REPUBLIC OF TURKEY
SİİRT UNIVERSITY
INSTITUTE OF SCIENCE

IMMOBILIZATION OF *Aspergillus oryzae* β-GALACTOSIDASE TO PRODUCE LACTOSE-FREE MILK

MASTER'S THESIS

Alan Yaseen TAHER
(163100801)

Department of Food Engineering

Thesis Supervisor: Asst. Prof. Dr. Yakup ASLAN
Thesis Co-Supervisor: Prof. Dr. İsa CAVİDOĞLU

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THESIS ACCEPTANCE AND APPROVAL

The thesis study titled **"Immobilization of *Aspergillus oryzae* β-galactosidase to produce lactose-free milk"** prepared by Alan Yaseen TAHER, has been accepted unanimously/by majority by the following jury as a MASTER'S/DOCTORAL THESIS at Siirt University Institute of Science and Technology Department of Food Engineering on 25.01.2018.

Jury Members	**Signature**
President Prof. Dr. Ender Sinan POYRAZOĞLU	.....
Supervisor Assist. Prof. Dr. Yakup ASLAN	.....
Member Assist. Prof. Seda DURSUN OĞUR	.....

I confirm the above results.

Assoc. Prof. Dr. Koray ÖZRENK
Director of Institute of Science

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Alan Yaseen TAHER

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PREFACE

In this thesis, *Aspergillus oryzae* β-Galactosidase enzyme was immobilized onto Eupergit CM by using covalently binding method and the immobilized enzyme was characterized and used for hydrolysis of semi-skimmed UHT cow's milk lactose.

First, I would like to express my deepest gratitude to my supervisor Assist. Prof. Dr. Yakup ASLAN for his guidance, patience, understands and excellent support throughout this study program. He showed me different ways to approach a research problem and taught to be persistent to accomplish any goal.

This project would not have been possible without the support of Siirt University Scientific Research Projects Coordinatorship (BAP).

There are too many people who have helped me during my study that I need to thanks them. However, there are some people that definitely deserve a place in my thesis because without them.

Last of all, I am greatly indebted to my family. Thanks to my parents and my wife they are all supported me for educating me for unconditional support and encouragement to pursuade my interests and also for believing in me. I owe all my achievements to them and I am really thankful to know that they will support me in anything I do.

Alan Yaseen TAHER
SİİRT-2018

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LIST OF ABREVIATIONS AND SYMBOLS

Abbreviation	Description
BSA	**:** Bovine Serum Albumin
g	**:** Gram
IU	**:** International Unit of Enzyme
L	**:** Liter
M	**:** Molarity
mg	**:** Milligram
mL	**:** Milliliter
^{o}C	**:** Degree of Celsius
pH	**:** -log [H^{+}]
rpm	**:** Rolling per minute
UHT	**:** Ultra High Temperature
UV	**:** Ultraviolet
V	**:** Volume
W	**:** Weight
µg	**:** Microgram
µL	**:** Microliter
µmol	**:** Micromole

