) buffer systems. Enzyme solutions were mixed with the buffer solutions mentioned above and incubated at 37 ºC for 30 minutes. Afterwards aliquots of the mixtures were taken to measure the residual β-galactosidase activity (%) with respect to control, under standard assay conditions.

5.2.10.2. Effect of Temperature on Activity and Stability

The effect of temperature on the activity of β-galactosidase was determined by performing the standard assay procedure at different temperatures ranging from 20 to 65ºC (20, 25, 30, 35, 40, 45, 50, 55, 60, 65 ^{o}C). Substrate (o-nitrophenol) was preincubated at the respective temperatures for 5 minutes. The relative activities (as percentages) were expressed as the ratio of the β-galactosidase activity obtained at certain temperature to the maximum activity obtained at the given temperature range. The thermo stability of the crude enzyme was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 20 to 65^{o}C for 30 minutes.

5.2.10.3. Effect of Metal Ions on Activity

The effect of metal ions (K^{+}, Na^{+}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cu^{+2} and Fe^{+2}) were tested on β-galactosidase activity in sodium phosphate buffer pH 7.0. The enzyme was preincubated for 30 min at 37 °C with various final concentrations (1-10mM) of metal ions prior to the substrate addition. Activity without added metal ions was taken as 100 % activity.

5.2.10.4. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

Thermal inactivation kinetics of β-galactosidase was studied by incubating the crude enzyme at different temperatures (45, 50, 55, and 60 °C) in the absence of the substrate. Aliquots were withdrawn at periodic intervals and cooled in an ice bath prior to assay as described above. The residual activity was expressed as % of the initial activity. From a semi natural logarithmic plot of residual activity versus time, the inactivation rate constants (k_d) were calculated (from slopes), and apparent half lives were estimated using the equation 5.4. The half-life ($t_{1/2}$) is known as the time where the residual activity reaches 50 %.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{5.4}$$

The temperature dependence of k_d was analyzed using the Arrhenius plot (Shuler and Kargi 2002). The inactivation energy was calculated from the Arrhenius equation as:

$$k_d = k_0 \exp\left(-\frac{E}{RT}\right) \tag{5.5}$$

or,

$$\ln (k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T} \tag{5.6}$$

The values of E and k_0 were estimated from the slope and intercept of the plot of ln (k_d) versus 1/T respectively. (5.6)

5.2.10.5. Estimation of Thermodynamic Parameters

The enthalpy of inactivation (ΔH^*) for each temperature was calculated according to equation 5.7

$$\Delta H^* = E_d - RT \tag{5.7}$$

where R (= 8.31447 $J.K^{-1}.mol^{-1}$) is the universal gas constant and T is the absolute temperature. The values for the Gibb's free energy (ΔG^*) of inactivation at different temperatures were calculated from the first-order constant of inactivation process by using equation 5.8

$$\Delta G^* = -RT \ln\left(\frac{k_d h}{\kappa T}\right) \tag{5.8}$$

where h (= 6.6262×10^{-34} Js) is the Plank constant, and κ (= 1.3806×10^{-23}) is the Boltzmann constant.

From equations (5.7) and (5.8) the entropy of inactivation (ΔS^*) of β-galactosidase was calculated from equation 5.9 (Ortega et al. 2004).

$$\Delta S^* = \frac{\left(\Delta H^* - \Delta G^*\right)}{T} \tag{5.9}$$

5.2.10.6. Determination of Kinetic Constants

The kinetic constants (V_{max} and K_m) were determined using Lineweaver –Burk double reciprocal (1/V versus 1/S) plot (Shuler and Kargi 2002) where different substrate concentrations (0.33-2mM) at pH 7.0 and 37 °C

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \tag{5.7}$$

With a slope of K_m/V_m and intercept of $1/V_m$ (equation 5.7) estimated K_m and V_m values were calculated as given in double-reciprocal plot.

5.2.10.7. Determination of Molecular Weight

The molecular weight of the subunits was estimated by SDS-PAGE using a vertical gel electrophoresis system. SDS-PAGE was done according to the procedure reported by Laemmli (1970). Crude enzyme extracts obtained from each strain was mixed in with sample buffer (10 ml glycerol, 5 ml 2-mercaptoethanol, 30 ml 10% SDS, 12.5 ml 0.5 M Tris buffer pH 6.8 and 100 mg bromophenol blue in a total volume of 100 ml). Reference marker including proteins with various subunit molecular weights (rabbit muscle myosin, 205 kDa; *E. coli* b-galactosidase, 116 kDa; rabbit muscle phosphorylase b 97.4 kDa; bovine albumin, 66 kDa; egg albumin, 45 kDa and bovine erythrocyte carbonic anhydrase 29 kDa) were used. (Sigma SDS 6H, High molecular weight marker kit). All samples and marker were boiled for 3–5 min and applied to the 10% resolving gel with 4% stacking gel. After running the gel, the proteins were stained by Coomassie brilliant blue for 6–8 h.

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Shake Flasks Experiments Results

6.1.1. Determination of Specific Growth Rates and Optimum Incubation Time

As β-galactosidase from lactic acid bacteria is an intracellular enzyme, it requires cell disruption or cell permeabilization for its release. The kinetics of cell disruption or permeabilization, however, is very closely related to the physiological state of the cells and growth conditions applied. For example cells harvested during the log phase of growth are more easily disrupted than those harvested during stationary phase. This is explained by the fact that, cells grown at lower specific growth rate such as in stationary phase direct their energy towards synthesis or strengthening of the cell wall structure rather than cell reproduction (Becerra et al. 2001). In order to determine the growth curves and the right incubation time where the β-galactosidase production is at its maximum level and where the disruption of the cells is easier, the strains (Lb 16, Lb 22b, Lb 77, St 74, St 77a and St 95/2) under study were subjected to fermentations where samples were taken for enzyme activity and cell counts at certain time points. The fermentation was carried out in 100 ml of MRS and M17 broth for *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* species respectively, with an inoculation ratio of 2.5 % under static conditions at 43 °C. Fermentation samples were taken and analyzed for enzyme activity and cell counts. The β-galactosidase enzyme was extracted using lysozyme enzyme treatment in this part of the study.

The effect of the incubation on the growth and enzyme production is presented in Figure 6.1 for *Lactobacillus delbrueckii subsp. bulgaricus* strains and in Figure 6.2 for *Streptococcus thermophilus* strains. The specific growth rates of *Lactobacillus delbrueckii subsp. bulgaricus* strains were determined from the growth curves as 0.43 h^{-1}, 0.54 h^{-1} and 0.56 h^{-1} for Lb 77, Lb 22b and Lb 16, respectively.

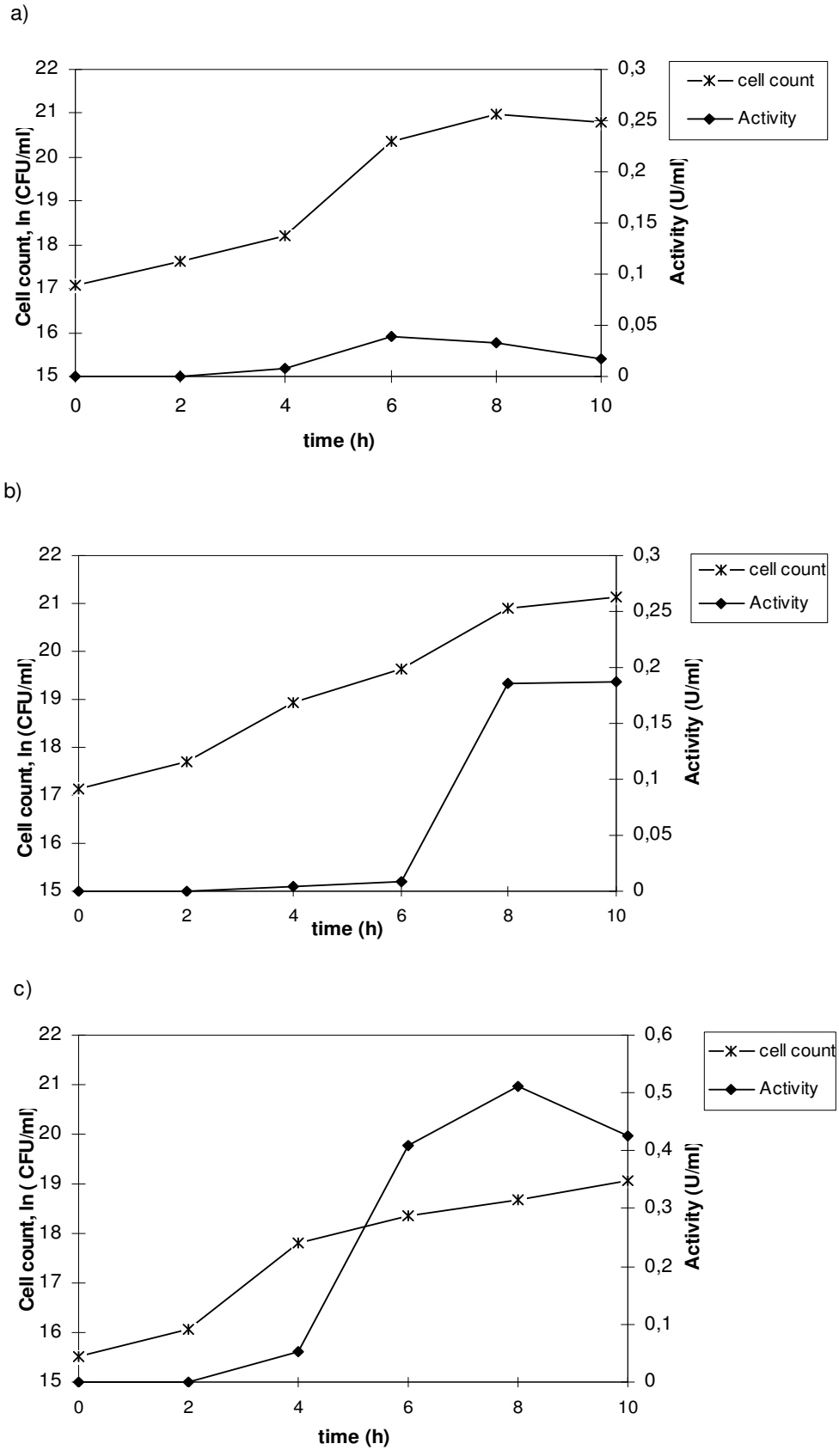
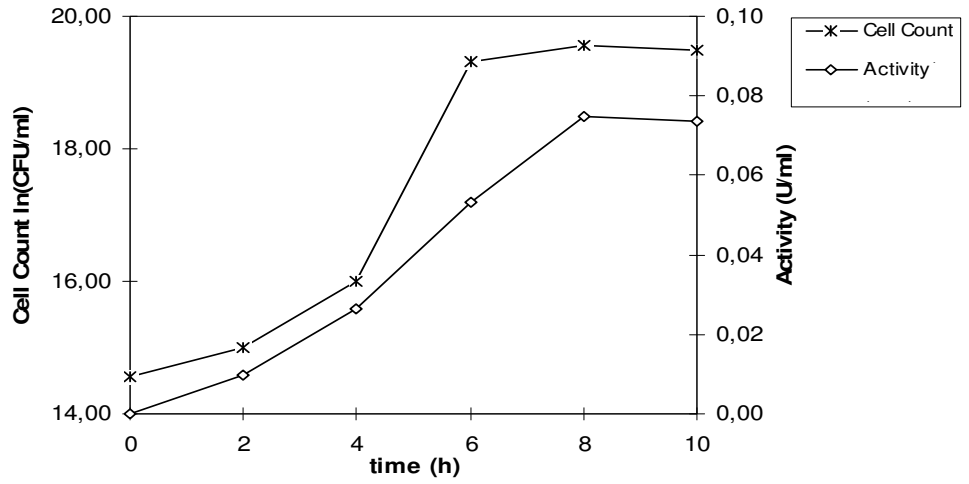
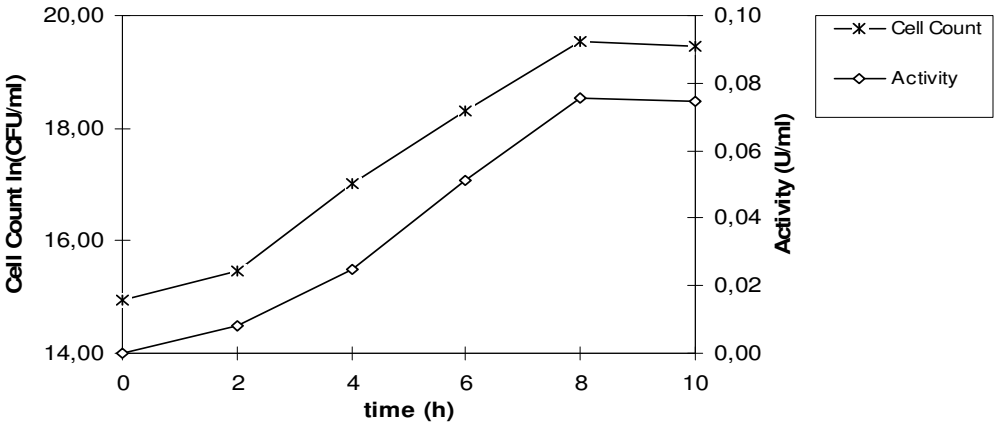


Figure 6.1. Growth and β-galactosidase profiles of *Lactobacillus bulgaricus* strains: a) LB 16, b)Lb 22b and c) Lb 77 in MRS growth medium at 43° C under static conditions.

a)



b)



c)

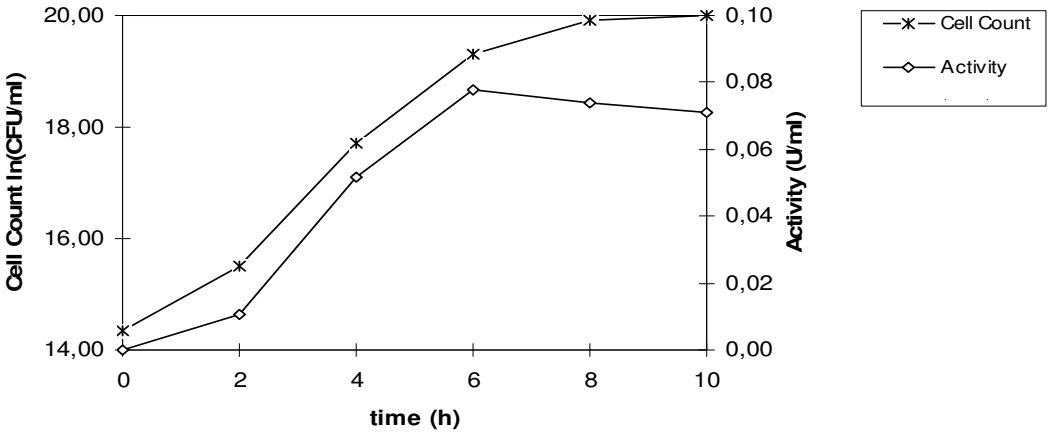


Figure 6.2. Growth and β-galactosidase profiles of *Streptococcus thermophilus* strains: a)St 77a, b) St 74 and c) St 95/2 in M17 growth medium at 43 ºC under static conditions

Similarly, the specific growth rates of *Streptococcus thermophilus* strains were determined as 0.635 h^{-1}, 0.732 h^{-1} and 0.893 h^{-1} for St 74, St 95/2 and St 77a, respectively. These were higher when compared to a study performed by El Demerdash et al. using *Streptococcus thermophilus* (El Demerdash et al. 2006).

Highest enzyme production was achieved at 8 hours for strains Lb 22b and Lb 77 and 6 hours for Lb 16 as seen from figure 6.3. There was an inverse relationship between the growth rates and the enzyme activities and a considerable difference among the strains, where Lb 77 seemed to be the promising strain under the conditions studied. This issue brought up the known phenomena that the lactase production is both growth and non growth - associated process which is indicated by the figures 6.1 and 6.3 and the incubation time of 8 hours corresponding to the early stationary growth phase. This has been stated by many researchers which confirm our findings (Rojaka et al., 2003).

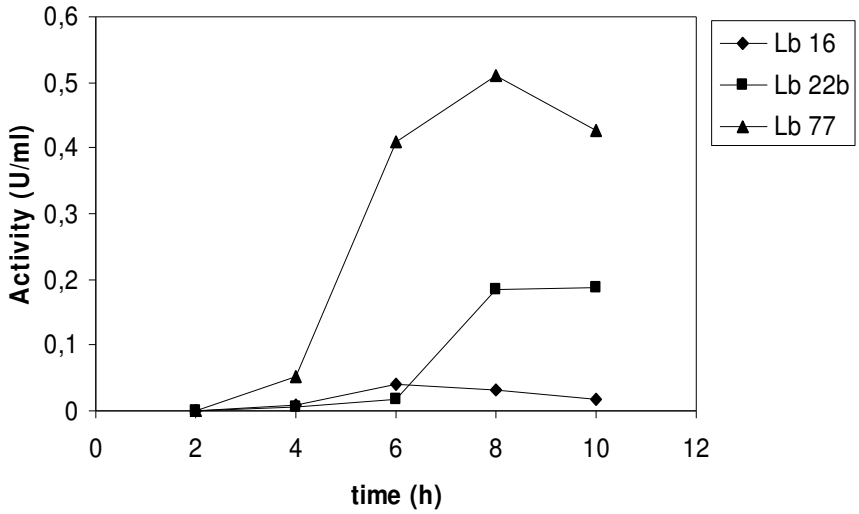


Figure 6.3. β-Galactosidase enzyme profiles for *Lactobacillus bulgaricus strains* (in MRS media at 43 ˚C under static conditions).

Similarly the highest enzyme production was achieved at 8 hours for strains St 74 and St 77a and 6 hours for St 95/2 as seen from Figure 6.4. Even though the specific growth rates were slightly different among the strains, the maximum β-galactosidase productivities were in close approximation such as $9.38x10^{-3}$, $9.5x10^{-3}$ and $9.75x10^{-3}$ U ml^{-1} h^{-1} for strains St 77a, St74 and St 95/2, respectively. As it is observed from the cell counts and β-galactosidase profiles, there is a proportional relationship presenting growth associated product formation kinetics. This finding was partly not in agreement with other researchers who reported a mixed (growth and non growth) growth

associated product formation kinetics for this enzyme in LAB cultures (Beshkova et al. 2002). This difference could be attributed to the unique characteristics of these strains which were isolated from traditional yogurt samples from mountain region. Therefore this might be indicative of the possible novel and unique properties of this enzyme which requires further characterization.

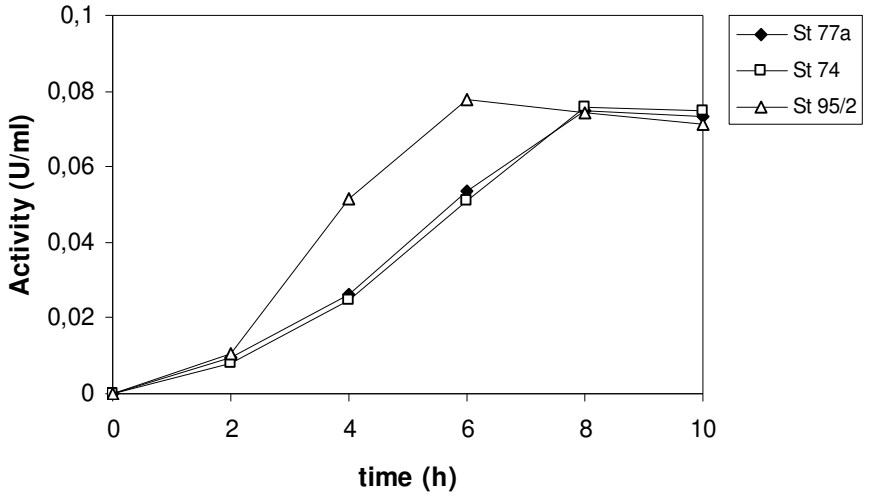


Figure 6.4. β-Galactosidase enzyme profiles for *Streptococcus thermophilus* strains (in M17 media at 43 ºC under static conditions)

Based on these findings incubation time of 8 hours was kept constant for all *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* strains in future studies, in order not to cause a discrepancy in further studies (difference in maximum enzyme activities between 6 and 8 hours was not significant for strain Lb 16 and St 95/2).

6.1.2. Full Factorial Design

6.1.2.1. Effects of Strain, Media and Agitation Speed on Biomass

In order to investigate the interactive effect of strain, media formulation and agitation speed on biomass and β-galacatosidase production, a total of 48 flasks (including the replicates) were prepared separately for *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* species according to the full factorial statistical design (Table B.1 and Table C.1) and analyzed .

According to results of full factorial design performed for *Lactobacillus delbrueckii subsp.* strains, the analysis of variance results (Table B.2), showed that the type of strain, media formulations, agitation speed, the interaction of strain with media, media with agitation speed and interaction between strain, media and agitation were significant at the significance level of $p<0.01$ on the biomass (cell dry weight) formation. The highest biomass was obtained in skim milk with all three *Lactobacillus delbrueckii subsp. bulgaricus* strains regardless of the agitation speed applied (Figure 6.5.). The other media formulations did not cause a significant effect.

In the comparison of the agitation speeds on the biomass, especially in skim milk a different tendency depending on the strain type was observed. For example for Lb 16, agitation at 150 rpm promoted cell growth more than with no agitation. However for Lb 77 no significant difference was observed, whereas for Lb 22 agitation at 150 rpm discouraged the growth by 23 % (Figure 6.5). This observation is very closely related to the type of strain and its tolerance to oxygen which is transferred by means of agitation. As it is known most of the lactic acid bacteria (LAB) are facultative anaerobes with a preference for anaerobic conditions. However researchers have not explicitly confirmed the detrimental effect of oxygen on cell growth, but have proved its effect on carbon metabolism (Beshkova et al. 2002). Therefore, it was decided to use this parameter as a variable in this study in order to investigate its effect.

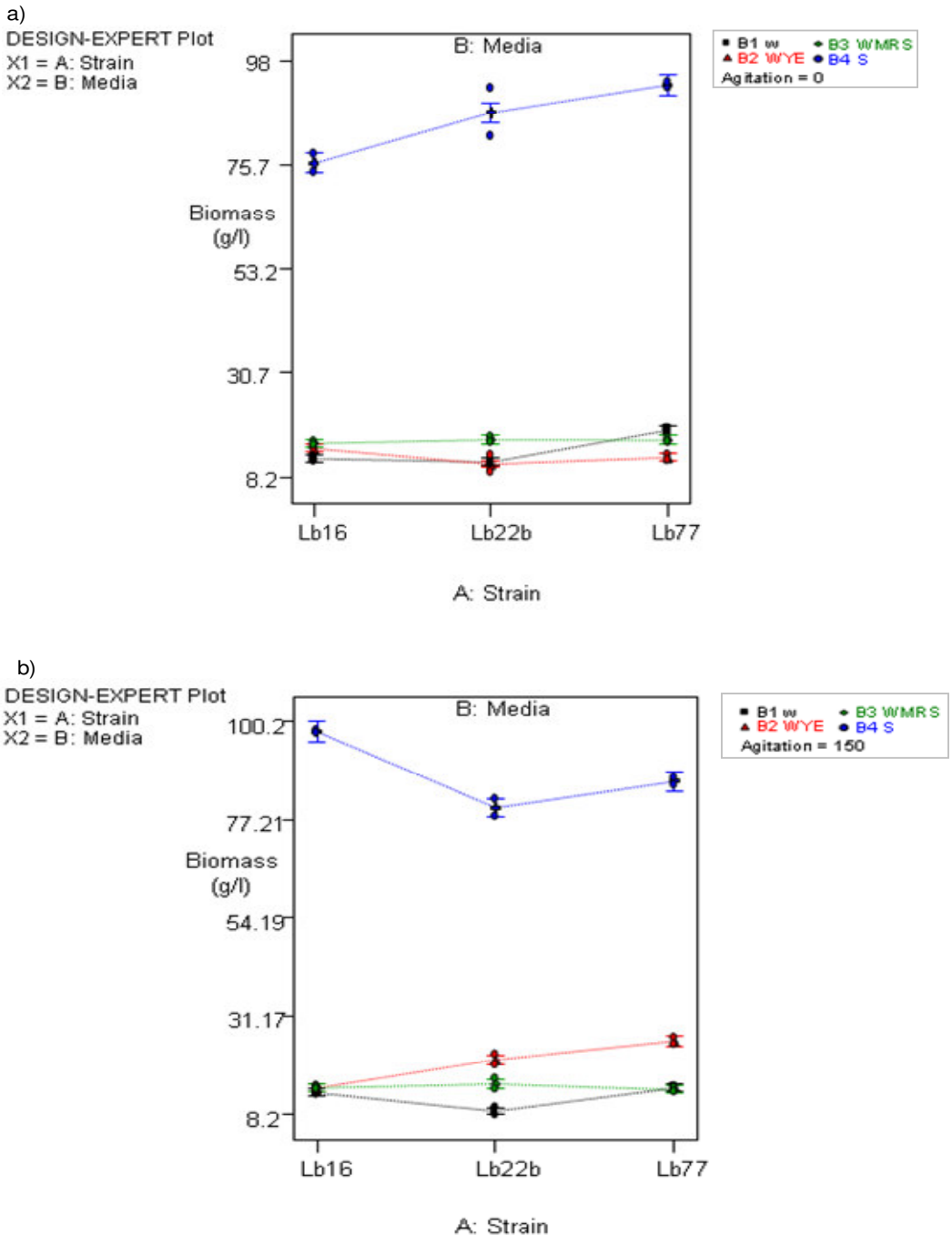


Figure 6.5. Effects of *Lactobacillus delbrueckii subsp. bulgaricus* strain types and different media formulations on biomass; incubation at 43° C for 8 hours under a) static conditions and b) at 150 rpm agitation speed.