ÖZET

YÜKSEK LİSANS TEZİ

LAKTOZSUZ SÜT ÜRETIMI İÇIN *Aspergıllus oryzae* β-GALAKTOZIDAZIN İMMOBILIZASYONU

Alan Yaseen TAHER

Siirt Üniversitesi Fen Bilimleri Enstitüsü
Gıda Mühendisliği Anabilim Dalı

Danışman : Yrd. Doç. Dr. Yakup ASLAN

II. Danışman : Prof. Dr. İsa CAVİDOĞLU

2018, 57 Sayfa

Bu tez çalışmasında, *Aspergillus oryzae* β-Galaktozidaz enzimi, immobilizasyon koşulları optimize edilerek Eupergit CM üzerinde % 100.00 immobilizasyon verimi ve % 129.82 aktivite verimi ile immobilize edildi. Serbest ve immobilize *Aspergillus oryzae* β-Galaktosidazın karakterizasyonu da çalışıldı. İmmobilizasyon sonrasında, optimum pH, 4.5'den 5.5'e kayarken, optimum sıcaklık (55 °C) değişmedi. Serbest ve immobilize enzimler için, kinetik sabitler de Lineweaver-Burk grafiği kullanılarak belirlendi. Serbest ve immobilze enzimin K_m değerleri sırasıyla 307.7 ve 234.2 g / L olarak belirlenirken, V_{max} değerleri sırasıyla 0.366 ve 0.415 g D-Glikoz / L.min olarak belirlendi. İmmobilize enzimin başlangıç aktivitesinde yirmi kez tekrarlanan kullanımdan sonra ve, optimum koşullar altında on beş gün depolandıktan sonra herhangi bir azalma olmamıştır. Ayrıca, İmmobilize enzim kullanılarak, yarım yağlı UHT inek sütündeki laktoz dört saat içinde tamamen hidroliz edildi. Sonuç olarak, bu tez çalışmasında elde edilen immobilize *Aspergillus oryzae* β-Galaktosidaz'ın laktozsuz sütün endüstriyel üretiminde kullanılabileceği söylenebilir.

Anahtar Kelimeler: *Aspergillus oryzae*, β-Galaktosidaz, Eupergit CM, immobilizasyon, laktozsuz süt, laktoz intoleransı

ABSTRACT
MASTER'S THESIS

IMMOBILIZATION OF *Aspergıllus oryzae* β-GALACTOSIDASE TO PRODUCE LACTOSE-FREE MILK

Alan Yaseen TAHER

The Graduate School of Natural and Applied Science of Siirt University
The Degree of Master of Science In Food Engineering

Supervisior: Asst. Prof. Dr. Yakup ASLAN

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2018, 57 Pages

In this thesis study, *Aspergillus oryzae* β-galactosidase enzyme was immobilized on Eupergit CM with 100 % immobilization yield and 129.82 % activity yield by optimizing the immobilization conditions. Characterization of free and immobilized *Aspergillus oryzae* β-Galactosidase was also studied. After immobilization, optimum pH shifted from 4.5 to 5.5, while the optimum temperature (55 ° C) did not change. For free and immobilized enzymes, kinetic constants were also determined by using the Lineweaver-Burk graph. The K_m values of the free and immobilized enzymes were determined to be 307.7 and 234.2 g / L, respectively, while the V_{max} values were determined to be 0.366 and 0.415 g D-Glucose / L.min respectively. There was no decrease in the initial activity of the immobilized enzyme after repeated 20 batch experiments and after storage for 15 days under optimum conditions. In addition, using the immobilized enzyme, the lactose in the semi-skimmed UHT cow's milk was completely hydrolyzed within four hours. In conclusion, it can be concluded that the immobilized *Aspergillus oryzae* β-Galactosidase obtained in this thesis can be used in the industrial production of lactose-free milk.

Keywords: *Aspergillus oryzae*, β-Galactosidase, Eupergit CM, immobilization, lactose-free milk, lactose intolerance

1. INTRODUCTION

Enzymes are attractive catalysts which increase the biochemical reactions as they are efficacious and specific under ambient conditions. The enzymatic hydrolysis of lactose into its constituent molecules glucose and galactose has received a particular interest from both the nutritional and technological viewpoints (Domingues et al., 2005). It can be applied to produce low lactose products to prevent lactose intolerance for the consumption by lactose intolerant persons (Furlan et al., 2000). Moreover it can be used to prevent lactose crystallization during the production of ice cream by the help of the hydrolysis with the enzyme lactase. In addition, β-galactosidase administrations is important in converting the waste cheese juice from the dairy industry into different value-added products (Linko et al., 1998). Additionally, galactooligosaccharides can also be formed by transgalactosylation activity of β-galactosidases. β-galactosidase, commercially named lactase, is commonly used for the production of low lactose milk and milk products.

Many enzymes consist of a protein. The major drawback of the free enzymes is their limited life time. Most enzymes are not stable under extreme conditions. Severe conditions such as high pressure, extreme pHs, and organic solvents can easily disrupt the three dimensional structures responsible for the activities of the enzymes.These factors can affect the structures of proteins and causes a decrease in catalytic activity of an enzyme. Moreover, seperation of enzymes from substrates and products present in same medium is so difficult and high cost. The using of immobilized enzymes instead of soluble enzymes resultid in easily removal of enzyme from reaction medium, many times usage in the repeated serial experiments and decrease in the product cost. (El-Masry et al., 2000; Tümtürk et al., 2007).