According to results of full factorial design performed for *Streptococcus thermophilus* strains, the analysis of variance results (Table C.2) showed that all linear terms (type of strain, media formulations and agitation speed) and interaction terms (interaction of strain with media and media with agitation speed and interaction between strain, media and agitation) were significant at the significance level of $p<0.0001$ on the

biomass formation. The highest biomass was obtained with M2 media (Figure 6.6) for all three *Streptococcus thermophilus* strains under static conditions. The other media formulations did not cause a significant effect. Looking into the component of this medium, one will observe that for biomass formation potassium phosphate at 2 % is necessary, provided that the whey concentration is not higher than 2 % under static conditions. This observation was attributed to the buffering characteristic of potassium phosphate and confirming a study conducted by Greenberg and Mahoney (1982), that the best growth conditions was observed near neutral pH. This issue was also checked by the final pH measurements which were determined as an average of 6.35 and 6.3 for M4 and M2, respectively. Since the next highest concentration of whey studied was 7 %, we could not exactly determine the inhibitory concentration of this compound. The high whey concentration may exhibits an inhibitory or toxic effect on the biomass formation of the strains under study. In another words, this could be due to substrate inhibition or due to accumulation of lactate or other by products formed (Buyukkileci and Harsa 2004). Comparisons among the four media (M1, M2, M3 and M4) using the strain St 77a at 150 rpm (Figure 6.6), revealed that M1 media promoted 2.78, 6.31 and 10.67 times more biomass than M2, M3 and M4, respectively. It is obvious that this organism does not exhibit essential need for extra potassium phosphate in the formulation and is adversely affected by the high whey concentration.

In the comparison of the agitation speeds on the biomass, different tendency depending on the strain type and media formulations was observed. Increasing the agitation speed to 150 rpm did not cause any effect on St 95/2 in M2 media whereas, it did cause profound effect on St 77a and St 74, namely it reduced the biomass formation 4.36 times and 3.37 for St 74 and St 77a, respectively. This finding can be related to the anaerobic preference of LAB cultures observed in many other studies as well (Beshkova et al., 2002). On the other hand, for strain St 77a agitation at 150 rpm promoted cell growth almost 5 times more than with no agitation when M1 was used as media. This demonstrates the interaction effect of agitation and media formulation which can be partly related to the mass transfer of nutrients, enhanced through agitation. However, for St 74 no significant difference was observed, whereas for St 95/2 agitation at 150 rpm prevented the growth by 50 %. In fact, attention should be paid, that not only the type of organism and media formulation is important but also the environment to which it is subjected to. The needs and preferences of these organisms to that

particular media may change accordingly. The complex nature of these organisms brings up the need of particular studies specific for each organism.

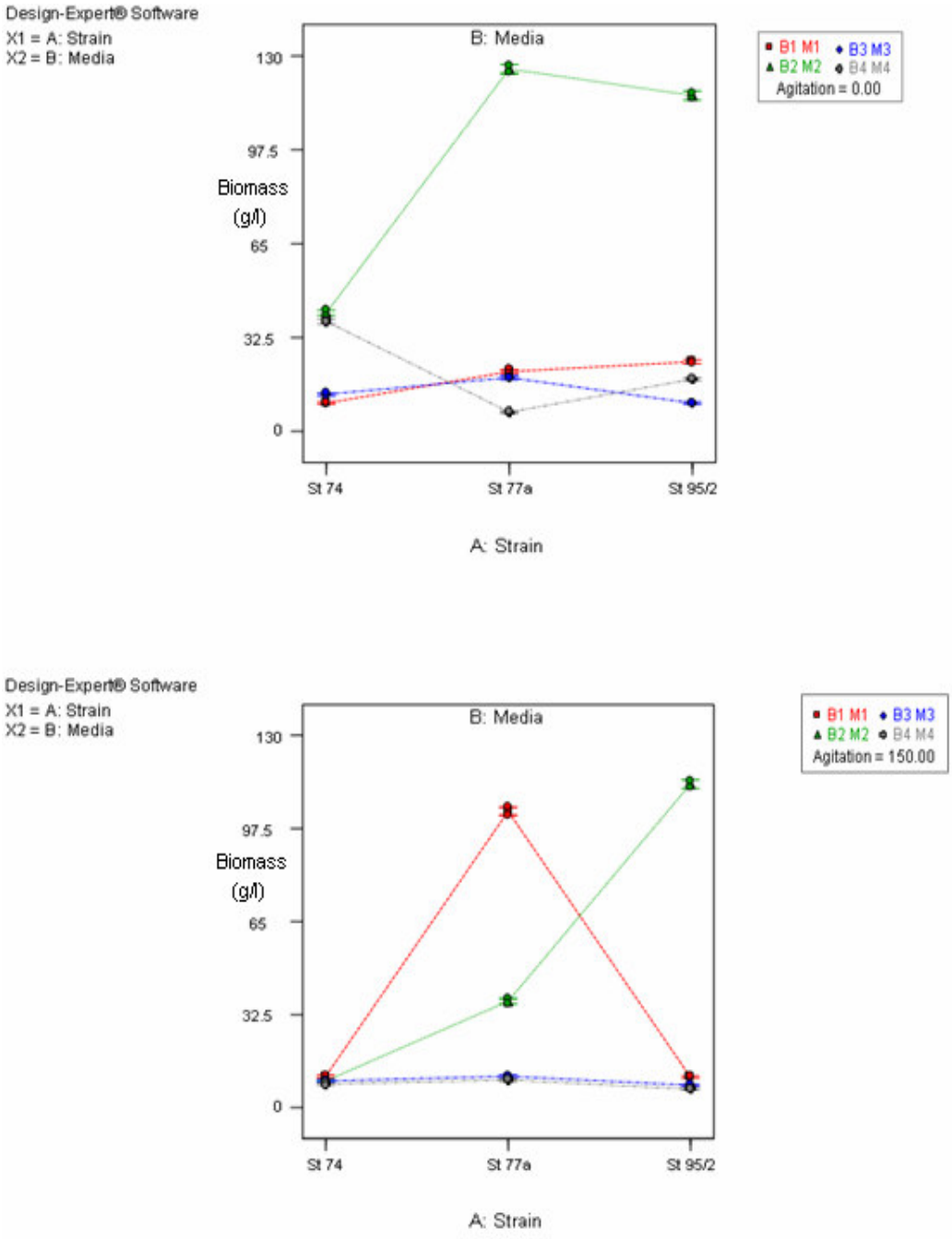


Figure 6.6. Effects of *Streptococcus thermophilus* strain types and different media formulations on biomass; incubation at 43 ºC for 8 hours under static conditions and at 150 rpm agitation speed.

6.1.2.2. Effects of Strain, Media and Agitation Speed on β-galactosidase Production

According to results of full factorial design performed for *Lactobacillus delbrueckii subsp. bulgaricus* strains, the analysis of variance results (Table B.3) showed that all terms were significant at $p<0.01$ significance level for both cell disruption methods with lysozyme and homogenizer. Only with glass bead extraction method (Table B.3) the only significant terms were the effect of type of strain, media formulation and the interaction between strain and agitation speed and the interaction between strain, media and agitation speed. Considering the interactive effect of strain and media formulations (Figure 6.7 and 6.8), skim milk was the best media formulation enhancing enzyme production in all three strains at both agitation speeds and with all three cell disruption methods used. The highest enzyme activity was observed for stain Lb 77 almost 1.79, 1.82 and 1.88 times more than Lb 22b and 5.0, 5.1 and 5.2 times more than Lb 16 at static environment by applying homogenizer, glass bead and lysozyme treatment, respectively. (Figure 6.7)

However this was reversed under agitation at 150 rpm, where Lb 77 was the strain with the lowest activity (Figure 6.8). This huge discrepancy was explained by the sole effect of agitation which was indirectly related to the oxygen supply. From these results it can be concluded that Lb 77 is a strain with the preference for anaerobic condition whereas strain Lb16 is on the contrary a strain with aerobic preference. Strain Lb 22 seems to exhibit an intermediate tolerance to oxygen. Of course the effect of agitation can not be only related to the effect of oxygen supply. It is also closely related to the nutrient mass transfer which is enhanced with agitation. This phenomenon however brings up the nutrient requirement of each strain and the more fastidious character of Lb 16 compared to Lb 77. Both oxygen and nutrient mass transfer are very much interactively affected by the intensity of the agitation speed as it is the case here.

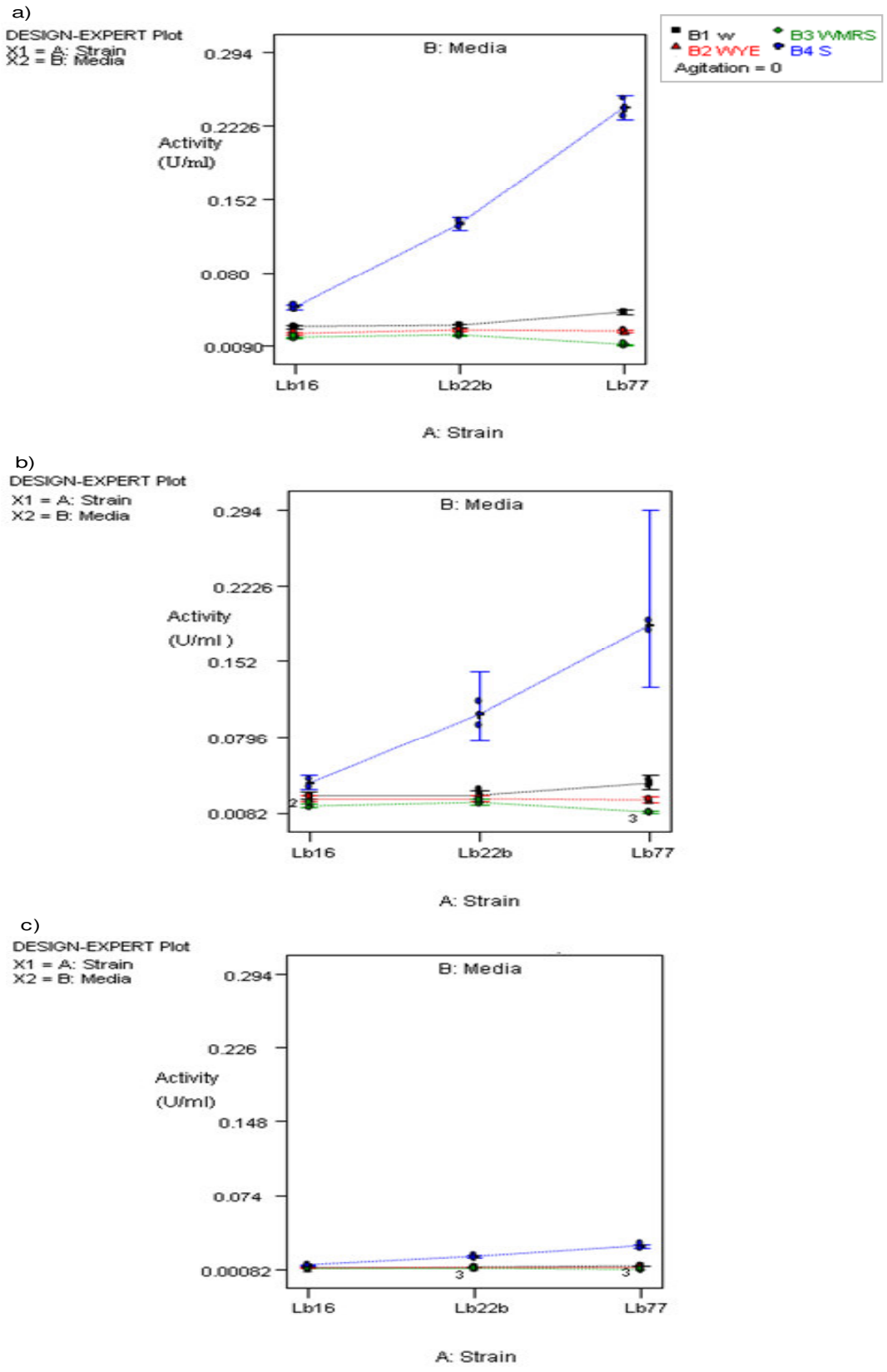


Figure 6.7. Effects of *Lactobacillus delbrueckii subsp. bulgaricus* strain types and different media formulations on β-galactosidase activity after incubation at 43° C for 8 hours at static condition and extracted using a) lysozyme, b) glass beads and c) homogenizer.

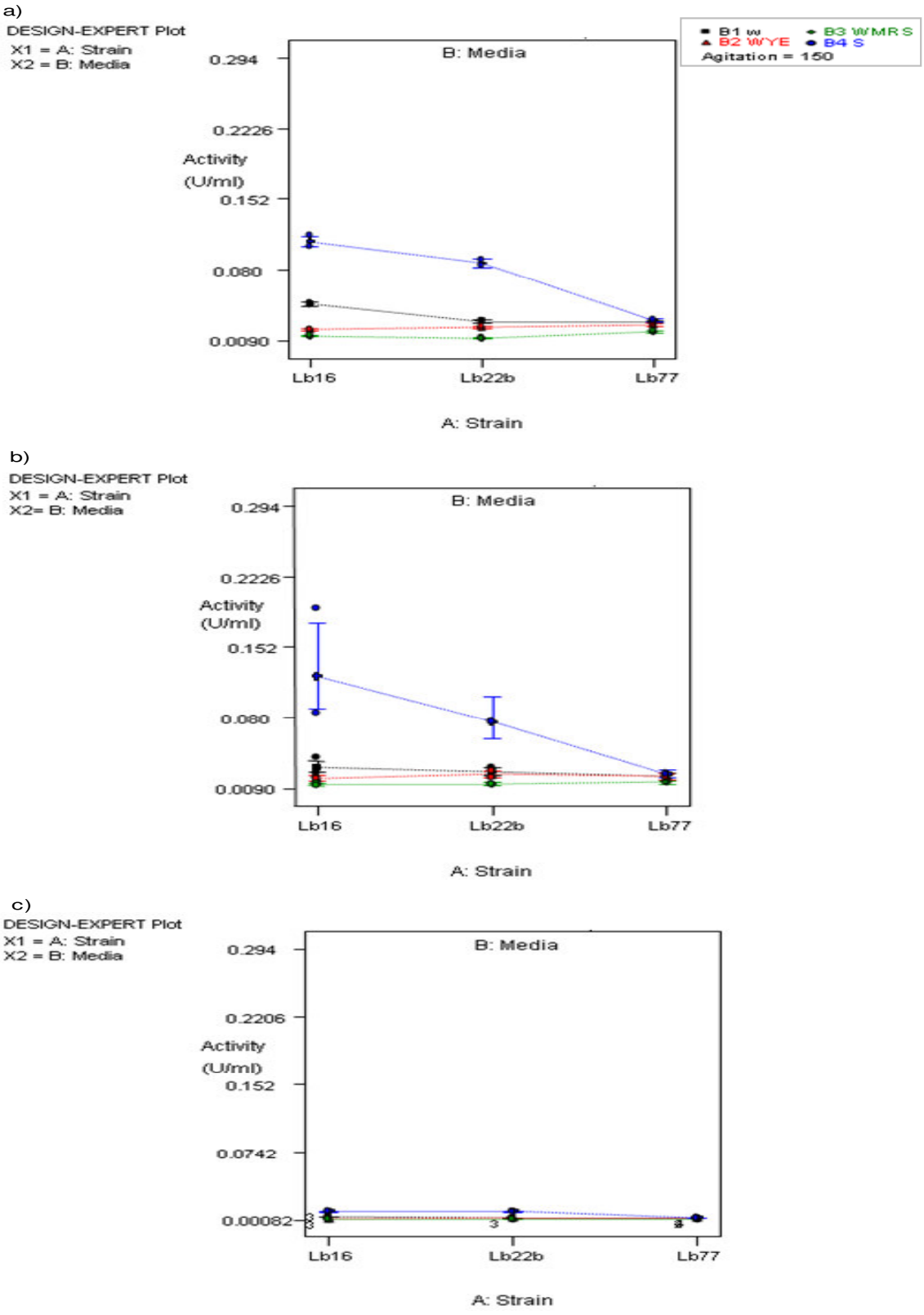


Figure 6.8. Effects of *Lactobacillus delbrueckii subsp. bulgaricus* strain types and different media formulations on β- galactosidase activity after incubation at 43 ˚C for 8 hours at 150 rpm and extracted using a) lysozyme, b) glass beads and c) homogenizer.

In a study performed by Vasiljevic and Jelen, the highest β-galactosidase activity was obtained when un-supplemented skim milk was used as cultivation media (Vasiljevic and Jelen 2001). However, lactic acid bacteria are fastidious microorganisms with complex growth requirements, particularly for amino acids (Gaudreau et al. 2005). This shows that β-galactosidase production is mainly affected by the type of carbohydrate and by the amount of nitrogen source. In order to encourage the growth of lactobacilli in whey, complex nutrients such as skim milk powder, malt sprouts, corn step liquor, yeast extract, meat extract or hydrolyzed whey proteins must be used (Bury et al. 1999). It seemed none of the formulations used in this study except for skim milk was adequate to supply the essential nutrients. This once again proved the fastidious nature of lactobacillus strains and their need for richer medium.

MRS supplementation with whey basal medium resulted in nearly 25.17 times lower activity than the activity obtained with skim milk. Similar results were obtained in previous studies as well. In a study performed by Hickey et al., they observed a significant decline in β-galactosidase activity of several *Lactobacillus bulgaricus* strains upon addition of small amounts of glucose to growing culture (Hickey et al. 1986). In another study carried out by Smart et al., they found that 19 out of 21 lactobacilli strains contained very low and non-detectable β-galactosidase activity when cultivated on media containing glucose or glucose + lactose (Smart et al. 1993). Vasiljevic and Jelen explained this by partial repression of the lac operon caused by glucose present in MRS, which resulted in lower β-galactosidase activity of the culture (Vasiljevic and Jelen 2001).

The highest β-galactosidase activity level obtained in this study using Lb 77 strain especially using skim milk under static conditions was comparable to many studies in the literature (Montanari et al. 2000, Gaudreau et al. 2005) and very promising for many industrial applications.

According to results of full factorial design performed for *Streptococcus thermophilus* strains, the analysis of variance results (Table C.3) showed that all factors and interaction effects were significant ($p<0.0001$) on β-galactosidase production for all extraction methods applied. Considering the interactive effect of strain and media formulation (Figure 6.9), M2 media was the best media formulation enhancing enzyme production in all three strains at static conditions.

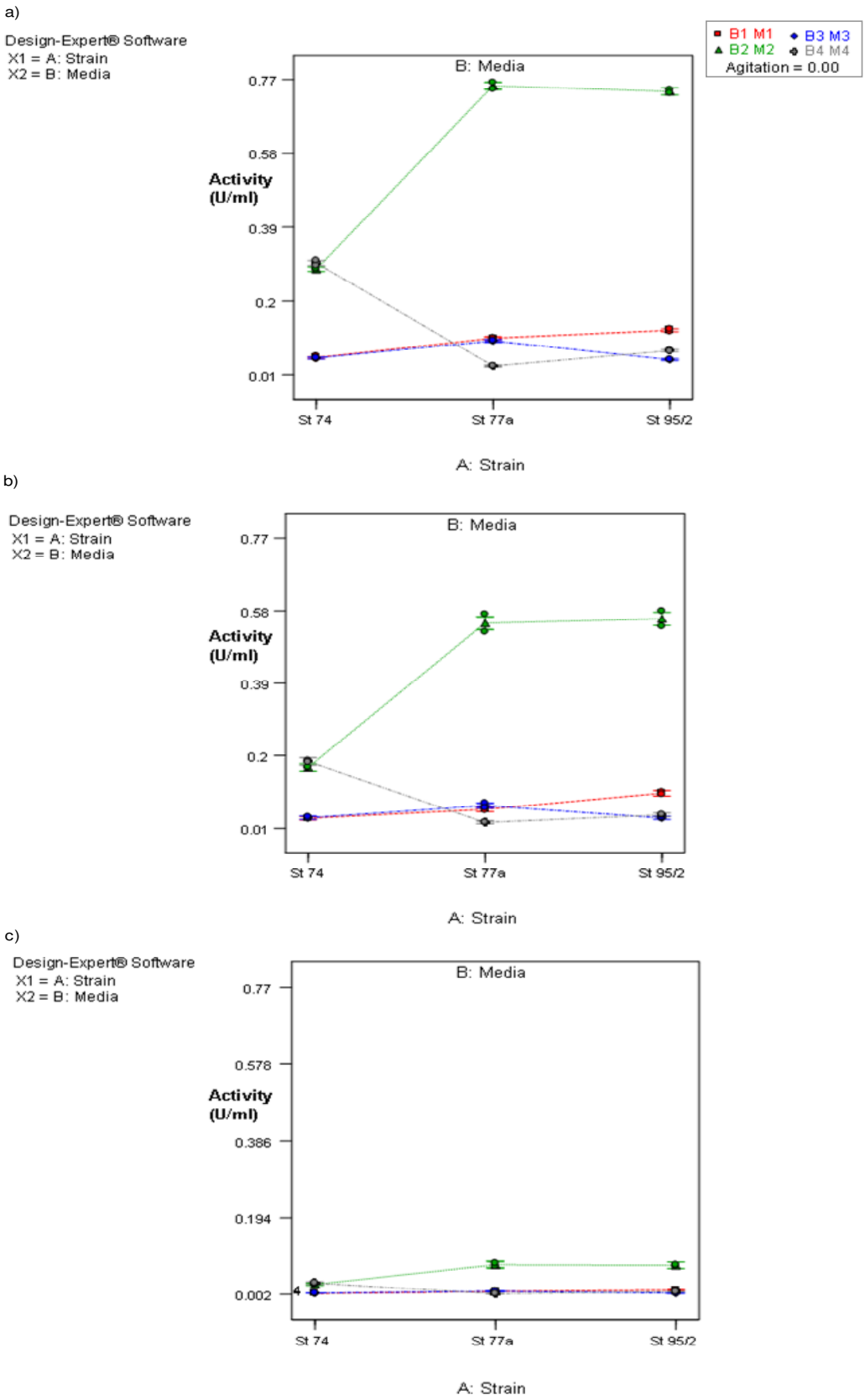


Figure 6.9. Effects of *Streptococcus thermophilus* strain types and different media formulations on β-galactosidase activity after incubation at 43 ℃ for 8 hours at static condition and extracted using a) lysozyme, b) glass beads and c) homogenizer.

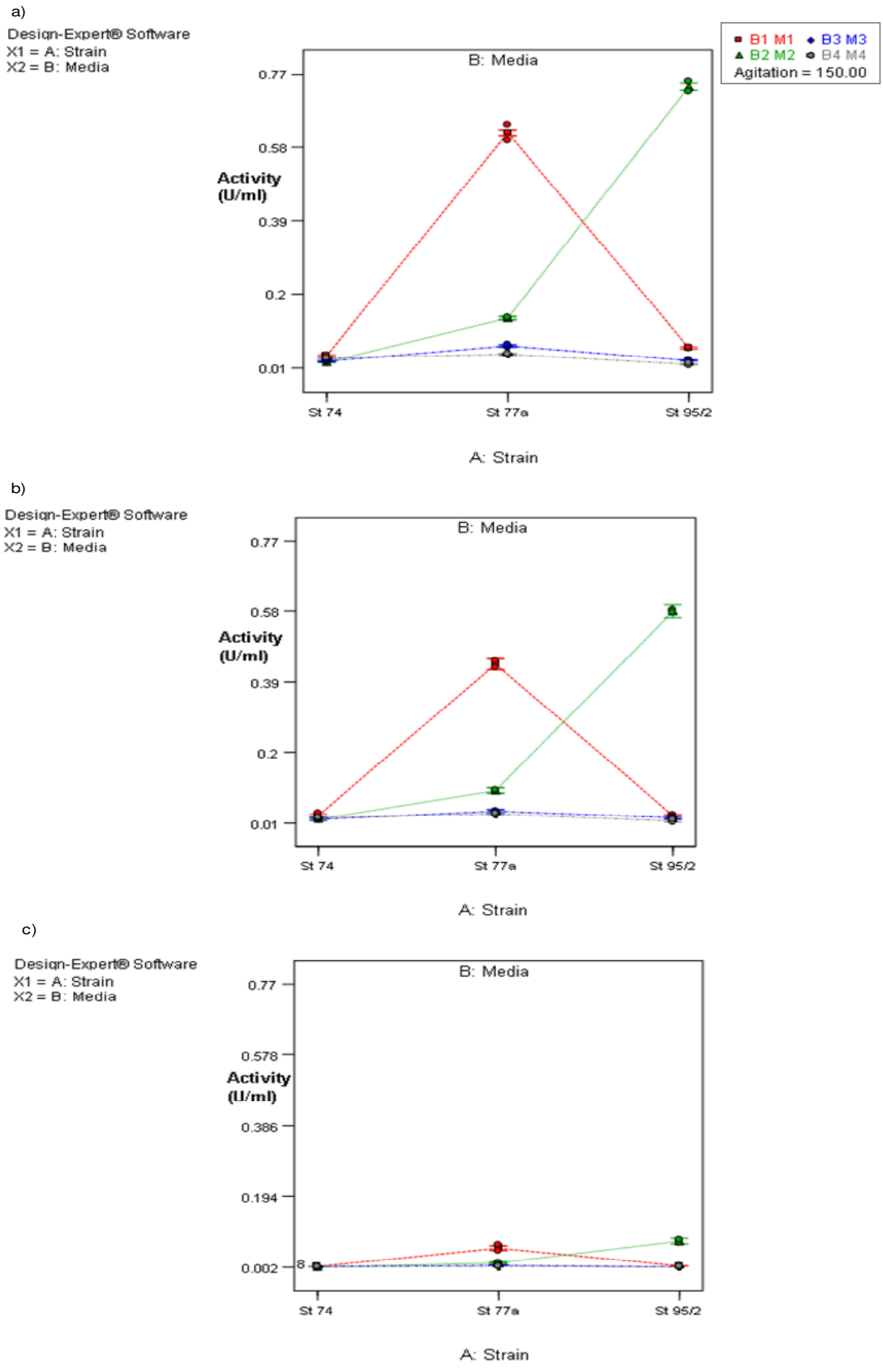


Figure 6.10. Effects of *Streptococcus thermophilus* strain types and different media formulations on β- galactosidase activity after incubation at 43 ºC for 8 hours at 150 rpm and extracted using a) lysozyme, b) glass beads and c) homogenizer.

Under these circumstances strains St 95/2 and St 77a exhibited high enzyme activity almost in the same range, whereas strain St 74 exhibited the lowest activity regardless of the type of extraction method applied. This observation could be partly related to the presence of potassium phosphate that could have an influence on the enzyme synthesis for the two strains (St 95/2 and St 77a) but being not effective in strain St 74. Its effect could be attributed to the buffering capacity which plays an important role in LAB cultures. Besides, this finding emphasizes the different characters of the three strains used. On the other hand, high enzyme activity was obtained with St 77a cultivated in media M1 under agitation speed of 150 rpm (Figure 6.10).

These conditions promoted enzyme synthesis for St 77a, 10 times and 15 times more than St 95/2 and St 74, respectively considering lysozyme treatment method. These values were 14.7 and 14.36 times for glass bead treatment and 9.36 and 10.48 times for homogenization method, respectively. Again these observations are partly in agreement with the vital role of potassium phosphate discussed above for the two strains. The role of agitation which would also contribute to this observation will be discussed below.

In the investigation of the interactive effect of agitation speed and media formulations on enzyme activity, it was observed that strain St 95/2 was not affected by the agitation regardless of the type of media used (Figure 6.9 and Figure 6.10). This was an important finding, pointing out the facultative anaerobic nature of this strain which would give flexibility to the manufacturer in industrial scale. Agitation plays a vital role in shake flask systems since it becomes the means of oxygen and nutrient supply to cells. However, the same finding was not valid for the other two strains. Furthermore, increasing the agitation speed to 150 rpm reduced the enzyme activity in M2 media for strain St 74 by 11.23, 8.12 and 10.57 times considering lysozyme, glass bead and homogenization treatment, respectively (Figure 6.9 and Figure 6.10). The same values were determined for strain St 77a as 5.47, 5.63 and 5.39, respectively (Table C.1). As it is observed the strains are differently affected by the degree of agitation. From here it can be concluded that strain St 74 is more fastidious and with the preference for anaerobic conditions compared to the strains St 77a and St 95/2.

It is seen that media M3 and media M4 resulted in lowest enzyme activity due to its composition with a whey concentration of 7 % which might be detrimental to enzyme synthesis. Since whey is a complex media, it is difficult to determine which of its component has toxic effect. This requires further study which is not considered at

this point. The highest β-galactosidase activity level obtained in this study using St 95/2 and St 77a strains especially using media M2 under static conditions was comparable to many studies in the literature (Greenberg and Mahoney 1982, Somkuti et al. 1996, Somkuti et al. 1998) and very promising for many industrial applications. Discovering new strains with novel characteristics is a difficult and tedious task to perform; therefore such strains are very valuable with economic significance.

6.1.2.3. Effects of Strain, Media and Agitation Speed on Lactic Acid Production

According to results of full factorial design performed for *Lactobacillus delbrueckii subsp.* strains, the analysis of variance results (Table B.4), showed that the type of strain, media formulations, agitation speed, the interaction of the factors were significant at the significance level of $p<0.01$ on lactic acid production. Skim milk promoted lactic acid production for Lb 22b under the static conditions where highest lactic acid production was obtained with Lb 22b.

When Lb 22b was cultivated in skim milk, it produced 1.88, 2.029 and 2.30 times more lactic acid than when it was cultivated in whey, whey + MRS and whey+ yeast extract respectively. However increasing the agitation speed to 150 rpm reduced the lactic acid production in all *Lactobacillus delbrueckii subsp. bulgaricus* strains.

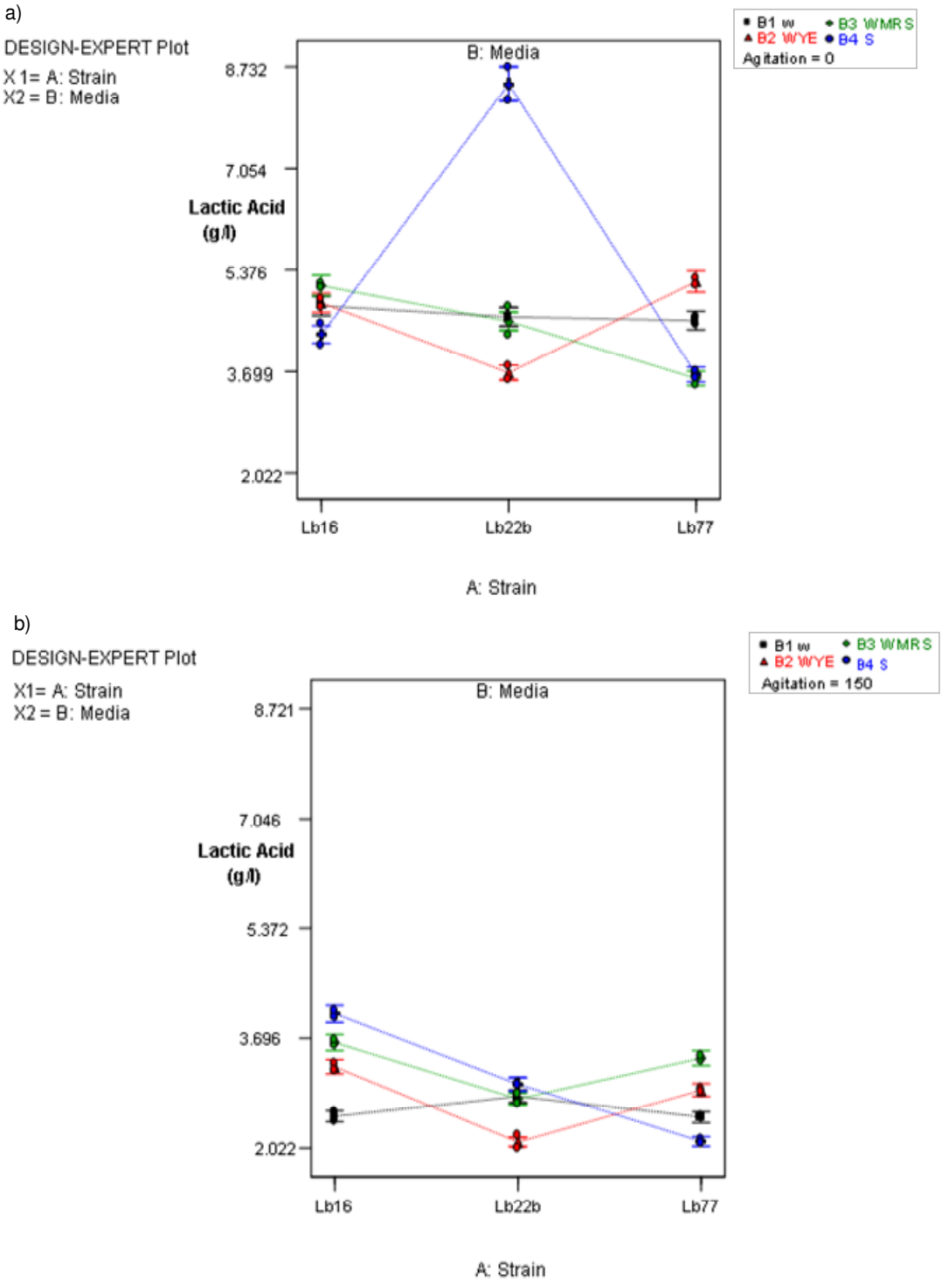
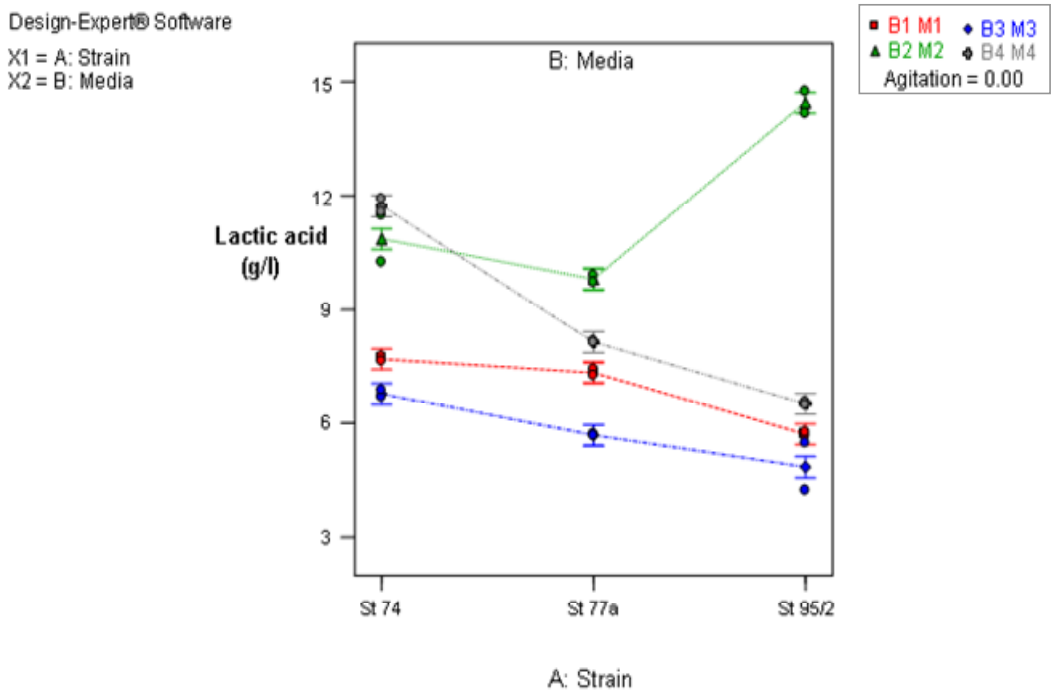


Figure 6.11. Effects of *Lactobacillus delbrueckii subsp. bulgaricus* strain types and different media formulations on lactic acid production; incubated at 43˚ C for 8 hours under a) static conditions and b) at 150 rpm agitation speed

According to results of full factorial design performed for *Streptococcus thermophilus* strains, analysis of variance results showed that all the linear and the interaction terms were significant ($p<0.0001$) on lactic acid formation, except the interaction between media and agitation speed (Table C.4). Even though M2 media formulation promoted lactic acid production for almost all three strains under both static and agitation conditions, M4 promoted 40 % more lactic acid for St 74 specifically under static conditions (Figure 6.12). Besides the preference for more anaerobic conditions, the need for higher whey concentration (7 %) of this strain might have been another parameter responsible in lactic production. However, in the comparison of the strains in M2 media, strain St 95/2 showed the highest potential for lactic acid production almost 24.78 % and 32 % more than St 74 and St 77a, respectively under static conditions. However increasing the agitation speed to 150 rpm reduced the lactic acid formation by 9.3%, 22 % and 22.5 % for St 95/2, St 77a and St 74, respectively, in M2 media (Figure 6.12). As a result, it can be deduced that strain St 95/2 in M2 media with preference for static environment is the potential strain for maximum lactic acid production. In a study performed by Nancib et al. (1999) on the production of lactic acid by *Streptococcus thermophilus,* they determined a maximum lactic acid amount of 2.36 mg/ml using date products, where our results obtained in this study was way above these. Similarly, comparing the current results with a study conducted by Vasiljevic and Jelen (2001) in the investigation of the production of β-galactosidase for lactose hydrolysis in milk and dairy products using lactic acid bacteria, our results were almost 10 to 15 times higher with respect to lactic acid production. Similarly our findings were in good correlation with a study conducted by Büyükkileci and Harsa (2004) and Altıok et al., (2006) who investigated the lactic acid formation characteristics of *Lactobacillus caseii* (NRLL B 441). Clearly, these findings demonstrate that, the strain St 95/2 can be considered as a potential candidate for lactic acid production with high commercial value.

a)



b)

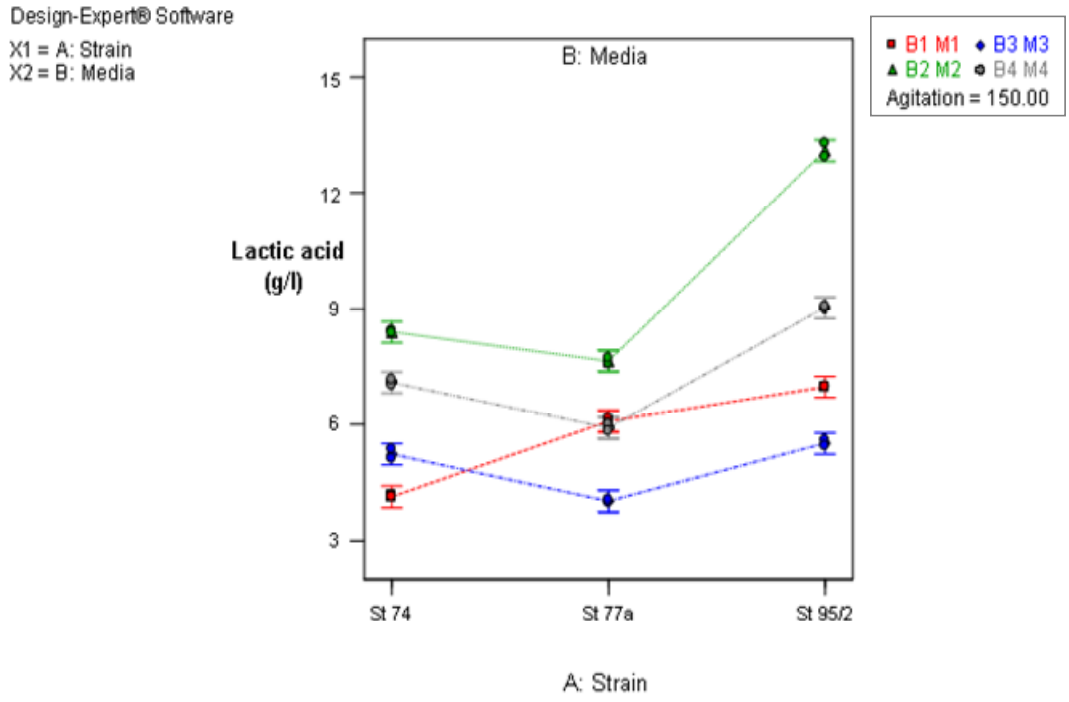


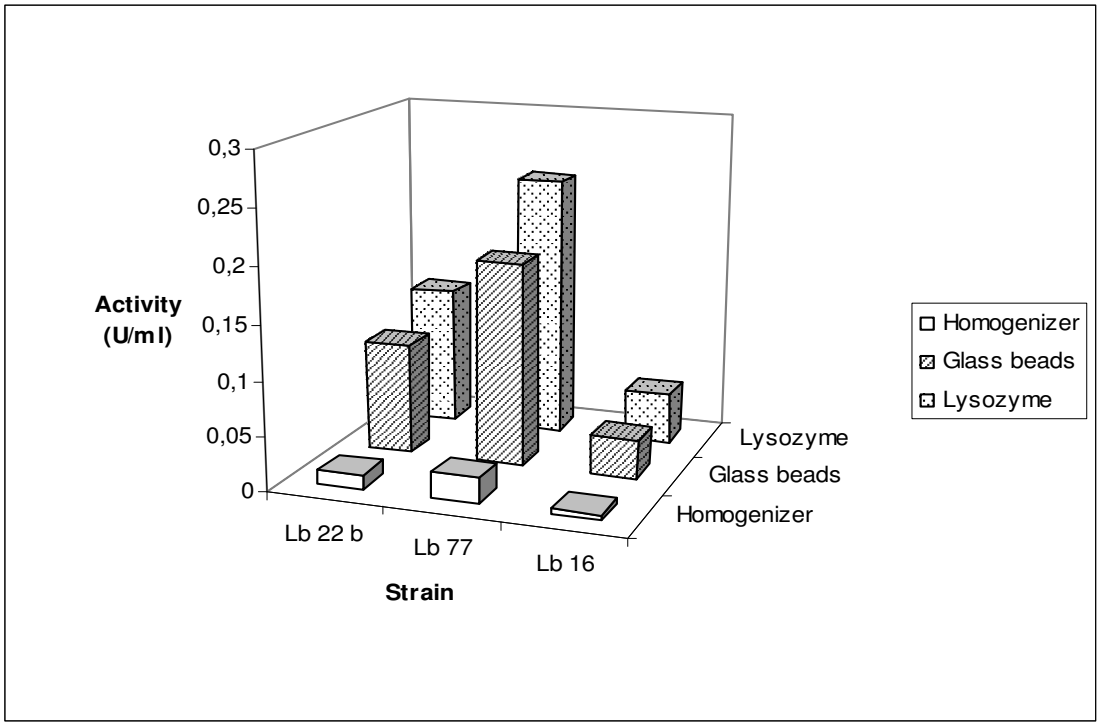
Figure 6.12. Effects of *Streptococcus thermophilus* strain types and different media formulations on lactic acid production, incubated at 43° C for 8 hours under a) static conditions and b) at 150 rpm agitation speed

6.1.2.4. Comparison of Cell Disruption Method

The disruption of cells is the first and important stage in the extraction and preparation of intracellular products. In this study, three different β-galactosidase extraction methods such as cell disruptions using lysozyme enzyme, glass beads and homogenizer were compared. Lysozyme treatment was used in enzymatic lyses. It is mainly based on the digestion of the peptidoglycan layer of the bacterial cell wall. Enzymatic cell lyses can be carried out on any scale but it is not suitable for large-scale preparations. Two methods that are currently used for large scale cell disruption are high speed bead mill and high pressure homogenizer (Bury et al. 2001). Most of the studies are focused mainly in the comparison of sonication, chemical and mechanical treatment (Geciova et al. 2000, Bury et al., 2001). Therefore this study will be one of the limited studies highlighting a comparison of mechanic methods and enzymatic treatment. In this study the highest β-galactosidase enzyme activity was achieved when enzyme extraction was performed by using lysozyme enzyme for both *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* strains (Figure 6.13 and 6.14).

This treatment resulted into 19.07, 10.07 and 9.77 times more β-galactosidase activity than homogenizer and 2.5, 1.3 and 1.29 times more β-galactosidase activity than glass bead extraction for Lb 22b, Lb 77 and Lb 16 respectively. In addition, the ratio of lysozyme treatment over glass bead was determined as 1.32, 1.67 and 1.38 with respect to total enzyme activity for St 95/2, St 74 and St 77a, respectively. Under the same conditions enzyme extraction by glass beads resulted into higher enzyme activity compared to the extraction done by using the homogenizer. The lowest activity was obtained in the extraction method using the homogenizer, almost 10 times less than lysozyme treatment. This could be explained by the insufficient cooling during homogenization process which was accounted due to heat generation of hydraulic and mechanic shear.

a)



b)

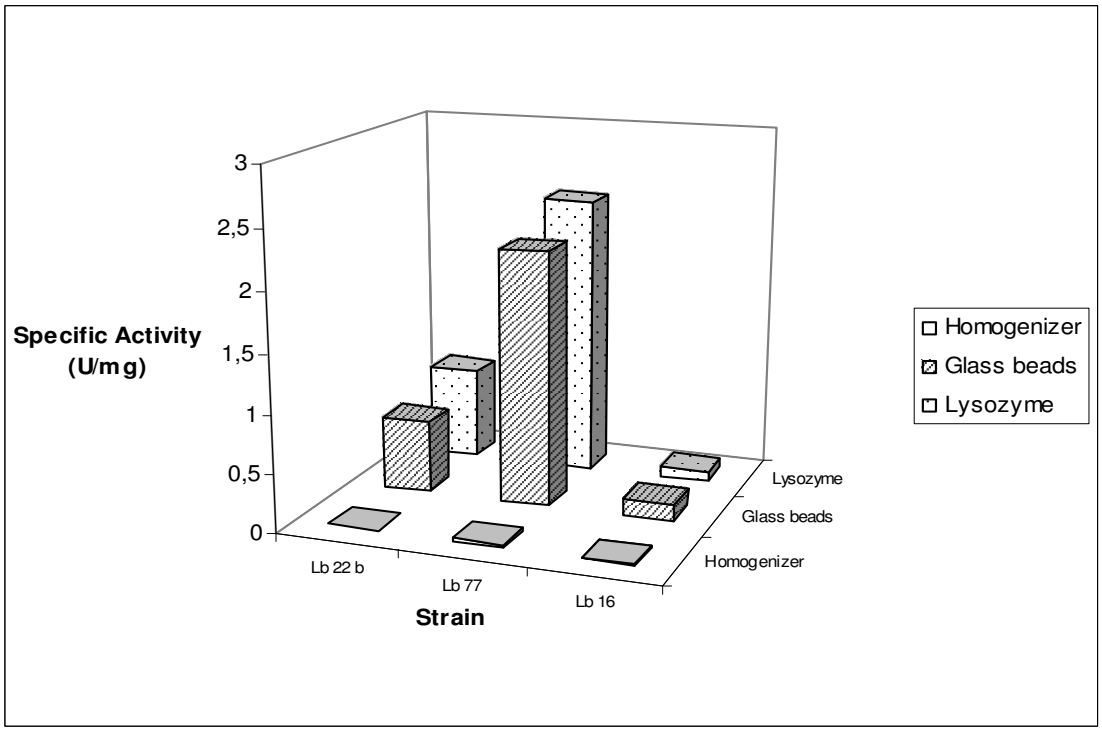
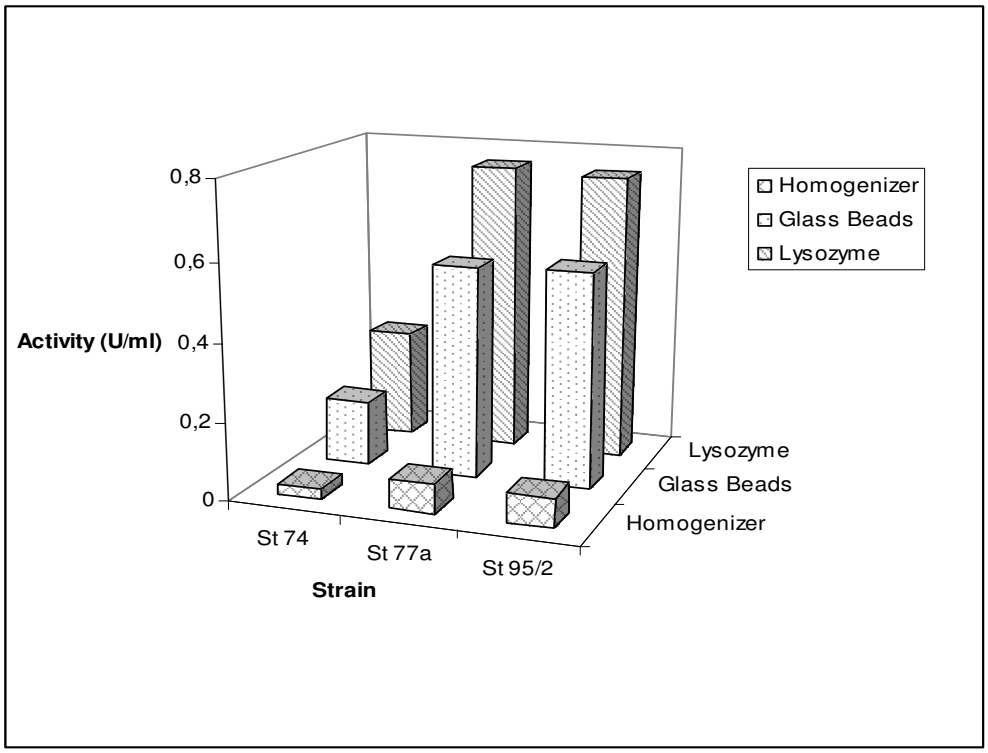


Figure 6.13. Comparison of the extraction methods for *Lactobacillus delbrueckii subsp. bulgaricus* species: (Skim milk was used as cultivation media under static conditions at 43 ˚C incubated for 8 hours) a) activity (U/ml), b) specific activity (U/mg protein)

a)



b)

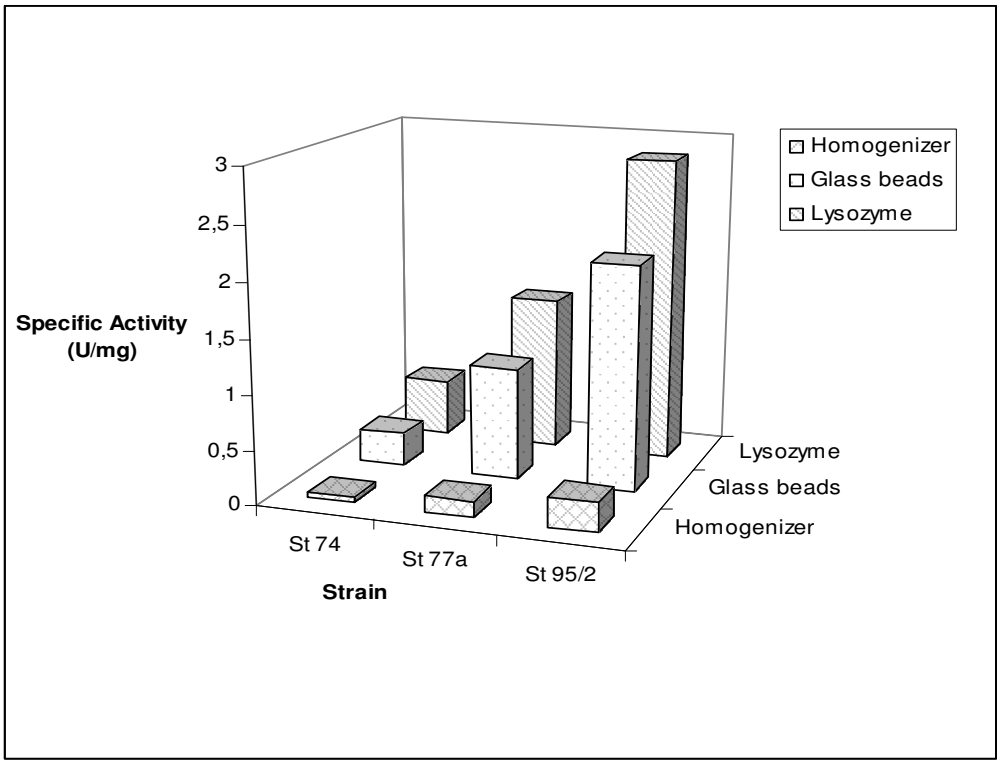


Figure 6.14. Comparison of the extraction methods for *Streptococcus thermophilus* species: (Media 2 was used as cultivation media under static conditions at 43 ºC incubated for 8 hours) a) activity (U/ml), b) specific activity (U/mg protein)

The overall comparison of specific activities (U/mg protein) using the above mentioned methods is presented in figure 6.13 and figure 6.14. In fact specific activity is an indirect indication of the purity of the enzyme and it is desired to be as high as possible. From these figures it is observed that similar to the activity data; highest specific activity was obtained with lysozyme enzyme treatment as well. Similarly, this treatment resulted into 51, 80 and 12 times more specific β-galactosidase activity than the homogenizer and 1.3, 1.1 and 0.6 times more specific β-galactosidase activity than glass bead extraction for Lb 22b, Lb 77 and Lb 16, respectively. These results clearly show that the lysozyme enzyme treatment is more effective in extracting β-galactosidase with higher purity and less side activities compared to the other two methods and this is more pronounced in strain Lb 77 followed by strains Lb 22b and Lb 16.

Considering specific activities, similar conclusion as above were withdrawn for *Streptococcus thermophilus* species as well. In the comparison of the effectiveness of each method on specific activities, it was determined that lysozyme treatment was 5.39 and 1.98 times more effective on St 95/2 than on St 77a and St 74, respectively. Similarly, these values were 6.62 and 2.01 times considering glass bead and 5.3 and 1.89 times considering homogenization treatments. The differences accounted could be related to the different cell wall structure of different strains. Overall, it was deduced that glass bead cell disruption method could be an alternative to lysozyme treatment where economics of the process would be a concern. Geciova et al. (2002) also determined in a study that glass bead treatment was more effective than homogenization treatment in extracting galactosidase by *Streptococcus thermophilus* whereas, Bury et al. (2001) determined that they were equally effective. With this respect our results confirmed the findings of Geciova et al. (2002). Of course one should not overlook that many parameters such as pH, temperature, age of the culture, shape of cells, structure of cell wall and cultivation medium play a significant role on the disruption characteristics of each method and that comparisons should be made under similar conditions (Geciova et al. 2002). In literature the focus is given mainly to Lactobacillus strains but rarely to *Streptococcus thermophilus* which are also known with their high potential for β-galactosidase production.

6.1.3. Central Composite Design

6.1.3.1. Optimisation of Fermentation Parameters for *Lactobacillus delbrueckii subsp. bulgaricus* 77

Twenty experiments were performed according to face-centered central composite design in order to determine the effects of inoculum, temperature and skim milk concentration on the responses (biomass, β-galactosidase activity, and lactic acid) using strain Lb 77. The respective low and high levels with the coded levels in parenthesis for the factors were defined as 35(-1) and 43(1) °C for temperature, 2 %(-1) and 4 %(1) (v/v) for inoculum and 8 %(-1) and 16 %(1) (w/v) for skim milk concentration. The experimental runs with response variables are presented in Table D.1. The results of ANOVA for all three responses (β-galactosidase activity, lactic acid and biomass) revealed that the individual models were highly significant. The coefficients of models in coded units and their significance levels are presented in (Table D.5). The coefficient determinations were 0.9895, 0.9006, 0.9689 for enzyme, lactic acid and biomass, respectively, indicating a good agreement between experimental and predicted values which can describe up to 98.95%, 95.90% and 96.89% variability of the responses. "The predicted R^2" of 0.8510, 0.8075 and 0.7605 for each of the responses (in the same order as above) were in reasonable agreements with "Adj. R^2"of 0.9800, 0.8820 and 0.9409.

According to the central composite design (Table D.1) and response surface graphics (Figure 6.15), maximum β-galactosidase activity, biomass and lactic acid could be attained at higher temperatures and inoculum ratios at a skim milk concentration of 8-8.5%. Therefore, the optimum conditions predicted by the software for maximum β-galactosidase activity (1.36 U/ml), biomass (69.03g/l) and lactic acid (13.06 g/l) were to use a temperature of 43 ºC, at an inoculum concentration of 4.0 % and skim milk concentration of 8.0% (Table F.6).

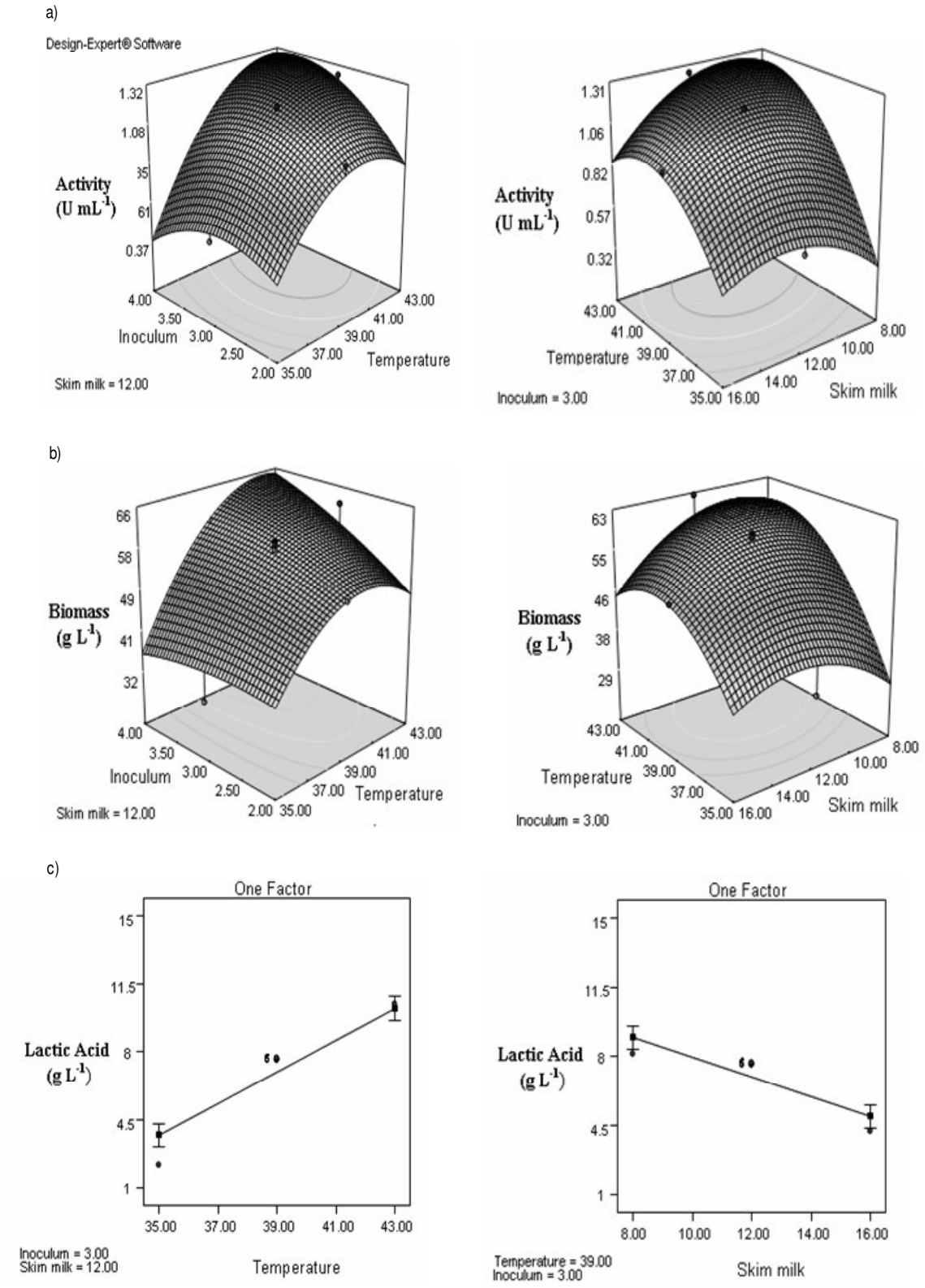


Figure 6.15. Results of optimisation study performed for Lb77: Response surface plots showing the effects of temperature, inoculum and skim milk concentration on a) β-galactosidase activity, b) biomass c) lactic acid

6.1.3.2. Optimisation of Fermentation Parameters for *Streptococcus thermophilus* 95/2

In order to investigate the effects of temperature, inoculum ratio, whey and CSL concentration on the responses (biomass, β-galactosidase activity, and lactic acid), a face-centered central composite design with 30 experiments was performed using strain St 95/2. The respective low and high levels with the coded levels in parenthesis for the factors were defined as 35(-1) and 45 (1) °C for temperature, 2% (-1) and 4% (1) (v/v) for inoculum, 0% (-1) and 5% (1) for whey and 0(-1) and 5(1) for corn steep liquor (CSL) concentration. In order to get normally distributed data, natural logarithm transformations were applied to β-galactosidase activity and biomass results, and square root transformation was applied to lactic acid results. The experimental design and the results are presented in Table E.1. The results of ANOVA for all three responses (β-galactosidase activity, lactic acid and biomass) revealed that the individual models were highly significant. The coefficients of models in coded units and their significance levels are presented in Table E.5. The coefficient determinations were 0.9499, 0.9848 and 0.9550 for enzyme, lactic acid and biomass, respectively, indicating a good agreement between experimental and predicted values which can describe up to 94.99%, 98.48% and 95.50% variability of the responses. "The predicted $R^{2"}$ of 0.7870, 0.9134 and 0.7751 for each of the responses were in reasonable agreements with "Adj. R^2" of 0.9032, 0.9707 and 0.9130.

From the response surface graphics (Figure 6.16), maximum β-galactosidase activity could be attained at higher temperatures and inoculum ratios with whey and CSL concentrations of 2.5%. However, maximum lactic acid and biomass production could be obtained at higher temperatures and inoculum ratios with maximum whey and CSL concentrations (5%) (Figure 6.16) Based on these, optimum conditions for maximum β-galactosidase (2.03 U/ml), biomass (21.86 g/l) and lactic acid (22.76 g/l) production were defined as a temperature of 44 ºC with an inoculum ratio of 3.87% at whey and CSL concentrations of 5% and 4.16%, respectively (Table F.6).

Comparing the two optimisation studies conducted with the two strains, it was observed that St 95/2 produced 1.50 times more β-galactosidase and 1.76 times more lactic acid than Lb 77 at optimum conditions.

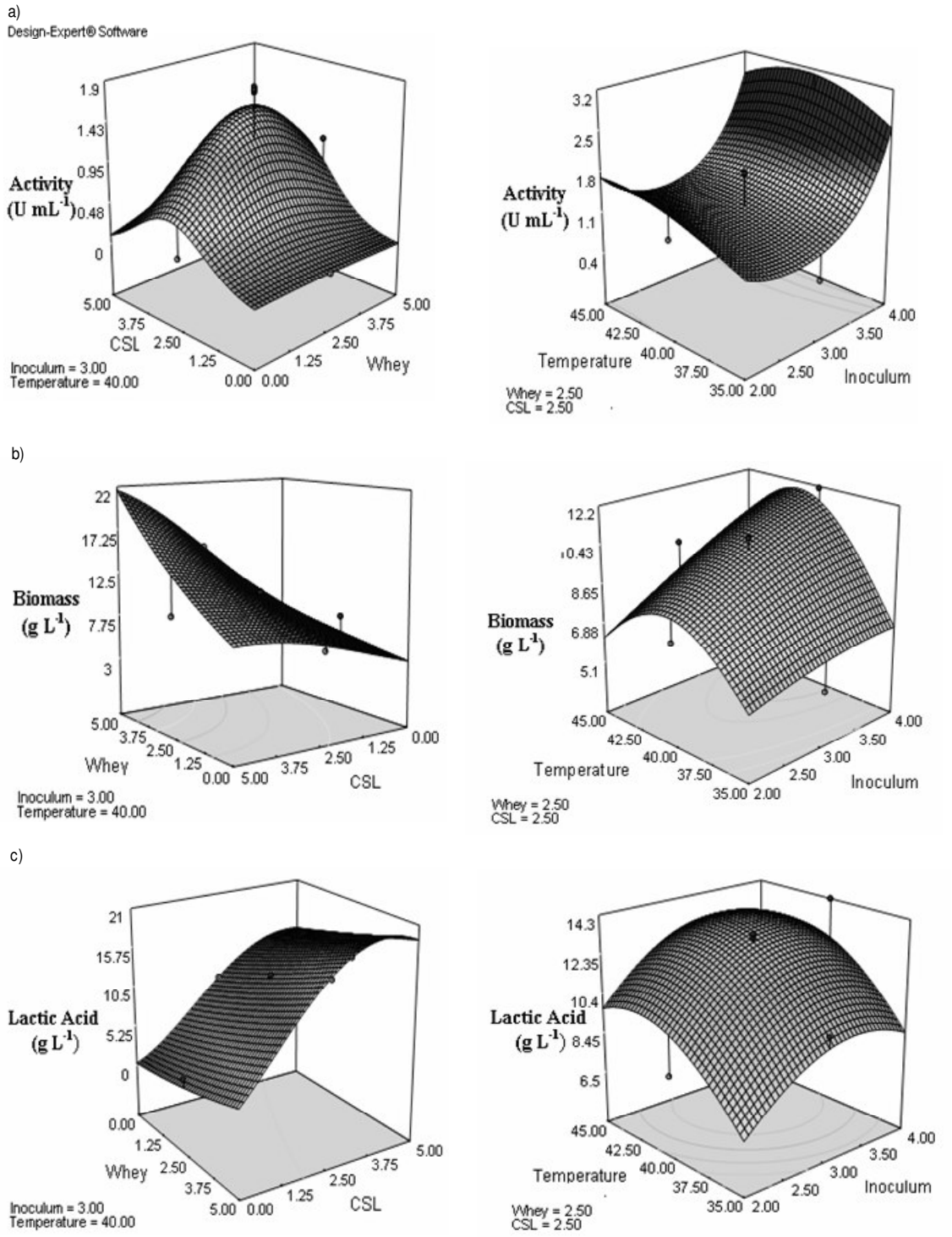


Figure 6.16. Results of optimisation study performed for St 95/2: Response surface plots showing the effects of temperature, inoculum, whey and CSL concentration on a) β-galactosidase activity, b) biomass c) lactic acid

6.1.3.3. Optimisation of Fermentation Parameters for Symbiotic Relationship of *Lactobacillus delbrueckii* subsp. *bulgaricus* 77 and *Streptococcus thermophilus* 95/2

As it is known, some strains grow better when they grow together. Therefore, symbiotic relationship of *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* is important in the yogurt production. These two organisms interact synergistically in the manufacture of yogurt. This synergistic interaction is based on the fact that *Streptococcus thermophilus* grows more rapidly than *Lactobacillus delbrueckii ssp. bulgaricus* in milk and ferments lactose to give lactic acid as the main product. Moreover, carbon dioxide is released by the breakdown of urea in the milk by urease and usually formic acid is produced. All these three metabolites are responsible in stimulating the growth of *Lactobacillus delbrueckii ssp. bulgaricus.* In contrast, *Lactobacillus delbrueckii ssp. bulgaricus* hydrolyzes casein through the cell wall bound proteinase to release free amino acids which are essential for further development of both species. As a result of this synergistic interaction, both species grow rapidly and ferment milk to yogurt (Tamime 2006).

In the literature, the effect of synergistic interaction is generally investigated in rheological and microbiological properties of dairy products using starter cultures (Kristo et al. 2003a, 2003b). There is no available literature to best of our knowledge that focuses on the effect of symbiotic relationship of these cultures on enzyme synthesis. Therefore, based on the results obtained from the optimisation studies described above, third optimisation study was performed to investigate the effect of symbiotic relationship of Lb 77 and St 95/2 on the response parameters. Therefore, effects of inoculum ratio of St 95/2, inoculum ratio of Lb 77 and media formulations were investigated on the responses of lactic acid, biomass and β-galactosidase activity by using central composite design. A face-centered central composite design with 26 experiments was performed. The respective low and high levels with the coded levels in parenthesis for the factors were defined as 1 %(-1) and 3 % (1) for inoculum ratio of Lb 77, 1% (-1) and 3% (1) for inoculum ratio of St 95/2. Two type of media formulations obtained at previous optimisation study described above were used (Media A and Media B). The experimental runs with response variables (β-galactosidase activity, lactic acid and biomass) are presented in Table F.1. The results of ANOVA for all three responses

(β-galactosidase activity, lactic acid and biomass) revealed that the individual models were highly significant. The coefficient determinations were 0.9768, 0.9607, 0.9348 for enzyme, lactic acid and biomass, respectively, indicating a good agreement between experimental and predicted values which can describe up to 97.68%, 96.07% and 93.48% variability of the responses. "The predicted R^2" of 0.9162, 0.8074 and 0.8568 for each of the responses (in the same order as above) were in reasonable agreements with "*Adj.* R^2"of 0.9658, 0.9422 and 0.9142. The coefficients of models in coded units and their significance levels are presented in Table F.5. The model equations for β-galactosidase enzyme ($Y_{\beta\text{-gal}}$), lactic acid (Y_L) and ln (biomass) (Y_b) with the coefficients in coded units of factors are given below:

$$Y_{\beta\text{-gal}}=1.22+0.14X_1+0.23X_2+0.45X_3+0.10X_1X_2+0.057X_1X_3+0.110X_2X_3-0.110X_1^2-0.017X_2^2$$

$$Y_L=14.95+0.91X_1+1.82X_2+6.22X_3+0.40X_1X_2-0.310X_1X_3+0.38X_2X_3-1.28X_1^2-0.94X_2^2$$

$$Y_b= 3.890+0.140X_1+0.2X_2-0.25X_3+0.098X_1X_2-0.017X_1X_3-0.022X_2X_3$$

From the response surface graphs (Figure 6.17), maximum β-galactosidase activity and lactic acid formation was attained in Media B with an inoculation ratio of 2:3 (St 95/2: Lb 77) whereas maximum biomass was achievable in Media A, with an inoculation ratio of 3:3 (St 95/2: Lb 77). Therefore the optimum conditions determined were such as, to use these two cultures together in a ratio of 2.6:3 (St 95/2:Lb 77) in Media B containing whey (5%), corn steep liquor (4%), potassium phosphate (2%) and peptone (2%) at 43 °C for 8 hours for maximum β-galactosidase (2.16 U/ml) and lactic acid (22.52 g/l) production. Under these circumstances maximum biomass achieved was around 52.82 g/l (Table F.3). Since the optimum condition for maximum biomass production in Media A resulted in lower enzyme activity and lactic acid formation, this condition was not considered in the validation experiments.

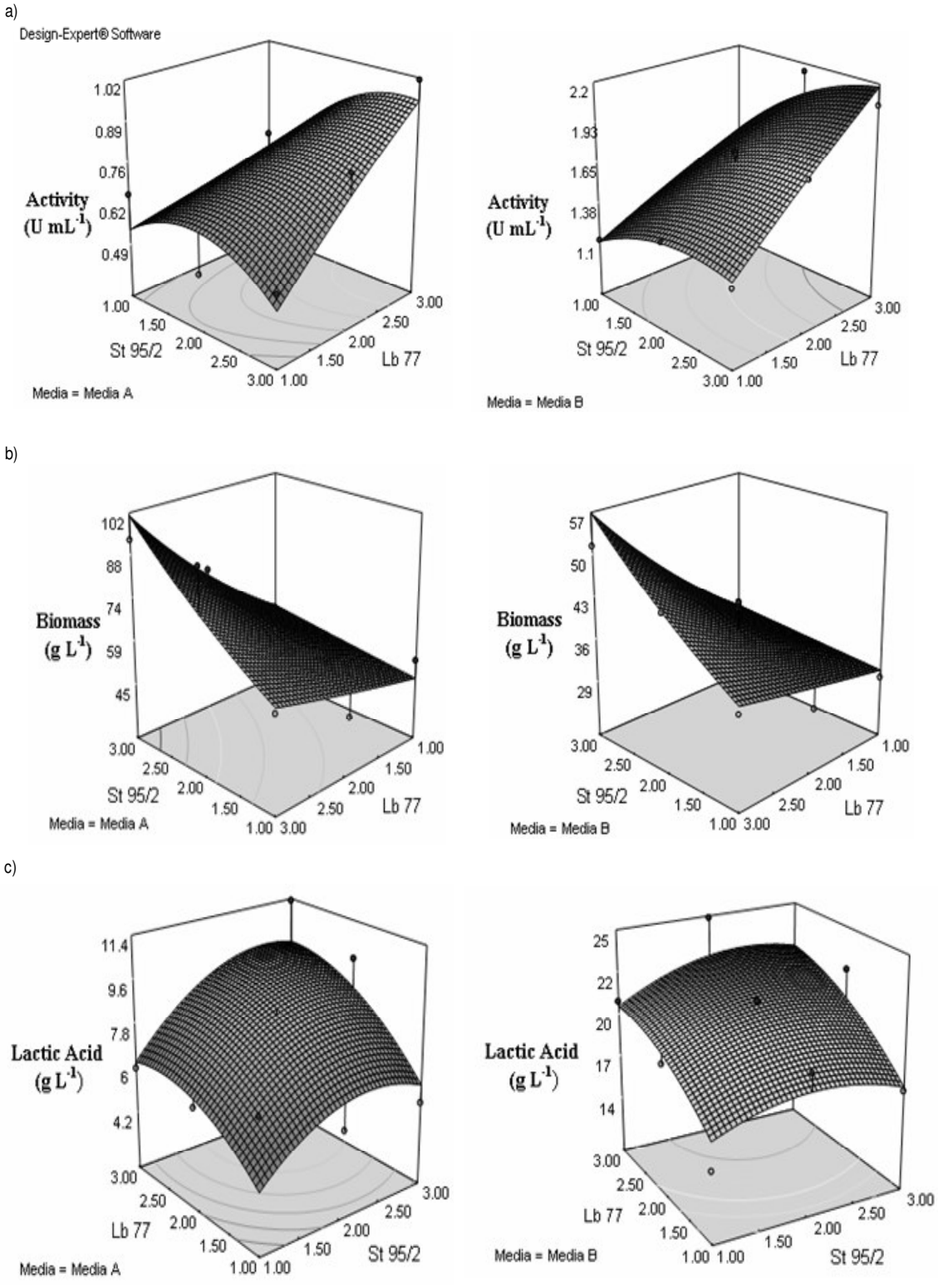


Figure 6.17. Results of optimisation study performed for Lb 77 and St 95/2: Response surface plots showing the effects of St 95/2 and Lb 77 on in Media A and B a) β-galactosidase activity, b) biomass, c) lactic acid

In the optimisation studies, significant lack of fit value was observed (Table D.2, Table D.3, Table D.4, Table E.2, Table E.3, Table E.4, Table F.2, Table F.3, and Table F.4). Significant lack of fit means that the variation of the replicates about their mean values is less than the variation of the design points about their predicted values. If the replicates were actually run more like repeated measurements, it is like that pure error has been underestimated (making lack of fit denominator artificially small). In this case, the lack of fit statistic is no longer a valid test. Hence, decisions about using model will have to be made based on other statistical criteria (WEB_1 2004). In these optimisation studies center points had less variation than the model points. Therefore it caused significant lack of fit value. In order to check the adequacy of the model confirmation runs were performed. Thus, total of three verification experiments that were repeated four times were carried out at the predicted optimum conditions for each optimisation. Results of these experiments are given in Table F.6. As it is seen from the results, there was a good correlation between the experimental and predicted values.

Overall, symbiotic relationship of these cultures provided 39% more β-galactosidase activity and 44 % more lactic acid compared to the results obtained by using Lb 77 only (these numbers are based on the average actual validation experiments). Similarly, this relationship provided 6.1% more β-galactosidase activity and 8.73% more lactic acid when St 95/2 was used alone under the defined optimum conditions obtained in section 6.1.3.2. Furthermore, the symbiotic relationship provided 32% less biomass 35.5% more lactic acid and 29.45% more enzyme yield, compared to the use of pure St 95/2 strain (Table F.6).Moreover, this relationship resulted into 65.7% more biomass, 19.46% more lactic acid and 17.13% more the enzyme yield compared when Lb 77 was used as a pure strain. The productivities (Table 6.1) under these circumstances were such as, symbiotic relationship provided 44.44% and 8.7% more lactic acid, 39.32% and 6.4% more β-galactosidase enzyme than Lb 77 and St 95/2, respectively. In large scales these percentages can be considered as significant improvements.

Table 6.1. Yield coefficients and productivities for optimisations studies at optimum conditions

	Yield			Productivity	
	Yx/s	**Yp1/s**	**Yp2/s**	**Activity U/ml.h**	**Lactic Acid g/l.h**
Optimisation of Lb 77	2.357 ± 0.196	0.4599 ± 0.028	46.976 ± 3.18	0.162 ± 0.0033	1.593 ± 0.058
Optimisation of St 95/2	0.542 ± 0.027	0.575 ± 0.035	55.18 ± 2.08	0.250 ± 0.0026	2.617 ± 0.088
Optimisation of Lb 77 & St 95/2	1.582 ± 0.06	0.714 ± 0.019	66.59 ± 0.59	0.267 ±0.0032	2.867 ± 0.089

Yx/s= Biomass yield coefficient (g biomass/g lactose); Yp1/s= Lactic acid yield coefficient (g lactic acid/g lactose);

Yp2/s= Activity yield coefficient (U activity/g lactose); Values are based on the mean ± SD of four validation experiments shown in Table F.3

In order to observe the contribution of each strain (considering the fraction of viable cell counts of individual strains in the bulk mixture) to the response parameters, the scatter plots between the output variables were generated using the Minitab software version 14 (Minitab Inc., Pennsylvania, US). As it can be observed 70-80 % of strain St 95/2 and 20-30 % of strain Lb 77 contributed to biomass, lactic acid and β-galactosidase activity (Figure 6.18).

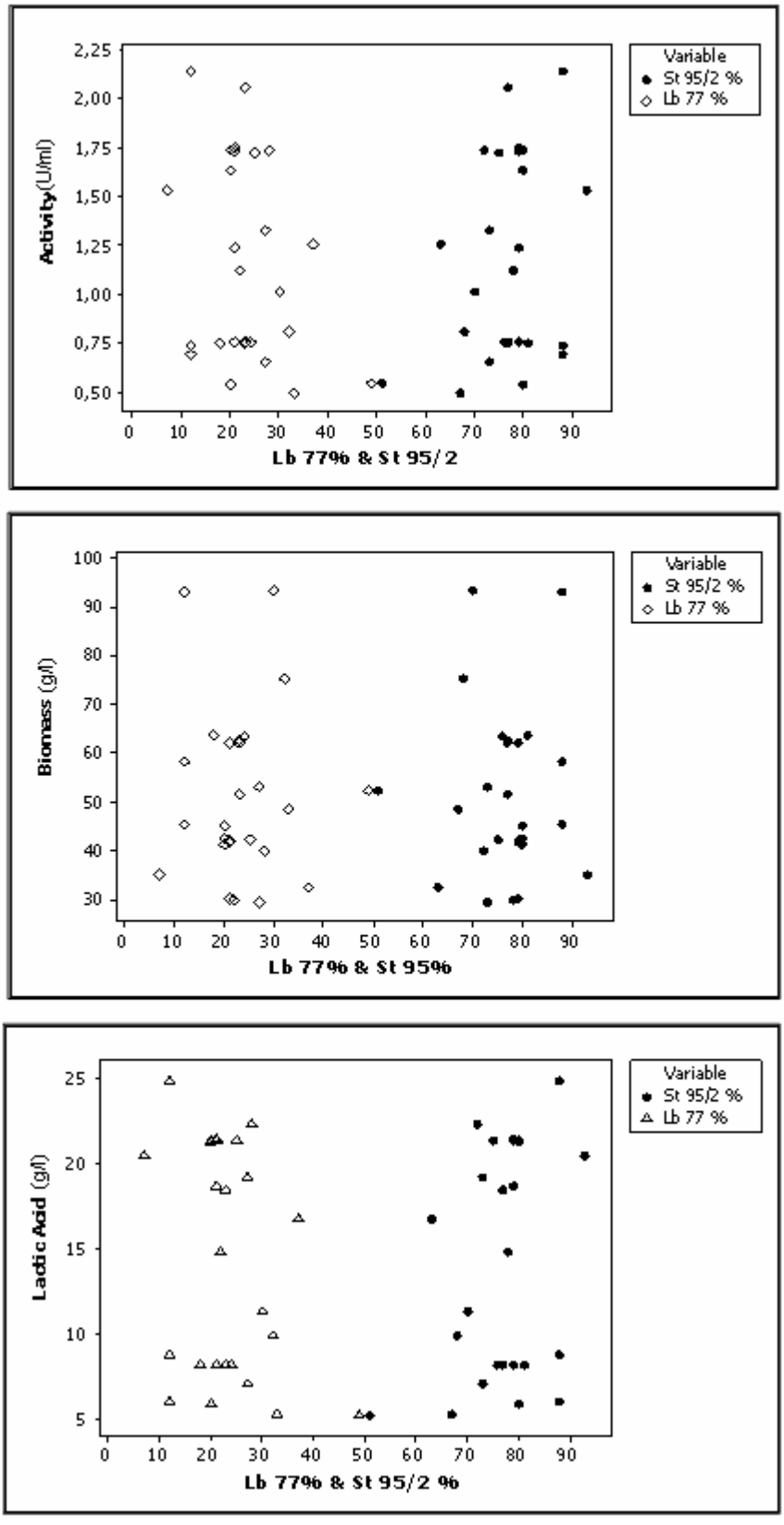


Figure 6.18. Scatter plots presenting the percent contribution Lb 77 and St 95/2 on β-galactosidase activity, biomass and lactic acid formation.

This issue was also partly confirmed by the individual and symbiotic specific growth rates of the strains under the optimum conditions. In fact, the specific growth rates of pure Lb 77 and St 95/2 under optimized conditions (first and second optimisation) were 0.44 and 0.717 h^{-1}, respectively. However the symbiotic relation under the symbiotic optimisation condition, increased the specific growth rates for both strains by 28% to 0.62 $h^{-1}$and 1.00 h^{-1}, respectively. The high biomass formation of Lb 77 (Table D.1) in spite of its lower specific growth rate can be attributed to possible exo-polysaccharide formation accounted in some LAB cultures (Tamime 2006). This might have been partly reflected in the biomass data of symbiotic growth.

This study clearly indicated that it is more efficient to use both strains in combination as opposed to traditional single organism use practiced often in industrial production of lactic acid and β-galactosidase enzyme. One may naturally have the concern on the extraction procedure of the intracellular enzyme of this mixed culture. Nevertheless, the extraction of the enzyme from both cultures demanded similar conditions demonstrated in our studies. In fact, these cultures are generally used as mixed cultures in the dairy industry therefore knowing their ratios, will simplify their application as starter culture without going into any strain separation technique. With this respect this study brings a new perspective to common industrial practice and opens up a new window to researchers working in this field.

In the comparison of the current optimisation results with various literature results conducted using pure strains revealed that, our results were considerably higher with respect to β-galactosidase activity (Montanari et al. 2000, Geciova et al. 2002, Gueimonde et al. 2002, Gaudreau et al. 2005). These findings were also in good correlation with a study conducted by Büyükkileci and Harsa (2004) and Altıok et al., (2006) who investigated the lactic acid formation characteristics of *Lactobacillus casei* NRRL B-441.

6.2. Bioreactor Experiment Results

Bioreactor studies were performed according to the results obtained from optimisation studies in shake flasks experiments.

6.2.1. *Lactobacillus delbrueckii subsp. bulgaricus 77*

For the *Lactobacillus delbrueckii subsp. bulgaricus 77* strain, optimized conditions were determined as 43 °C, skim milk concentration of 8% and inoculum ratio of 4 %. Two type of fermentation experiments were performed at these conditions for this strain using Minifors-Infors bioreactor with working volume of 2.5 L.

In the first experiment, pH adjustment was not considered, whereas in the second experiment pH was adjusted to 6.2 ± 0.1 by the automatic addition of 2N NaOH. Fermentation was carried out for 8 hours and samples were taken every 2 hours for the analysis of enzyme activity, lactic acid and lactose amount, biomass and cell count. The results of the experiments are presented in Table 6.2.

Table 6.2. Results of bioreactor experiments performed for Lb 77

Lb 77 (no pH adjustment)					
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.0022	10.25	15.65	0	46.56
2	0.0755	14.65	16.57	0.948	45.963
4	0.6205	40.3	17.82	3.391	40.83
6	0.8791	59.25	18.23	5.199	35.946
8	1.2807	68.65	19.24	8.873	26.958

Lb 77 (pH 6.2 ± 0.1)					
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0,0021	10,32	15,88	0	46,413
2	0,0095	15,19	16,38	0,881	44,785
4	0,3912	34,68	17,32	2,529	37,183
6	0,673	48,27	18,72	4,119	33,748
8	0,807	55,63	19,94	7,676	24,665

As it is seen from Table 6.2, activity was reduced 1.58 times at constant pH of 6.2 at the end of fermentation. Lactic acid and biomass results were also lower when compared to the results obtained at uncontrolled conditions. When the yields are compared at the end of 8 hour, it can be observed that higher values are obtained without pH adjustment. Yield coefficients of biomass, enzyme and lactic acid were determined as 3.50 g biomass/g lactose, 65.34 U activity/g lactose and 0.45 g lactic acid /g lactose, respectively for the condition where pH adjustment was not considered.

However, yield coefficients of biomass, enzyme and lactic acid were determined as 2.56 g biomass/g lactose, 37.11 U activity/g lactose and 0.35 g lactic acid/g lactose, respectively when pH was adjusted to 6.2.

6.2.2. *Streptococcus thermophilus 95/2*

For the *Streptococcus thermophilus 95/2* strain, optimized condition were determined as 44 °C, media including whey (5%), corn steep liquor (4%), peptone (2%) potassium phosphate (2%) and inoculum ratio of 3.9 % from the optimisation studies. Two type experiments as mentioned in section 6.2.1 were performed for St 95/2.

Fermentation was carried out for 8 hours and samples were taken every 2 hours for the analysis of enzyme activity, lactic acid and lactose amount, biomass and cell count. The results of the experiments are presented in Table 6.3

Table 6.3. Results of bioreactor experiments performed for St 95/2

St 95/2 (no pH adjustment)					
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.044	8.17	14.59	0	38.943
2	0.103	10.12	16.11	3.881	36.265
4	0.6918	14.48	17.79	7.674	25.738
6	1.3703	19.87	19.43	12.161	18.273
8	1.897	22.18	20.1	16.939	2.642

St 95/2 (pH 6.2 ± 0.1)					
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.052	7.65	15.64	0	37.14
2	0.163	10.8	16.36	3.881	35.176
4	0.625	12.9	18.38	6.861	26.329
6	1.3403	17.7	19.09	9.757	19.226
8	1.961	22.4	20.23	12.392	4.823

As it can be observed from the Table 6.3, at the end of 8 hours, activity was increased to 3.37 % when pH was adjusted to 6.2. However, lactic acid amount was reduced with pH adjustment. When the yields are compared at the end of fermentation, it can be observed that higher values are obtained with no pH adjustment for biomass and lactic acid production. Yield coefficients of biomass, enzyme and lactic acid were determined as 0.611 g biomass/g lactose, 52.257 U activity /g lactose and 0.466 g lactic acid/g lactose, respectively where pH adjustment was not considered. However, yield

coefficients of biomass, enzyme and lactic acid were determined as 0.693 g biomass/g lactose, 60.68 U activity/g lactose and 0.383 g lactic acid /g lactose, respectively at constant pH of 6.2.

6.2.3. *Lactobacillus delbrueckii* subsp. *bulgaricus 77 and Streptococcus thermophilus 95/2*

The effect of symbiotic relationship between *Lactobacillus delbrueckii subsp. bulgaricus 77* and *Streptococcus thermophilus 95/2* was investigated in shake flask experiments and it was found that using both strains together was more efficient than using single strains. In order to scale up to bioreactor, optimized condition determined for these strains were used. These conditions were determined as 43 °C, media including whey (5%), corn steep liquor (4%), peptone (2%), potassium phosphate (2%) and inoculum ratio of 3 % of Lb 77 and 2.6 % of St 95/2. Three different experiments were performed at these conditions for these two strains using Minifors-Infors bioreactor.

In the first experiment, pH adjustment was not considered, whereas in the second experiment pH was adjusted to 6.2 ± 0.1. These two experiments were performed in batch mode, whereas the third experiment was performed in fed-batch mode with the supplementation of 500 ml whey (5%) at the end of 4h hour. Fermentation was carried out for 8 hours and samples were taken every 2 hours for the analysis of enzyme activity, lactic acid and lactose amount, biomass and cell count. The results of the experiments are presented in Table 6.4

Table 6.4. Results of bioreactor experiments performed for Lb 77 & St 95/2.

Lb 77 & St 95/2 (no pH adjustment-Batch mode)						
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count Lb 77 (ln CFU/ml)**	**Cell count St 95/2 (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.103	10.8	15.7	17.03	0	38.295
2	0.322	12.6	16.21	18.4	4.915	36.024
4	0.788	24.3	18.23	19.81	8.571	24.753
6	1.962	31.8	19.97	21.37	12.697	13.676
8	2.549	48.7	20.71	23.62	19.672	0.6075

Lb 77 & St 95/2 (pH 6.2 ± 0.1-Batch mode)						
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count Lb 77 (ln CFU/ml)**	**Cell count St 95/2 (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.093	9.83	15.84	16.13	0	37.139
2	0.298	11.43	16.86	17.93	4.826	35.084
4	0.704	21.98	18.69	16.21	8.1117	24.771
6	1.987	29.74	19.65	20.83	10.195	15.411
8	2.449	43.65	20.31	22.72	14.472	3.543

Lb 77 & St 95/2 (no pH adjustment-FedBatch mode)						
Time (h)	**Activity (U/ml)**	**Biomass (g/l)**	**Cell count Lb 77 (ln CFU/ml)**	**Cell count St 95/2 (ln CFU/ml)**	**Lactic Acid (g/l)**	**Lactose (g/l)**
0	0.078	9.17	15.54	16.72	0	38.145
2	0.265	10.45	17.06	17.59	4.761	36.063
4	0.804	18.92	18.25	19.42	7.997	25.548
6	2.016	28.73	19.55	20.97	14.603	23.456
8	2.249	45.65	20.1	21.81	18.472	17.543

As it can be seen from Table 6.4, at the end of 8 hours, activity was reduced by 3.92 % when pH was adjusted to 6.2. Lactic acid amount was also reduced by 26% with pH adjustment. When the yields are compared at the end of fermentation, it can be observed that higher values are obtained with pH adjustment for biomass and enzyme production. Yield coefficients of biomass, enzyme and lactic acid were determined as 1.29 g biomass/g lactose, 67.64 U activity/g lactose and 0.52 g lactic acid/g lactose, respectively where pH adjustment was not considered. However, yield coefficients of biomass, enzyme and lactic acid were determined as 1.29 g biomass/g lactose, 72.90 U activity/g lactose and 0.44 g lactic acid /g lactose, respectively when pH was adjusted to 6.2.

On the other hand, when fermentation was performed in fed-batch mode, activity and lactic acid was reduced by 11.76 % and 6.10% respectively.

When the bioreactor experiments results were compared to the results of shake flasks experiments, it can be observed that yields coefficients were in agreement with each other. Furthermore, yield coefficients for enzyme production were increased in the bioreactor experiments.

6.3. Characterization of β-galactosidase

6.3.1. Effect of pH on Activity and Stability of β-galactosidase

As it is known, pH influences the velocity of an enzyme-catalyzed reaction. Therefore, it is important to know the effect of pH on activity and stability of an enzyme.

The effect of pH on β-galactosidase activity is presented in Figure 6.19 for the crude enzyme extracts obtained from Lb 77, St 95/2 and Lb77 & St 95/2 (mixed culture). As it is seen, optimum pH was determined as pH 7 for enzymes obtained from three sources and they also showed 80% activity between the pH 7.0 to 7.5. These findings are in accordance with several earlier literature studies. In a study performed by Greenberg and Mahoney (1982) optimum pH for β-galactosidase from *Streptococcus thermophilus* was found to be 7.0. Same findings were observed in another study performed by Kreft and Jelen (2000).

The pH stability profile at 37 °C is shown in Figure 6.20. The enzyme was essentially stable (85-90%) over the pH range of 7-9. Enzymes from *Lactobacillus bulgaricus* and *Streptococcus thermophilus* would appear to be stable over a wider pH range than *E. coli* β-galactosidase whose stability decreases sharply below pH 6 and more gradually above 8 (Greenberg and Mahaney 1982).

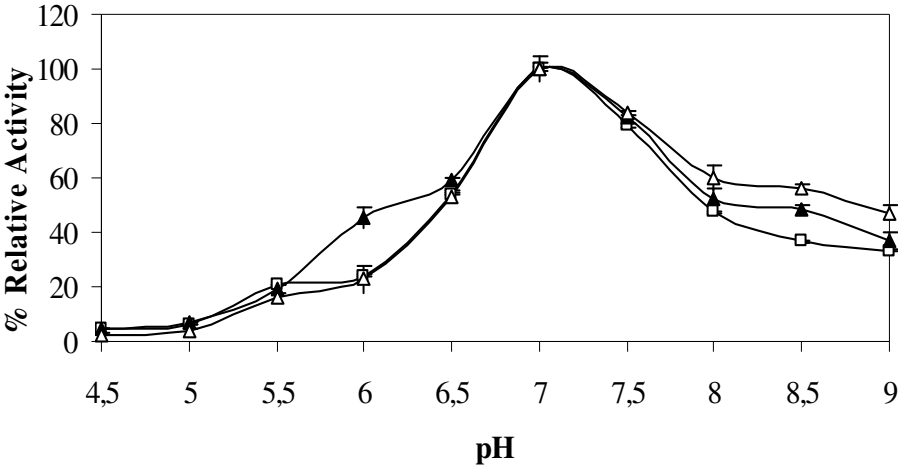


Figure 6.19. Effect of pH on activity of β-galactosidase; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

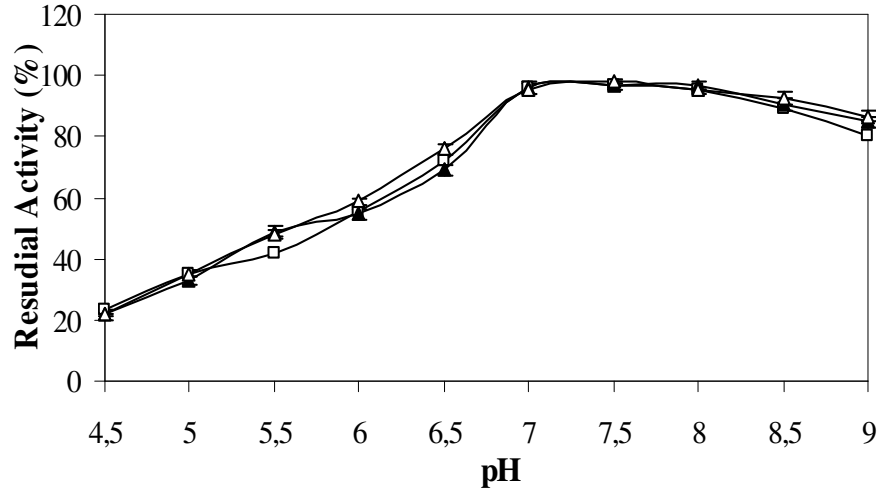


Figure 6.20. Effect of pH on stability of β-galactosidase; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

As it can be deduced from the results, enzymes from these sources seem to be well-suited for hydrolysis of lactose in milk since they show good activity and stability at pH values close to neutral.

6.3.2. Effect of Temperature on Activity and Stability of β-galactosidase

β-galactosidase from Lb 77, St 95/2 and mixed culture (Lb 77 & St 95/2) was found to have an optimum temperature of 45 °C, 50 °C and 50 C°, respectively. These enzymes retained 90-100% of its activity between 45-55ºC. Similar findings were reported in several literature studies. For example, Shah and Jelen 1991 found the optimum temperature of β-galactosidase from *Lactobacillus delbrueckii subsp. bulgaricus* 11842 as 45-50 °C, Similarly, maximum activity for β-galactosidase from *Streptococcus thermophilus* was determined at 55 °C (Greenberg and Mahoney 1982). As it can be observed from the Figure 6.21, enzyme from mixed culture (Lb 77 & St 95/2) shows same pattern with St 95/2.

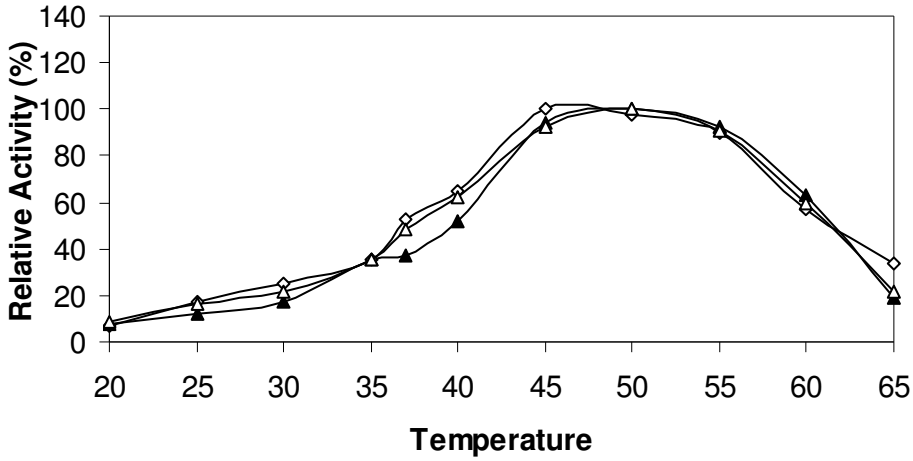


Figure 6.21. Effect of temperature on activity of crude β-galactosidase; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

Thermostability is the ability of an enzyme to resist against thermal unfolding in the absence of substrates (Bhatti et al. 2006). The thermostability of the crude β-galactosidases from all three sources was determined by measuring the residual activity of the enzyme after incubation at various temperatures ranging from 20 to 65ºC for 30 minutes. As it is shown in Figure 6.22, the β-galactosidase enzyme from Lb 77, St 95/2 and Lb 77& St 95/2 was stable at temperature between 20- 37 °C. However, at 40 °C β-galactosidase activity increased 6 %, 9% and 10% above the control, respectively. The activation of enzymes during heating occurs mostly during the initial stages of mild

heating (Yemenicioğlu et al. 1997, Rodriguez-Lopez et al. 1999, Yemenicioğlu, 2002) and is generally attributed to conformational changes in the enzyme active site (Rodriguez - Lopez et al. 1999).

However, after the incubation of the enzymes at 55 °C, β-galactosidase activity was reduced to 54.53 %, 57.25 % and 53.65% for Lb 77, St 95/2 and Lb 77 &St 95/2, respectively and almost all crude extracts lost 95% of its activity at 65 °C.

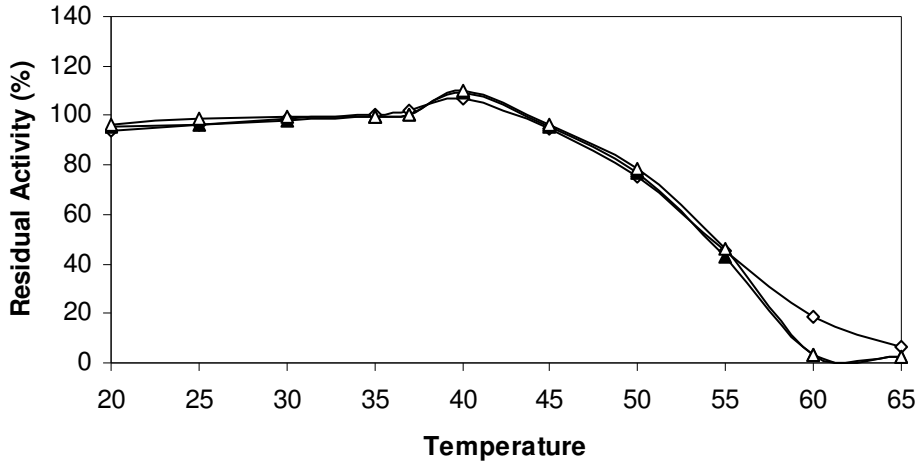


Figure 6.22. Effect of temperature on the stability of crude β-galactosidase; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

6.3.3. Effect of Metal Ions on β-galactosidase Activity

Various cations present in the solution may have effect as an activator or inhibitor during the hydrolysis process. The effects of metal ions were examined by adding the chlorides of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} and sulphates of Cu^{2+} and Fe^{2+} at different concentrations to the buffer solutions. As it can be observed from Table 6.5 Ca^{2+}, Zn^{2+} and Cu^{2+} had an inhibitory effect on the β-galactosidase enzyme obtained from Lb 77, St 95/2 and mixed culture (Lb 77 and St 95/2). Cu^{2+} had strong inhibitory effect on β-galactosidase even at low concentrations. However, Mg^{2+} and Mn^{2+} caused activation in all enzymes. In fact, the effects of these ions were concentration dependent. For example, increase in concentration of Ca^{2+} caused deactivation of the enzyme, whereas increase in the concentration of Mg^{2+} and Mn^{2+} caused activation.

Similarly increasing the concentration of Fe^{2+} from 2.5 mM to 10 mM resulted into, 2.83, 3.52 and 2.96 fold reduction in the β-galactosidase activities of the enzymes obtained from Lb 77, St 95/2 and mixed culture (Lb 77 & St 95/2), respectively.

Table 6.5. Effects of metal ions on the activity of crude β-galactosidase: from Lb 77, St 95/2 and Lb 77 & St 95%2

Strain Lb 77	**% Relative Activity**			
Control	100			
Metal ions	**1mM**	**2.5mM**	**5mM**	**10mM**
K^+	97.55	94.34	96.24	98.4
Na^+	98.4	99.64	91.27	88
Ca^{2+}	55	35	28	15
Mg^{2+}	97.73	104.24	105.35	108.43
Mn^{2+}	100.8	106.25	117.68	122.87
Zn^{2+}	55	40	25	ND
Cu^{2+}	25	18	5	ND
Fe^{2+}	90	85	75	30

Strain St 95/2	**% Relative Activity**			
Control	100			
Metal ions	**1mM**	**2.5mM**	**5mM**	**10mM**
K^+	93.91	99.25	97.69	98
Na^+	98.8	96.72	92.66	90.11
Ca^{2+}	48.18	26	12	10
Mg^{2+}	98.73	101.76	103.51	109.87
Mn^{2+}	101.76	107.9	108.53	130.21
Zn^{2+}	58	45	15	ND
Cu^{2+}	6.63	ND	ND	ND
Fe^{2+}	93	88	77	25

Strains (Lb 77&St 95/2)	**% Relative Activity**			
Control	100			
Metal ions	**1mM**	**2.5mM**	**5mM**	**10mM**
K^+	99.36	97.1	95.77	98.14
Na^+	94.91	93.88	90.19	89.26
Ca^{2+}	64.18	46	27	14
Mg^{2+}	99.39	103.29	107.18	110.93
Mn^{2+}	116.21	134.32	159.78	167.12
Zn^{2+}	56	40	21	ND
Cu^{2+}	5.29	ND	ND	ND
Fe^{2+}	91	83	72	28

In various studies reported in literature different results were obtained depending on the ion used and the source of β-galactosidase. The effect of monovalent cations (K^+ and Na^+) varies with the enzymes from different sources (Jurado et al. 2002). For the enzyme from *Streptococcus thermophilus* and ONPG as a substrate, no effect was found on one case (Somkuti and Steinberg 1979), however slight activation was observed in another (Rao and Dutta 1981). Greenberg and Mahoney found highest activity with K^+ in the presence of Mn^{2+} (Greenberg and Mahoney 1982). Similarly, presence or absence

of Mg^{2+} had shown different effects. In a study performed by Bhowmik et al. (1987) β-galactosidase from *L. acidophilus* was stimulated with Mg^{2+} whereas in another study it had no effect in the case of *L. kefiranofaciens* (Itoh et al. 1992). Similar results were reported in other studies as well (Batra et al. 2002, Chakraborti et al. 2000). These results were partly in agreement with our results.

6.3.4. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

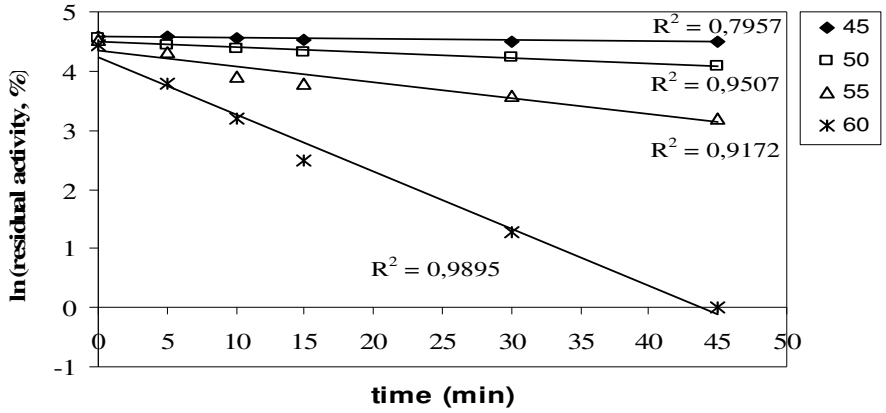
Inactivation is defined as a process where the secondary, tertiary or quaternary structure of a protein changes without breaking covalent bonds (Naidu and Panda 2003). Inactivation rate constants (k_d) of β-galactosidase enzymes obtained from Lb 77, St 95/2 and mixed culture of Lb 77 and St 95/2 which are presented in Table 6.6 at 45, 50, 55 and 60 ºC were calculated from the slope of semi natural logarithmic plot of residual activity versus time (Figure 6.23). Similarly the half-life values were calculated using inactivation rate constants. These values are presented in the same Table 6.6.

Table 6.6. Kinetic parameters for thermal inactivation of β-galactosidase: from *Lb 77, St 95/2* and mixed culture of *Lb 77 & St 95/2*

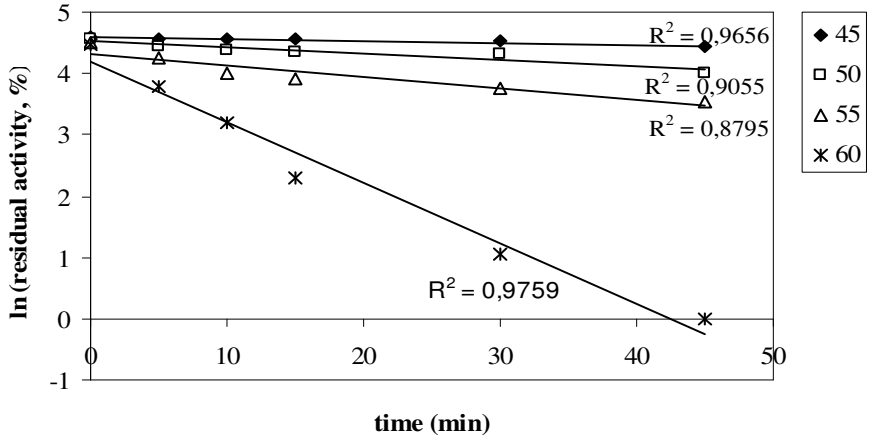
	Kd			$t_{1/2}$ (min)		
T (°C)	**Lb 77**	**St 95/2**	**Lb 77&St 95/2**	**Lb 77**	**St 95/2**	**Lb 77&St 95/2**
45	0.0021	0.0033	0.0025	330.07	210.04	277.25
50	0.0092	0.0104	0.0089	75.34	66.65	77.88
55	0.027	0.0187	0.0194	25.67	37	35.73
60	0.097	0.099	0.0995	7.14	7	6.96

The half life of β-galactosidase enzyme for all three sources at 45 ºC were higher than the half-life values at temperatures of 50, 55 and 60 ºC at pH 7. This issue once again reveals the thermal stability of this enzyme at 45 ºC and its easy inactivation at higher temperatures such as 50, 55 and 60ºC. Comparison of half-life of the enzymes at 45 °C revealed that the enzyme obtained from Lb 77 was more stable than the enzymes obtained from the other sources (St 95/2 and mixed culture.)

a)



b)



c)

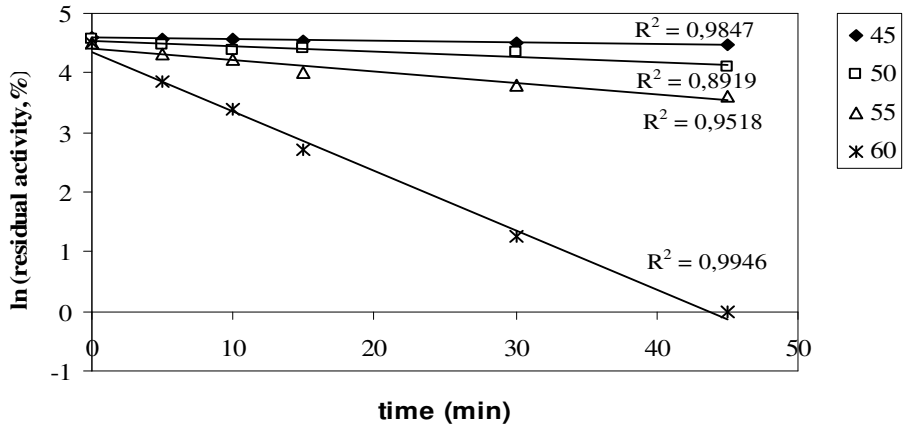


Figure 6.23. First order plots of the thermal denaturation of β-galactosidase; from a) Lb 77 b) St 95/2 c) Lb 77 & St 95/2

Inactivation energy (E_d) of crude β-galactosidase from Lb 77, St 95/2 and mixed culture of Lb 77 and St 95/2 were found to be 51.288, 44.01 and 48.25 kcal mol^{-1}, respectively (Figure 6.24). These values are in the range (40-70 kcal mol^{-1}) estimated for many microbial enzymes (Kargi and Shuler 2002).

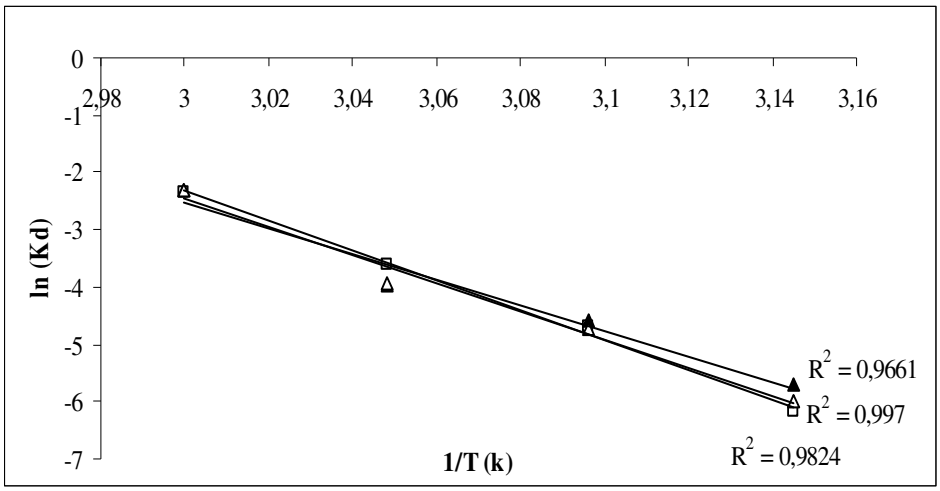


Figure 6.24. Arrhenius plots for the determination of the inactivation energy of β-galactosidase enzymes; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

6.3.5. Estimation of Thermodynamic Parameters

To exploit industrial potentials of β-galactosidase, it is necessary to investigate new microbial strains and to understand the structure-stability relationship of this enzyme. Estimation of thermodynamic parameters helps to understand the probable mechanism of denaturation which is very important to enzymatic processes. The change in enthalpy (ΔH^*) and entropy (ΔS^*) for the thermal inactivation of β-galactosidase enzymes which have been calculated using transition state theory (Bhatti et al. 2006, Ortega et al. 2004) are presented in Table 6.7. A positive ΔH^* and ΔS^* were determined at the temperature ranges studied for all enzymes. As the ΔH^* and ΔS^* are heat and entropy change respectively, these two parameters also provide a measure of the number of non-covalent bonds broken and the net enzyme/solvent disorder change associated with the formation of the transition state (Ortega et al. 2004). As it is seen from the Table 6.7, with the increase of temperature a slight decrease in ΔH^* whereas a remarkable increase in ΔS^* was observed. This also suggests the thermal denaturation of

the enzymes which could be due to disruption of non-covalent linkages including hydrophobic interactions.

It is reported that formation of unfolded enzyme is confirmed by the increase in the entropy or disorder of inactivation. The increase in ΔS^* also indicates an increase in number of protein molecules in transition activated stage, which in turn results in lower values of ΔG^*. A large positive ΔS^* would suggest that the inactivation is highly accompanied by an unfolding of the polypeptide chain into a less highly ordered, more random structure (Segel 1975). ΔS^* values are also known to provide information regarding the degree of solvation and the degree of compactness of protein molecule. Furthermore, positive entropy values suggest that enzyme unfolding might be the rate determining step for the irreversible thermal inactivation of the enzyme. It is reported that the numerical values of ΔH^* and ΔS^* are highly influenced by the factors including the solvent and structural effects (Naidu and Panda 2003).

Table 6.7. Thermodynamic parameter values of thermal deactivation of β-galactosidase enzymes; from Lb 77, St 95/2 and Lb 77 & St 95/2 at different temperatures.

T (^oK)	**ΔH^* (kJ mol^{-1})**			**ΔG^* (kJ mol^{-1})**			**ΔS^*(kJ $mol^{-1}K^{-1}$)**		
	Lb 77	St 95/2	Lb 77 & St 95/2	Lb 77	St 95/2	Lb 77 & St 95/2	Lb 77	St 95/2	Lb 77 & St 95/2
318	213.8	183.096	200.98	105.178	103.98	104.72	0.339	0.248	0.302
323	213.76	183.055	200.94	102.91	102.57	102.99	0.343	0.249	0.303
328	213.72	183.013	200.9	101.605	102.61	102.51	0.341	0.245	0.299
333	213.68	182.971	200.86	99.655	99.599	99.585	0.342	0.250	0.304

As it is seen from the Table 6.7, ΔS^* are close to zero which implied that thermal deactivation did not imply any relevant variation in the enzyme tertiary structure. In other words, most of the hydrogen bonds responsible for the active structure of catalytic site of enzyme were still present in the activated complex. The effect of temperature on the rate of the denaturation can be explained by usual thermodynamic values. The rate of denaturation of these enzymes was slow at low temperature but it increased rapidly with the increase in the temperature. Along with the temperature increase from 45 °C to 55 °C, the half lives of these enzymes were decreased from 330, 210.04, 277.25 to 25.67, 73, and 35.73 minutes for Lb 77, St 95/2 and Lb 77 & St 95/2, respectively. These enzymes were found to be more stable at 45 °C than the other temperatures studied.

6.3.6. Calculation of Kinetic Constants

The kinetic parameters of β-galactosidase from Lb 77, St 95/2 and mixed culture (Lb 77& St 95/2) for hydrolysis towards o-nitrophenyl-β-D galactopyranoside (ONPG) at pH 7 and 37 ºC were obtained by a typical double reciprocal Lineweaver Burk plot (Figure 6.25). The apparent K_m value and V_m value for hydrolyzing ONPG is presented in Table 6.8

Table 6.8. Kinetic constants of β-galactosidase enzymes (Lb 77, St 95/2 and Lb 77 & St 95/2)

	Vmax (µmol/min)	**Km (mM)**
Lb 77	2.461	1.72
St 95/2	2.994	0.981
Lb 77& St 95/2	3.44	3.671

The estimated Km value of the enzyme from St 95/2 is 1.75 and 3.74 times lower than the *Lactobacillus bulgaricus 77* and mixed culture (Lb 77 & St 95/2) β-galactosidase, respectively. This indicates that β-galactosidase from *Streptococcus thermophilus 95/2* has a higher affinity for ONPG compared to the other two. The values for Km for these enzymes are of same order of magnitude for β-galactosidase from other microbial sources. In a study performed by Greenberg and Mahoney (1982), they found a similar K_m value (0.98 mM) by using *Streptococcus thermophilus* strains. In another study about Km value of β-galactosidase from *Lactobacillus thermophilus* was found to be 1.7mM (Greenberg and Mahoney 1982).

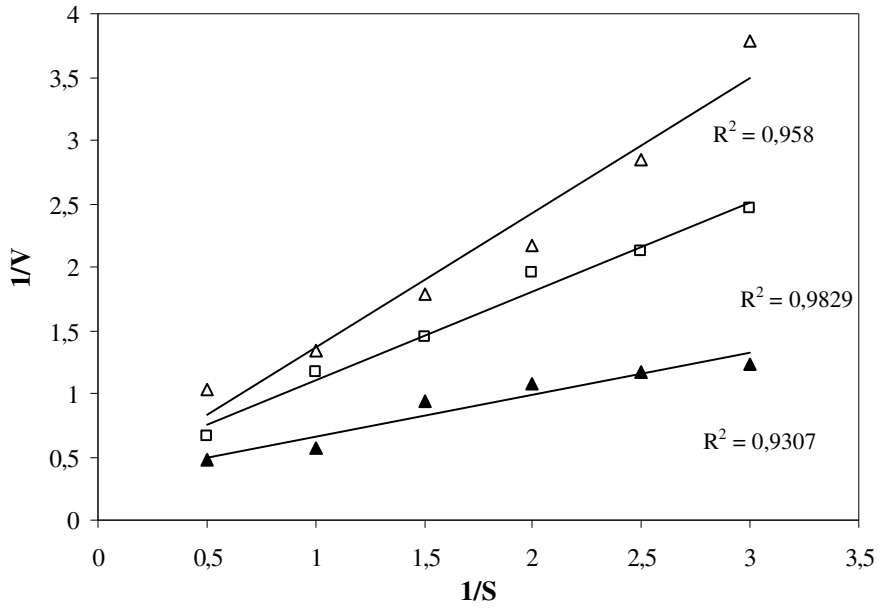


Figure 6.25. Double reciprocal plots to determine the kinetic constants for β-galactosidase enzyme; from Lb 77(□), St 95/2(▲) and Lb 77 & St 95/2 (Δ)

6.3.7. Determination of Molecular Weight

SDS-PAGE profiling (Figure 6.26) revealed that the crude enzyme from Lb 77, St 95/2 and mixed culture of Lb 77 and St 95/2 composed of eight, seven and seven main fractions, respectively. As it can be seen from the Figure 6.26; the sum of subunits gave 535.6, 543.5 and 541.9 kDa for the crude extracts from Lb 77, St 95/2 and Lb 77 & St 95/2 respectively. Since the crude enzymes were not purified it is difficult to say if these bands are different β-galactosidases or other enzymes. This issue remains a task to perform in future studies. It is also not possible to compare these bands with commercial β-galactosidase enzyme as there is not any available commercial enzyme from lactic acid bacteria.

However, evaluation of these crude extracts by SDS-PAGE is important in order to compare the bands whether all extracts show the same pattern or not. As it is seen from the Figure 6.26, crude extract from mixed culture showed the same pattern with the crude extract from St 95/2, whereas two different bands with the molecular weights of 47.3 and 43.3 kDa were obtained from the crude extract of Lb 77.

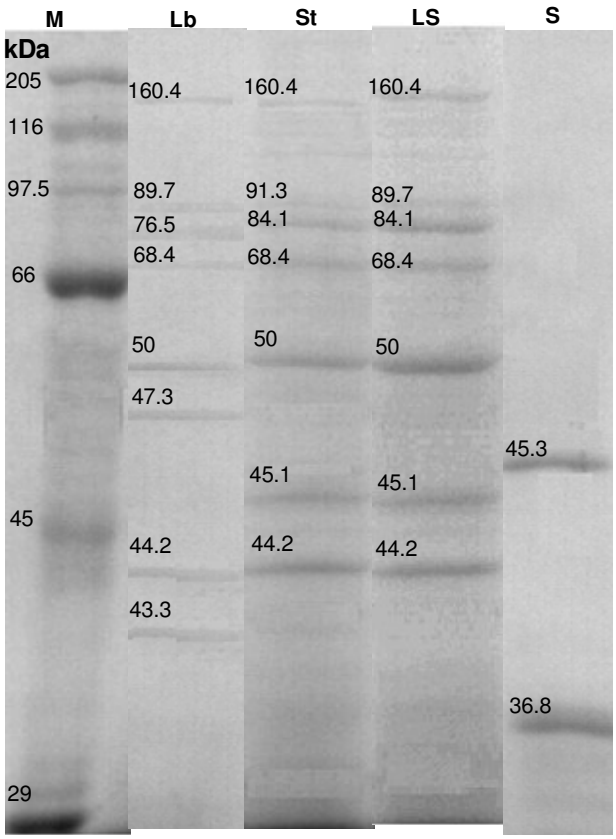


Figure 6.26. SDS-PAGE profiles of crude β-galactosidase extracts: Lane M, marker proteins; lane Lb, β-galactosidase from Lb 77; lane St, β-galactosidase from St 95/2, lane LS, β-galactosidase from Lb 77 & St 95/2, and lane S, commercial β-galactosidase from *Saccharomyces fragilis*

In literature, wide variations in molecular weights of β-galactosidases from microbial sources have been reported. Many β-galactosidases containing numerous subunits with various molecular weights have been described (Berger et al. 1997).

Among β-galactosidases from thermophiles, molecular weights of 150, 240, 440 and 700 kDa were reported by Berger et al. (1997). The presence of isoenzymes has been noted for *Thermoanaerobacter* (Lind et. al 1989), *Bacillus subtilis* (Rahim and Lee 1991), and some *Bifidobacterium species* (Berger et al. 1997). Hirata et al. (1984) described three isoenzymes for *B. stearothermophilus* having molecular weights of 120, 95 and 70 kDa, whereas *Kluyveromyces lactis* contained four isoenzymes with molecular masses of 630, 550, 41 and 19 kDa (Mbuyi et al. 1988). Variations have been encountered with the β-galactosidase from *E.coli*, which is a tetramer composed of four identical subunits (135 kDa) with molecular masses about 540 and 747 kDa for β-galactosidase from *E.coli* ML 308. In a study performed by Chakraborti et al. (2000),

the molecular masses of subunits of β-galactosidase from *Bacillus* sp MTCC 3088 were estimated to be 115, 86.5, 72.5 and 41.2 kDa by SDS-PAGE. In other studies, the molecular weight of β-galactosidase from *Streptococcus thermophilus* was estimated to be 530 kDa by gel-permeation chromatography. β-galactosidases from *Streptococcus lactis* and *Lactobacillus thermophilus* were reported with the a molecular weight of 540 kDa (McFeters et al, 1969).

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, new *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* strains isolated from traditional yogurt samples were introduced and their potentials as β-galactosidase producer were exploited.

Statistical analysis of the full factorial design revealed that factors of strain types, fermentation media formulation and agitation speed and their interactions had significant effect on the β-galactosidase activity, lactic acid and biomass formation. Among the three *Lactobacillus delbrueckii subsp. bulgaricus* strains, strain Lb 77 showed such a potential using skim milk as growth media under static conditions. Similarly, strain St 95/2 and St 77a showed such a potential using media M2 as growth media under static conditions. Besides, strain St 95/2 also resulted in high lactic acid production. Therefore; Lb 77 and St 95/2 was used in the further optimisation studies.

Since β-galactosidase enzyme is an intracellular enzyme, efficiency of different cell disruption methods was also investigated. Among the methods, lysozyme treatment seemed to be the most efficient extraction method followed by glass bead treatment. The efficiency of glass beads which could be increased by further optimisation of process parameters was promising.

As a result of the optimisation studies, optimum conditions for the production of biomass (if the strains should be considered as starter cultures) lactic acid and β-galactosidase of new strains (St 95/2 and Lb 77) isolated from traditional yogurts were determined using response surface methodology. The optimisation study provided the optimum regions of response parameters for pure as well as for mixed strains, which will give the end user the flexibility to produce the product of interest either using single strains or combined strains. In fact the symbiotic relationship of these two strains provided higher product yields and brought a new vision to the enzyme industry which has been lacking so far. Furthermore, new strains with potential lactic acid and β-galactosidase producing capabilities have been introduced.

Characterization experiments indicated that the optimum pH and temperature of crude β-galactosidase extracts from Lb 77, St 95/2 and mixed culture of Lb77 & St 95/2 were 7 and 45-50^{o}C, respectively. This showed that these enzymes are well suited for

the hydrolysis of lactose in milk since they showed good stability at the pH of milk. Besides, the cations in milk are present at levels which would enhance enzyme activity. These enzymes are more heat stable than the β-galactosidase from *Kluyveromyces lactis*. The half-life of β-galactosidase enzyme for all three sources at 45 ºC were higher than the half-life values at temperatures of 50, 55 and 60 ºC at pH 7. This issue once again reveals the thermal stability of this enzyme at 45 ºC. Furthermore the calculated inactivation energies of crude β-galactosidase from Lb 77, St 95/2 and mixed culture of Lb 77 and St 95/2 were found to be 51.288, 44.01 and 48.25 kcal mol^{-1}, respectively.

Characterization studies showed unique thermophilic characteristics of these enzymes that are always in demand by the industry. These strains will not only be new sources for these products but also be new starter cultures with potential application in the dairy industry with possible unique characteristics. As it is known discovering new species with novel characteristics is a tedious task to perform. The mixed preparations of cultures investigated in this study can also be considered as potential probiotics, if they are introduced into the market for lactose intolerant individuals due to their ability to yield high amount of lactose hydrolyzing β-galactosidase enzyme *in vivo*.

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APPENDIX A

CHEMICALS USED

Table A.1. Chemicals used

NO	CHEMICAL	CODE
1	Citric acid	Riedel 33114
2	Sodium phosphate dibasic	Sigma S 7907
3	Sodium chloride	Riedel 31434
4	Hydrochloric acid	Riedel 07102
5	Acrylamide	Sigma A 3553
6	N,N'-Methylene-bis-acrylamide	Sigma M 7279
7	Trizma base	Sigma T 6066
8	Trizma HCL	Sigma T 5941
9	Glycerol	Sigma G 8773
10	Bromphenol blue	Sigma B 8026
11	2-mercaptoethanol	Sigma M 7154
12	Ammonium persulphate	Sigma A 3678
13	Temed	Sigma T 9281
14	Lysozyme	Sigma L 6876
15	Brilliant blue R	Sigma B 7920
16	SDS-protein standards	Sigma SDS 6H
17	Sodium acetate	Merck
18	O-Nitrophenyl B-D-galactopyranoside	Sigma N 1127
19	Acedic Acid	Merck 35710467
20	M17 agar	Merck 15108
21	M17 broth	Merck 15029
22	MRS agar	Fluka 69964
23	MRS broth	Fluka 69966
24	Peptone	Merck 07214
25	Yeast extract	Fluka 70161
26	Corn steep liquor	Sigma C 4648
27	Tryptone	Merck 07213
28	Copper(II) sulfate-5-hydrate	Riedel 12849
29	Sodium carbonate	Riedel 13418
30	Sodium hydroxide	Riedel 06203
31	Magnesium chloride	Fluka 63063
32	Sulfuric acid 95-97 %	Riedel 07208
33	Sodium dodecyl sulphate	Riedel 62862
34	Sodium phosphate monobasic	Sigma S 8282
35	Potassium dihydrogen phosphate	Fluka 60218
36	Potassium phosphate dibasic	Sigma P 5504
37	Protein standard	Sigma P 5619
38	Calcium chloride	Riedel
39	Manganese(II) chloride-4-hydrate	Riedel 12022

Table A.1. Chemicals used (cont.)

40	Zinc chloride	Riedel 31422
41	Coomassie (R) brilliant blue G 250	Fluka 27815
42	A-lactose monohydrate	Sigma L 3625
44	ortho-phosphoric acid 85 %	Riedel 04107
45	Whey powder	Pınar
46	Skim milk powder	Pınar

APPENDIX B

FULL FACTORIAL DESIGN RESULTS OF LB

Table B.1. Full factorial design and experimental results of biomass, lactic acid and β-galactosidase activity extracted by using lysozyme, glass beads and homogenizer for Lb.

Combination	Strain	Media	Agitation	Biomass (g/l)[a]	Lactic Acid (g/l)[b]	Activity[c] (U/ml) (Lysozyme)	Activity[d] (U/ml) (Glass beads)	Activity[e] (U/ml) (Homogenizer)
1	Lb22b	WYE	0	11	3.79	0.0246	0.0212	0.0024
2	Lb77	WYE	0	12.4	5.23	0.0238	0.0204	0.0026
3	Lb16	WYE	150	14.4	3.31	0.0194	0.0194	0.0018
4	Lb22b	S	0	92	8.72	0.1278	0.1022	0.0134
5	Lb22b	S	150	82	3.01	0.0874	0.0762	0.0098
6	Lb22b	W	0	11.4	4.62	0.0288	0.0252	0.003
7	Lb77	WMRS	150	14	3.36	0.0178	0.0148	0.0016
8	Lb16	W	150	13.2	2.44	0.0452	0.0318	0.0044
9	Lb22b	W	150	8.98	2.83	0.028	0.0262	0.0022
10	Lb16	W	0	12.1	4.80	0.0274	0.024	0.003
11	Lb77	W	150	14.4	2.50	0.0278	0.0206	0.0026
12	Lb77	W	0	18.2	4.60	0.042	0.0366	0.0042
13	Lb16	WYE	0	14.3	4.90	0.022	0.0216	0.0022
14	Lb16	WMRS	150	14	3.59	0.0134	0.0128	0.0012
15	Lb16	S	150	98.2	4.02	0.109	0.1378	0.0098
16	Lb22b	WMRS	0	16.2	4.30	0.0194	0.0184	0.0018
17	Lb16	WMRS	0	15.4	5.15	0.0172	0.0152	0.0018
18	Lb77	S	150	87	2.15	0.03	0.024	0.0028
19	Lb22b	WYE	150	21	2.02	0.022	0.024	0.0024
20	Lb77	S	0	92.4	3.71	0.24	0.186	0.0024
21	Lb77	WMRS	0	16	3.70	0.104	0.009	0.001
22	Lb16	S	0	78	4.49	0.046	0.0362	0.0048
23	Lb22b	WMRS	150	15.3	2.85	0.0112	0.0132	0.0012
24	Lb77	WYE	150	25	2.93	0.024	0.0206	0.0026

[a,b,c,d,e] Mean standard deviations for biomass, lactic acid, lysozyme, glass bead and homogenization treatments were determined as 1.16 g/l, 0,15 g/l 0.002 U/ml, 0.003 U/ml and 0.0004 U/ml, respectively. Mean values and standard deviations were calculated based on two replications

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON BIOMASS FOR LB

Table B.2. Results of full factorial design presenting the effects and significance level of factors on biomass for Lb.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	47993.71	23	2086.68	481.07	< 0.0001	Significant
Strain	35.61	2	17.80	4.10	0.0299	
Media	47162.34	3	15720.78	3624.33	< 0.0001	
Agitation	118.44	1	118.44	27.31	< 0.0001	
Strain x Media	86.08	6	14.35	3.31	0.0170	
Strain x Agitation	26.16	2	13.08	3.01	0.0687	
Media x Agitation	258.83	3	86.28	19.89	< 0.0001	
Strain x Media x Agitation	306.26	6	51.04	11.77	< 0.0001	
Residual	99.76	23	4.34			
Cor Total	48121.69	47				

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON LACTIC ACID FOR LB

Table B.3. Result of full factorial design presenting the effects and significance level of factors on lactic acid for Lb.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	4.810	23	0.209	199.704	< 0.0001	significant
Strain	0.225	2	0.113	107.497	< 0.0001	
Media	0.108	3	0.036	34.488	< 0.0001	
Agitation	2.778	1	2.778	2652.989	< 0.0001	
Strain x Media	0.941	6	0.157	149.713	< 0.0001	
Strain x Agitation	0.169	2	0.085	80.885	< 0.0001	
Media x Agitation	0.146	3	0.049	46.566	< 0.0001	
Strain x Media x Agitation	0.442	6	0.074	70.335	< 0.0001	
Residual	0.024	23	0.001			
Cor Total	4.835	47				

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON β-GALACTOSIDASE ACTIVITY FOR LB

Table B.4. ANOVA results of β-galacosidase activity of Lb using a) lysozyme, b) glass beads and c) homogenizer as extraction methods

a)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	27.63	23	1.20	503.67	< 0.0001	significant
Strain	0.04	2	0.02	8.66	0.0016	
Media	20.69	3	6.90	2892.20	< 0.0001	
Agitation	0.34	1	0.34	144.12	< 0.0001	
Strain x Media	0.46	6	0.08	32.33	< 0.0001	
Strain x Agitation	1.14	2	0.57	238.60	< 0.0001	
Media x Agitation	0.60	3	0.20	84.19	< 0.0001	
Strain x Media x Agitation	4.35	6	0.72	303.75	< 0.0001	
Residual	0.05	23	0.002			
Cor Total	27.71	47				

b)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	354.97	23	15.43	35.48	< 0.0001	Significant
Strain	5.88	2	2.94	6.76	0.0049	
Media	272.37	3	90.79	208.73	< 0.0001	
Agitation	1.51	1	1.51	3.48	0.0751	
Strain x Media	6.48	6	1.08	2.48	0.0533	
Strain x Agitation	7.96	2	3.98	9.16	0.0012	
Media x Agitation	2.62	3	0.87	2.01	0.1407	
Strain x Media x Agitation	58.14	6	9.69	22.28	< 0.0001	
Residual	10.00	23	0.43			
Cor Total	366,22	47				

c)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	71.69	23	3.12	9.39	< 0.0001	Significant
Strain	3.12	2	1.56	4.70	0.0195	
Media	33.82	3	11.27	33.98	< 0.0001	
Agitation	2.22	1	2.22	6.68	0.0166	
Strain x Media	4.43	6	0.74	2.22	0.0773	
Strain x Agitation	4.68	2	2.34	7.06	0.0041	
Media x Agitation	6.14	3	2.05	6.17	0.0031	
Strain x Media x Agitation	17.28	6	2.88	8.68	< 0.0001	
Residual	7.63	23	0.33			
Cor Total	79.46	47				

APPENDIX C

FULL FACTORIAL DESIGN RESULTS OF ST

Table C.1. Full factorial design and experimental results of biomass, lactic acid and β-galactosidase activity extracted by using lysozyme, glass beads and homogenizer for St

Combination	Strain	Media	Agitation	Biomass (g/l) [a]	Lactic Acid (g/l) [b]	Activity [c] (U/ml) (Lysozyme)	Activity [d] (U/ml) (Glassbeads)	Activity [e] (U/ml) (Homogenizer)
1	St 74	M1	0	9,8	7,692	0,0539	0,037	0,0043
2	St 77a	M1	150	103,5	6,082	0,6177	0,438	0,0543
3	St 95/2	M4	150	6,5	9,042	0,0192	0,0156	0,0031
4	St 95/2	M1	150	10,7	6,977	0,0612	0,0298	0,0058
5	St 77a	M1	0	20,7	7,196	0,1042	0,0603	0,0095
6	St 77a	M4	0	6,7	8,146	0,0321	0,0259	0,0035
7	St 95/2	M3	0	9,8	4,851	0,0485	0,0376	0,005
8	St 95/2	M2	0	116,3	14,439	0,739	0,5582	0,0749
9	St 77a	M3	0	18,6	5,684	0,0973	0,0705	0,0101
10	St 95/2	M1	0	24,1	5,704	0,1247	0,1026	0,0131
11	St 95/2	M3	150	7,8	5,516	0,029	0,0247	0,0028
12	St 95/2	M2	150	113	13,095	0,737	0,5792	0,0727
13	St 74	M3	0	12,8	6,769	0,0528	0,0381	0,0053
14	St 74	M3	150	9,3	5,237	0,0272	0,0202	0,0033
15	St 77a	M2	150	37,2	7,653	0,1376	0,0972	0,0142
16	St 74	M1	150	11	4,143	0,0411	0,0305	0,0041
17	St 77a	M4	150	9,7	5,909	0,0452	0,0345	0,0045
18	St 74	M2	0	41	10,86	0,2819	0,1689	0,0275
19	St 74	M4	0	38,1	11,729	0,2978	0,1844	0,0308
20	St 95/2	M4	0	18,1	6,509	0,0717	0,0458	0,0092
21	St 77a	M3	150	16,4	4,029	0,0667	0,0418	0,0071
22	St 74	M4	150	8,2	7,089	0,035	0,024	0,0035
23	St 74	M2	150	9,4	8,41	0,0251	0,0208	0,0026
24	St 77a	M2	0	125,4	9,81	0,7537	0,5471	0,0766

[a,b,c,d,e] Mean standard deviations for cell dry weight, lactic acid, lysozyme, glass bead and homogenization treatments were determined as 0.93 g/l, 0.18g/l, 0.005 U/ml, 0.006 U/ml and 0.001 U/ml, respectively. Mean values and standard deviations were calculated based on two replications

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON BIOMASS FOR ST

Table C.2. Result of full factorial design presenting the effects and significance level of factors on biomass for St

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Source
Model	0.3727	23	0.0162	3496.17	< 0.0001	Significant
Strain	0.0262	2	0.0131	2824.43	< 0.0001	
Media	0.1664	3	0.0555	11963.00	< 0.0001	
Agitation	0.0097	1	0.0097	2083.34	< 0.0001	
Strain x Media	0.0930	6	0.0155	3343.14	< 0.0001	
Strain x Agitation	0.0062	2	0.0031	672.67	< 0.0001	
Media x Agitation	0.0287	3	0.0096	2060.48	< 0.0001	
Strain x Media x Agitation	0.0427	6	0.0071	1534.16	< 0.0001	
Residual	0.0001	23	0.0000			
Cor Total	0.3728	47				

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON LACTIC ACID FOR ST

Table C.3. Result of full factorial design presenting the effects and significance level of factors on lactic acid production for St

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	339.54	23	0.33	206.84	< 0.0001	significant
Strain	16.89	2	14.76	118.36	< 0.0001	
Media	199.28	3	8.45	930.72	< 0.0001	
Agitation	22.25	1	66.43	311.76	< 0.0001	
Strain x Media	56.66	6	22.25	132.31	< 0.0001	
Strain x Agitation	30.54	2	9.44	213.92	< 0.0001	
Media x Agitation	2.10	3	15.27	9.81	0.0002	
Strain x Media x Agitation	11.82	6	0.70	27.60	< 0.0001	
Residual	1.64	23	1.97			
Cor Total	341.51	47	0.07			

ANOVA RESULTS PRESENTING THE EFFECTS OF FACTORS ON β-GALACTOSIDASE ACTIVITY FOR ST

Table C.4. Result of full factorial design presenting the effects of the factors on β-galactosidase activity for St using a) lysozyme, b) glass beads and c) homogenizer as extraction methods

a)

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	254.00	23	11.04	18.33	< 0.0001	significant
Strain	16.28	2	8.14	13.51	0.0001	
Media	70.03	3	23.34	38.74	< 0.0001	
Agitation	32.65	1	32.65	54.18	< 0.0001	
Strain x Media	66.83	6	11.14	18.48	< 0.0001	
Strain x Agitation	35.45	2	17.72	29.41	< 0.0001	
Media x Agitation	10.80	3	3.60	5.97	0.0036	
Strain x Media x Agitation	21.97	6	3.66	6.08	0.0006	
Residual	13.86	23	0.60			
Cor Total	268.47	47				

b)

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	328.74	23	14.29	8.20	< 0.0001	Significant
Strain	12.90	2	6.45	3.70	0.0405	
Media	79.26	3	26.42	15.15	< 0.0001	
Agitation	38.17	1	38.17	21.89	0.0001	
Strain x Media	97.30	6	16.22	9.30	< 0.0001	
Strain x Agitation	33.46	2	16.73	9.60	0.0009	
Media x Agitation	14.21	3	4.74	2.72	0.0681	
Strain x Media x Agitation	53.44	6	8.91	5.11	0.0018	
Residual	40.10	23	1.74			
Cor Total	369.30	47				

c)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2311.02	23	100.48	20.16	< 0.0001	significant
Strain	194.61	2	97.30	19.52	< 0.0001	
Media	574.44	3	191.48	38.42	< 0.0001	
Agitation	325.30	1	325.30	65.27	< 0.0001	
Strain x Media	608.77	6	101.46	20.36	< 0.0001	
Strain x Agitation	274.86	2	137.43	27.57	< 0.0001	
Media x Agitation	119.20	3	39.73	7.97	0.0008	
Strain x Media x Agitation	213.85	6	35.64	7.15	0.0002	
Residual	114.63	23	4.98			
Cor Total	2429.30	47				

APPENDIX D

RESULTS OF OPTIMISATION STUDY OF LB 77

Table D.1. Results of face centered central composite design for optimisation of fermentation parameters for Lb 77

Run	Temperature (°C)	Inoculum (%)	Skim milk (%)	Activity U mL^{-1}	Biomass g L^{-1}	Lactic acid g L^{-1}
1[a]	39	3	12	1.186	58.35	7.69
2	43	3	12	1.258	62.35	10.45
3[a]	39	3	12	1.179	57.5	7.62
4	35	4	16	0.180	29.85	1.98
5	35	4	8	0.239	32.3	6.06
6	35	2	8	0.121	26.9	5.41
7[a]	39	3	12	1.185	57.5	7.65
8[a]	39	3	12	1.191	58.9	7.63
9	39	3	16	0.957	49.3	4.21
10	35	2	16	0.492	39.3	1.54
11	43	2	8	0.814	42.4	9.27
12	43	4	16	0.873	45.7	7.58
13	43	2	16	0.457	38.8	7.99
14	43	4	8	1.341	69.2	14.44
15	39	3	8	0.997	50.35	8.14
16[a]	39	3	12	1.191	59.2	7.58
17[a]	39	3	12	1.183	58.6	7.61
18	35	3	12	0.549	32.55	2.19
19	39	4	12	1.020	57.6	8.42
20	39	2	12	0.987	52.4	5.97

[a] The average and the standard deviation among the repeated experiments performed at all center points were 1.186 ± 0.005 U mL^{-1}, 58.34 ± 0.712 g L^{-1}, and 7.63 ± 0.037 g L^{-1} for activity, biomass and lactic acid respectively.

ANOVA RESULTS FOR THE OPTIMISATION STUDY PEFORMED BY USING LB 77

Table D.2. Analysis variance results for enzyme activity

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	2.85	9	0.32	104.43	< 0.0001	significant
Temperature	1.00	1	1.00	329.01	< 0.0001	
Inoculum	0.06	1	0.06	20.16	0.0012	
Skim milk	0.03	1	0.03	10.04	0.0100	
Temperature x Inoculum	0.16	1	0.16	53.09	< 0.0001	
Temperature x Skim milk	0.16	1	0.16	53.09	< 0.0001	
Inoculum x Skim milk	0.04	1	0.04	12.04	0.0060	
Temperature x Temperature	0.19	1	0.19	62.72	< 0.0001	
Inoculum x Inoculum	0.07	1	0.07	24.22	0.0006	
Skim milk x Skim milk	0.10	1	0.10	32.62	0.0002	
Residual	0.03	10	0.0030			
Lack of Fit	0.03	5	0.0061	288.36	< 0.0001	Significant
Pure Error	0.00010	5	0.000021			
Cor Total	2.89	19				

Table D.3. Analysis variance results for lactic acid

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	152.98	3	50.99	48.33	< 0.0001	Significant
Temperature	105.99	1	105.99	100.45	< 0.0001	
Inoculum	6.90	1	6.90	6.54	0.0211	
Skim milk	40.09	1	40.09	38.00	< 0.0001	
Residual	16.88	16	1.06			
Lack of Fit	16.87	11	1.53	1033.38	< 0.0001	Significant
Pure Error	0.01	5	0.0015			
Cor Total	169.87	19				

Table D.4. Analysis variance results for biomass

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	2768.18	9	307.58	34.64	< 0.0001	Significant
Temperature	951.60	1	951.60	107.16	< 0.0001	
Inoculum	121.45	1	121.45	13.68	0.0041	
Skim milk	33.12	1	33.12	3.73	0.0823	
Temperature x Inoculum	178.13	1	178.13	20.06	0.0012	
Temperature x Skim milk	171.59	1	171.59	19.32	0.0013	
Inoculum x Skim milk	150.95	1	150.95	17.00	0.0021	
Temperature x Temperature	227.05	1	227.05	25.57	0.0005	
Inoculum x Inoculum	6.49	1	6.49	0.73	0.4126	
Skim milk x Skim milk	123.87	1	123.87	13.95	0.0039	
Residual	88.80	10	8.88			
Lack of Fit	86.27	5	17.25	34.07	0.0007	Significant
Pure Error	2.53	5	0.51			
Cor Total	2856.99	19				

ESTIMATED COEFFICIENTS FOR ACTIVITY, LACTIC ACID AND BIOMASS

Table D.5. Estimated coefficients for a) β-galactosidase activity, b) lactic acid and c) biomass

a) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	1.18	1	0.019	0.0001
Temperature	0.32	1	0.017	0.0001
Inoculum	0.08	1	0.017	0.0012
Skim milk	-0.06	1	0.017	0.0100
Temperature x Inoculum	0.14	1	0.019	0.0001
Temperature x Skim milk	-0.14	1	0.019	0.0001
Inoculum x Skim milk	-0.07	1	0.019	0.0060
Temperature x Temperature	-0.26	1	0.033	0.0001
Inoculum x Inoculum	-0.16	1	0.033	0.0006
Skim milk x Skim milk	-0.19	1	0.033	0.0002

b) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	6.97	1	0.230	0.0001
Temperature	3.26	1	0.325	0.0001
Inoculum	0.83	1	0.325	0.0211
Skim milk	-2.00	1	0.325	0.0001

c) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	57.62	1	1.024	0.0001
Temperature	9.76	1	0.942	0.0001
Inoculum	3.49	1	0.942	0.0041
Skim milk	-1.82	1	0.942	0.0823
Temperature x Inoculum	4.72	1	1.054	0.0012
Temperature x Skim milk	-4.63	1	1.054	0.0013
Inoculum x Skim milk	-4.34	1	1.054	0.0021
Temperature x Temperature	-9.09	1	1.797	0.0005
Inoculum x Inoculum	-1.54	1	1.797	0.4126
Skim milk x Skim milk	-6.71	1	1.797	0.0039

APPENDIX E

RESULTS OF OPTIMISATION STUDY OF ST 95/2

Table E.1. Results of face centered central composite design for optimisation fermentation parameters for St 95/2

Run	Temperature (°C)	Inoculum (%)	Whey (%)	CSL (%)	Activity U mL^{-1}	Biomass g L^{-1}	Lactic Acid g L^{-1}
1[a]	40	3	2,5	2,5	1.797	10.75	13.45
2[a]	40	3	2,5	2,5	1.810	10.80	13.40
3	35	3	2,5	2,5	0.485	5.15	9.89
4	45	2	5	5	0.755	13.50	19.92
5[a]	40	3	2,5	2,5	1.792	10.76	13.28
6	45	2	5	0	0.014	3.55	1.49
7	35	4	0	0	0.091	2.50	0
8[a]	40	3	2,5	2,5	1.765	10.78	13.36
9	40	3	5	2,5	0.964	14.55	16.69
10	40	3	2,5	5	0.336	9.35	13.46
11	35	2	5	0	0.011	2.95	1.05
12	35	4	5	0	0.061	4.85	2.54
13	45	4	5	5	1.196	22.00	22.75
14	40	3	0	2,5	0.196	6.40	9.56
15[a]	40	3	2,5	2,5	1.794	10.75	13.49
16	45	2	0	0	0.056	2.70	0
17	35	4	5	5	0.628	14.90	10.62
18	45	4	0	0	0.062	3.40	0
19	45	3	2,5	2,5	1.133	9.60	11.75
20[a]	40	3	2,5	2,5	1.828	10.78	13.45
21	45	2	0	5	0.17	4.80	11.78
22	40	2	2,5	2,5	1.160	7.30	7.86
23	35	2	5	5	0.316	12.50	8.38
24	35	2	0	5	0.097	6.85	7.81
25	35	2	0	0	0.062	2.05	0
26	45	4	0	5	0.473	7.35	12.14
27	40	4	2,5	2,5	2.027	12.10	14.28
28	40	3	2,5	0	0.029	6.95	2.85
29	35	4	0	5	0.206	5.20	10.54
30	45	4	5	0	0.022	6.72	3.71

[a] The average and the standard deviation among the repeated experiments performed at all center points were 1.796 ± 0.0211 U mL^{-1}, 10.77 ± 0.02 g L^{-1}, and 13.41 ± 0.076 g L^{-1} for activity, biomass and lactic acid respectively

ANOVA RESULTS FOR THE OPTIMISATION STUDY PEFORMED BY USING ST 95/2

Table E.2. Analysis variance results for enzyme activity

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	73.80	14	5.27	20.32	< 0.0001	Significant
Whey	0.18	1	0.18	0.68	0.4228	
CSL	23.38	1	23.38	90.10	< 0.0001	
Inoculum	2.06	1	2.06	7.95	0.0129	
Temperature	0.35	1	0.35	1.36	0.2623	
Whey x CSL	5.33	1	5.33	20.56	0.0004	
Whey x Inoculum	0.06	1	0.06	0.25	0.6271	
Whey x Temperature	0.0015	1	0.0015	0.0058	0.9402	
CSL x Inoculum	0.0059	1	0.0059	0.0226	0.8825	
CSL x Temperature	1.07	1	1.07	4.13	0.0602	
Inoculum x Temperature	0.14	1	0.14	0.55	0.4688	
Whey x Whey	1.20	1	1.20	4.64	0.0479	
CSL x CSL	12.12	1	12.12	46.71	< 0.0001	
Inoculum x Inoculum	0.87	1	0.87	3.36	0.0867	
Temperature x Temperature	0.06	1	0.06	0.21	0.6499	
Residual	3.892	15	0.26			
Lack of Fit	3.891	10	0.39	3016.39	< 0.0001	Significant
Pure Error	0.000645	5	0.0001			
Cor Total	77.69	29				

Table E.3. Analysis variance results for lactic acid

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	55.78	14	3.98	69.610	< 0.0001	significant
Whey	4.69	1	4.69	81.954	< 0.0001	
CSL	33.51	1	33.51	585.533	< 0.0001	
Inoculum	0.65	1	0.65	11.358	0.0042	
Temperature	1.26	1	1.26	22.061	0.0003	
Whey x CSL	0.70	1	0.70	12.173	0.0033	
Whey x Inoculum	0.13	1	0.13	2.267	0.1529	
Whey x Temperature	0.47	1	0.47	8.166	0.0120	
CSL x Inoculum	0.0006	1	0.0006	0.011	0.9191	
CSL x Temperature	0.73	1	0.73	12.782	0.0028	
Inoculum x Temperature	0.01	1	0.0063	0.111	0.7438	
Whey x Whey	0.00003	1	0.00003	0.0005	0.9830	
CSL x CSL	2.13	1	2.13	37.244	< 0.0001	
Inoculum x Inoculum	0.22	1	0.22	3.904	0.0669	
Temperature x Temperature	0.23	1	0.23	4.025	0.0632	
Residual	0.86	15	0.06			
Lack of Fit	0.86	10	0.09	869.09	< 0.0001	significant
Pure Error	0.000493	5	0.0001			
Cor Total	56.64	29				

Table E.4. Analysis variance results for biomass

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	9.974	14	0.71	22.74	< 0.0001	significant
Whey	2.410	1	2.41	76.93	< 0.0001	
CSL	4.176	1	4.18	133.29	< 0.0001	
Inoculum	0.462	1	0.46	14.74	0.0016	
Temperature	0.262	1	0.26	8.37	0.0111	
Whey x CSL	0.203	1	0.20	6.49	0.0223	
Whey x Inoculum	0.093	1	0.09	2.96	0.1057	
Whey x Temperature	0.010	1	0.01	0.33	0.5769	
CSL x Inoculum	0.035	1	0.04	1.12	0.3068	
CSL x Temperature	0.025	1	0.03	0.81	0.3830	
Inoculum x Temperature	0.088	1	0.09	2.81	0.1145	
Whey x Whey	0.001	1	0.0011	0.03	0.8565	
CSL x CSL	0.104	1	0.10	3.31	0.0888	
Inoculum x Inoculum	0.006	1	0.01	0.18	0.6775	
Temperature x Temperature	0.294	1	0.29	9.38	0.0079	
Residual	0.470	15	0.03			
Lack of Fit	0.470	10	0.05	13636.21	< 0.0001	significant
Pure Error	0.000017	5	0.000003			
Cor Total	10.444	29				

ESTIMATED COEFFICIENTS FOR ACTIVITY, LACTIC ACID AND BIOMASS

Table E.5. Estimated coefficients for a) β-galactosidase activity, b) lactic acid and c) biomass

a) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	0.22	1	0.158	0.0001
Whey	0.10	1	0.120	0.4228
CSL	1.14	1	0.120	0.0001
Inoculum	0.34	1	0.120	0.0129
Temperature	0.14	1	0.120	0.2623
Whey x CSL	0.58	1	0.127	0.0004
Whey x Inoculum	0.06	1	0.127	0.6271
Whey x Temperature	-0.01	1	0.127	0.9402
CSL x Inoculum	0.02	1	0.127	0.8825
CSL x Temperature	0.26	1	0.127	0.0602
Inoculum x Temperature	-0.09	1	0.127	0.4688
Whey x Whey	-0.68	1	0.316	0.0479
CSL x CSL	-2.16	1	0.316	0.0001
Inoculum x Inoculum	0.58	1	0.316	0.0867
Temperature x Temperature	-0.15	1	0.316	0.6499

b) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	3.62	1	0.074	0.0001
Whey	0.51	1	0.056	0.0001
CSL	1.36	1	0.056	0.0001
Inoculum	0.19	1	0.056	0.0042
Temperature	0.26	1	0.056	0.0003
Whey x CSL	-0.21	1	0.060	0.0033
Whey x Inoculum	0.09	1	0.060	0.1529
Whey x Temperature	0.17	1	0.060	0.0120
CSL x Inoculum	-0.01	1	0.060	0.9191
CSL x Temperature	0.21	1	0.060	0.0028
Inoculum x Temperature	-0.02	1	0.060	0.7438
Whey x Whey	0.003	1	0.149	0.9830
CSL x CSL	-0.91	1	0.149	0.0001
Inoculum x Inoculum	-0.29	1	0.149	0.0669
Temperature x Temperature	-0.30	1	0.149	0.0632

c) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	2.33	1	0.055	0.0001
Whey	0.37	1	0.042	0.0001
CSL	0.48	1	0.042	0.0001
Inoculum	0.16	1	0.042	0.0016
Temperature	0.12	1	0.042	0.0111
Whey x CSL	0.11	1	0.044	0.0223
Whey x Inoculum	0.08	1	0.044	0.1057
Whey x Temperature	0.03	1	0.044	0.5769
CSL x Inoculum	-0.05	1	0.044	0.3068
CSL x Temperature	-0.04	1	0.044	0.3830
Inoculum x Temperature	0.07	1	0.044	0.1145
Whey x Whey	-0.02	1	0.110	0.8565

APPENDIX F

ANOVA RESULTS OF THE OPTIMISATION STUDY PERFORMED BY USING LB 77 AND ST 95/2

Table F.1. Results of optimisation study performed by using Lb 77& St 95/2

Run	St 95/2 (%)	Lb 77 (%)	Media	Activity U mL^{-1}	Biomass g L^{-1}	Lactic Acid g L^{-1}
1[a]	2	2	Media A	0.748	62.54	8.18
2[b]	2	2	Media B	1.748	41.85	21.33
3[b]	2	2	Media B	1.723	42.30	21.35
4[a]	2	2	Media A	0.754	62.05	8.16
5[a]	2	2	Media A	0.758	63.40	8.17
6	3	2	Media A	0.810	75.15	9.85
7[a]	2	2	Media A	0.752	63.60	8.18
8	3	3	Media B	2.058	51.60	18.43
9	1	2	Media A	0.538	45.18	5.88
10	2	3	Media B	2.144	45.25	24.86
11[a]	2	2	Media A	0.754	62.10	8.15
12	1	3	Media A	0.693	58.25	6.02
13	1	1	Media A	0.655	53.05	7.07
14	1	3	Media B	1.531	34.90	20.45
15	1	2	Media B	1.235	30.05	18.64
16	3	3	Media A	1.013	93.20	11.31
17	3	2	Media B	1.739	39.90	22.31
18	2	1	Media B	1.327	29.30	19.16
19[b]	2	2	Media B	1.740	42.45	21.30
20	2	3	Media A	0,734	93.10	8.75
21	3	1	Media A	0.544	52.35	5.17
22	2	1	Media A	0.495	48.40	5.29
23[b]	2	2	Media B	1.734	41.65	21.39
24	1	1	Media B	1.124	29.75	14.76
25	3	1	Media B	1.256	32.45	16.69
26[b]	2	2	Media B	1.637	41.25	21.33

[a] The average and the standard deviation among the repeated experiments performed at all center points using Media A were 0.753 ± 0.004 U mL^{-1}, 62.34 ± 0.724 g L^{-1}, and 8.61 ± 0.013 g L^{-1} for activity, biomass and lactic acid respectively.

[b] The average and the standard deviation among the repeated experiments performed at all center points using Media B were 1.7164 ± 0.045 U mL^{-1}, 41.90 ± 0.487 g L^{-1}, and 21.34 ± 0.033 g L^{-1} for activity, biomass and lactic acid respectively.

ANOVA RESULTS FOR THE OPTIMISATION STUDY PEFORMED BY USING LB 77 AND ST 95/2

Table F.2. Analysis variance results for enzyme activity

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	6.53	8	0.816	89.36	< 0.0001	Significant
St 95-2	0.22	1	0.224	24.48	0.0001	
Lb 77	0.64	1	0.640	70.10	< 0.0001	
Media	5.31	1	5.314	581.66	< 0.0001	
St 95/2 x Lb 77	0.085	1	0.085	9.32	0.0072	
St 95/2 x Media	0.039	1	0.039	4.30	0.0536	
Lb 77 x Media	0.137	1	0.137	14.94	0.0012	
St 95/2 x St 95/2	0.069	1	0.069	7.58	0.0136	
Lb 77 x Lb 77	0.0015	1	0.002	0.17	0.6874	
Residual	0.16	17	0.009			
Lack of Fit	0.15	9	0.016	15.79	0.0003	Significant
Pure Error	0.008	8	0.001			
Cor Total	6.69	25				

Table F.3. Analysis variance results for lactic acid

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	1082.68	8	135.33	51.98	< 0.0001	Significant
St 95-2	9.99	1	9.99	3.84	0.0668	
Lb 77	39.10	1	39.10	15.02	0.0012	
Media	1007.18	1	1007.18	386.82	< 0.0001	
St 95/2 x Lb 77	1.30	1	1.30	0.50	0.4887	
St 95/2 x Media	1.18	1	1.18	0.45	0.5094	
Lb 77 x Media	1.74	1	1.74	0.67	0.4250	
St 95/2 x St 95/2	9.07	1	9.07	3.48	0.0794	
Lb 77 x Lb 77	4.84	1	4.84	1.86	0.1904	
Residual	44.26	17	2.60			
Lack of Fit	44.26	9	4.92	7351.08	< 0.0001	Significant
Pure Error	0.01	8	0.001			
Cor Total	1126.94	25				

Table F.4. Analysis variance results for biomass

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	2.405	6	0.40	45.38	< 0.0001	significant
St 95-2	0.249	1	0.25	28.14	< 0.0001	
Lb 77	0.473	1	0.47	53.56	< 0.0001	
Media	1.597	1	1.60	180.75	< 0.0001	
St 95/2 x Lb 77	0.078	1	0.08	8.78	0.0080	
St 95/2 x Media	0.003	1	0.0035	0.39	0.5381	
Lb 77 x Media	0.006	1	0.0059	0.67	0.4234	
Residual	0.168	19	0.0088			
Lack of Fit	0.167	11	0.0152	112.87	< 0.0001	significant
Pure Error	0.001	8	0.0001			
Cor Total	2.573	25				

ESTIMATED COEFFICIENTS FOR ACTIVITY, LACTIC ACID AND BIOMASS

Table F.5. Estimated coefficients for a) β-galactosidase activity, b) lactic acid and c) biomass

a) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	1.22	1	0.028	0.0001
St 95-2	0.14	1	0.028	0.0001
Lb 77	0.23	1	0.028	0.0001
Media	0.45	1	0.019	0.0001
St 95/2 x Lb 77	0.10	1	0.034	0.0072
St 95/2 x Media	0.06	1	0.028	0.0536
Lb 77 x Media	0.11	1	0.028	0.0012
St 95/2 x St 95/2	-0.11	1	0.041	0.0136
Lb 77 x Lb 77	-0.02	1	0.041	0.6874

b) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	14.95	1	0.47	0.0001
St 95-2	0.91	1	0.47	0.0668
Lb 77	1.81	1	0.47	0.0012
Media	6.22	1	0.32	0.0001
St 95/2 x Lb 77	0.40	1	0.57	0.4887
St 95/2 x Media	-0.31	1	0.47	0.5094
Lb 77 x Media	0.38	1	0.47	0.4250
St 95/2 x St 95/2	-1.28	1	0.69	0.0794
Lb 77 x Lb 77	-0.94	1	0.69	0.1904

c) Term	Coefficient Estimate	df	Standard Error	p-value Prob > F
Constant	3.89	1	0.018	0.0001
St 95-2	0.14	1	0.027	0.0001
Lb 77	0.20	1	0.027	0.0001
Media	-0.25	1	0.018	0.0001
St 95/2 x Lb 77	0.10	1	0.033	0.0080
St 95/2 x Media	-0.02	1	0.027	0.5381
Lb 77 x Media	-0.02	1	0.027	0.4234

RESULTS OF EXPERIMENTAL AND PREDICTED VALUES FOR ACTIVITY, LACTIC ACID AND BIOMASS AT OPTIMUM CONDITIONS

Table F.6. Results of experimental and predicted values for activity, lactic acid and biomass at optimum conditions

a) Validation experiments for first optimization

Running order	Inoculum (% (v/v))	Temperature (°C)	Skim milk (% (w/v))	Activity (UmL^{-1})	Lactic Acid (gL^{-1})	Biomass (gL^{-1})
1	4.0	43	8	1.303 (1.363)	12.99 (13.06)	65.26 (69.03)
2	3.9	43	8	1.269 (1.360)	12.05 (12.94)	64.98 (68.99)
3	3.99	43	8.15	1.294 (1.322)	12.87 (12.96)	63.22 (69.15)
4	4.0	43	8.63	1.332 (1.370)	13.07 (12.87)	67.32 (69.02)

b) Validation experiments for second optimization

Running order	Inoculum (% (v/v))	Temperature (°C)	Whey (% (w/v))	CSL (% (w/v))	Activity (UmL^{-1})	Lactic Acid (gL^{-1})	Biomass (gL^{-1})
1	3.87	44	5	4.16	1.986 (2.027)	20.85 (22.76)	19.76 (21.86)
2	3.85	44	5	4.07	2.015 (2.032)	21.55 (22.77)	20.15 (21.52)
3	3.94	44	5	4.34	1.992 (2.043)	19.98 (22.76)	20.28 (20.19)
4	3.9	44	5	4.17	2.032 (2.045)	21.37 (22.76)	18.94 (19.32)

c) Validation experiments for third optimization

Running order	St 95/2 (% (v/v))	Lb 77 (% (v/v))	Media	Activity (UmL^{-1}	Lactic Acid (gL^{-1})	Biomass (gL^{-1})
1	2.67	3	Media B	2.162 (2,144)	23.85 (22.52)	53.16 (52.82)
2	2.6	3	Media B	2.108 (2.134)	22.61 (22.50)	48.82 (52.24)
3	2.6	3	Media B	2.127 (2.135)	22.18 (22.47)	49.15 (52.15)
4	2.67	3	Media B	2.153 (2.144)	23.12 (22.53)	52.02 (52.82)

APPENDIX G

STANDART CALIBRATION CURVE FOR ACTIVITY

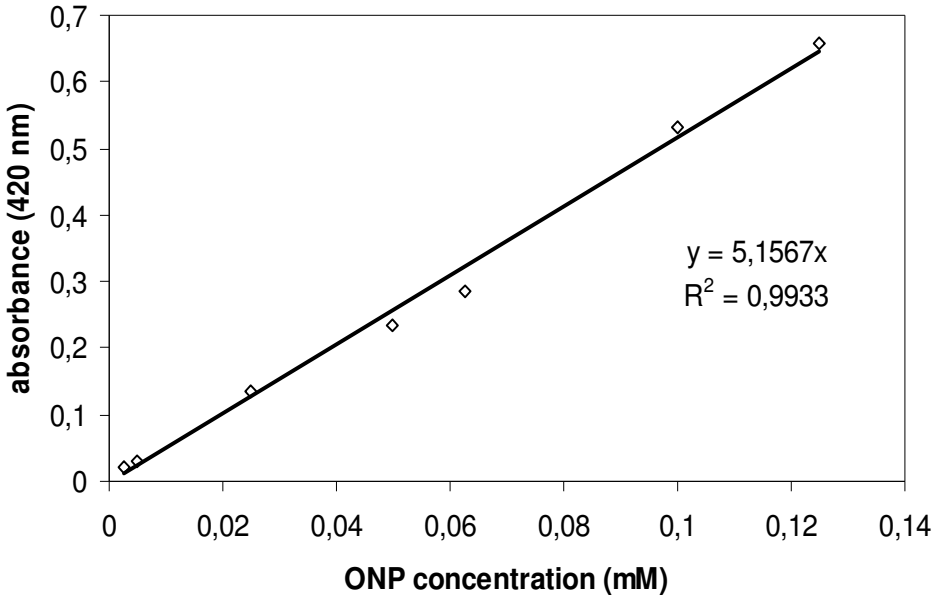

Figure G.1. Standard calibration curve for activity

APPENDIX H

STANDARD CALIBRATION CURVE FOR TOTAL PROTEIN

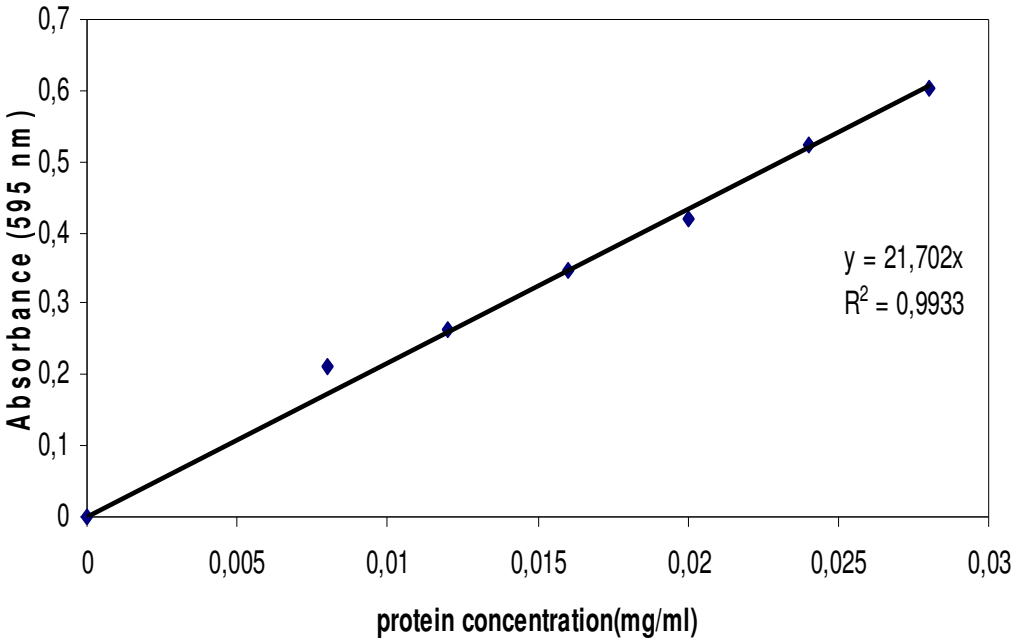

Figure H.1. Standard calibration curve for total protein