Immobilization means preparing the water-insoluble biocatalyst derivatives by binding the enzymes to a water-insoluble solid polymer or trapping it in a gel. Due to these advantages, immobilized enzymes are more preferred in recent industrial applications. Numerous natural and synthetic materials are used as immobilization matrices.

β-galactosidases (lactases) are obtained from only microorganisms for industrial applications, although present in many living systems (Vasiljevic and Jelen, 2001). Major enzymes of commercial interest which might be immobilized are *Kluyveromyces*

lactis, *Kluyveromyces fragilis*, *Escherichia coli*, *Aspergillus oryzae* and *Aspergillus niger* (Holsinger and Kligerman, 1991).

The various methods used for immobilization of enzymes include adsorption, entrapment, cross-linking and covalent attachment (Tanaka and Kawamoto, 1999). It is known that several immobilization process parameters such as enzyme concentration (Finocchiaro et al., 1980; Betancor et al., 2008) or the glutaraldehyde concentration used during enzyme immobilization influence the activity and the thermo-stability of the enzyme in reaction (Betancor et al., 2006). However, no systematic study has been conducted till date to optimize the immobilization of β-galactosidase enzyme in order to use them efficiently.

Covalent immobilization can easily performed by the direct reaction of specific functional groups available on the support with functional groups such as amino, sulphydryl, carboxyl and hydroxyl present in the side chains of amino acids on the enzyme surface.

The major advantage of covalent immobilization is the preventing the leakage of enzyme molecules from the supports under operational conditions that responsible for the decreased activity in the repeated usage.

Another big advantage of this method is the increase in the stability against to denaturants such as temperature, extreme pHs, and chemical reagents.

The covalent attachement may resulted in the drastic decreased enzymatic activity due to binding of enzyme molecules to the supports via amino acids involved in the active site. This is the only disadvantage of this method (Novick and Rozzell, 2005).

With this perspective, in this thesis study *Aspergillus oryzae* β-galactosidase will be immobilized onto Eupergit CM by optimizing the various parameters affect the immobilization and activity yields. Furthermore, characterization of the free and the immobilized enzyme also will be studied. Lastly, obtained immobilized enzyme will be used to hydrolyze semi-skimmed UHT cow's milk lactose.

2. LITERATURE REVIEW

2.1. Lactose

Lactose, the major carbohydrate of milk, is a disaccharide with low relative solubility, which is hydrolyzed into glucose and galactose that are its component. The monosaccharides are joined by a β-(1-4) glycosidic linkage between the anomeric C-1 of the β -D-galactose and the C-4 of D-glucose (Figure 2.1) (Miller et al., 1993).

Lactose in solid form can be crystalline or amorphous. Crystalline lactose can present in one of two different forms: β-lactose and α-lactose monohydrate. Although both forms can be crystallized, the physico-chemical intercourses between the different forms of lactose are so complicated. Crystals of α-lactose can be formed by concentrated an aqueous lactose solution to supersaturation and allowing it to crystallize at temperatures lower than 93.5 ^{o}C. α-lactose is corporated with one water molecule; crystallizes as a monohydrate. These crystals are very hard and brittle. β-lactose; the anomer of α-lactose is formed when a very concentrated solution of lactose is allowed to crystallized at temperatures above 93.5 ^{o}C. Since no water is associated with the molecule in this case, its designation is banhydride. Amorphous lactose is very hygroscopic and can cause caking problems while and after spray drying, in powders which have high lactose content (Schuck ve Dolivet, 2002).

In milk, lactose is present in two isomeric forms called α- and β-lactose as well (Figure 2.2). The molecular structures of α- and β-lactose differ in the orientation of a hydrogen and a hydroxyl group on carbon atom in the glucose moiety. Both forms change into one another (Anonym, 2008).

The sweet syrup prepared from whey by lactose hydrolysis can be used as a source of sugar and, in some cases, of protein in soft drinks, in ice cream, in feedstuffs for cattle instead of molasses, in dairy desserts, or as basis for further fermentation to alcohol. Hydrolyzed demineralized lactose syrup was produced by Valio Process in Finland (Gekas and López-Leiva, 1985). In addition, lactose may be used to partially replace skim milk powder in some baked products, such as muffins and biscuits, without significantly affecting product quality yet providing an effective means of cost reduction. In recent years lactose is applied in dried vegetable process in order to reduce sweetness and prevent discoloration.

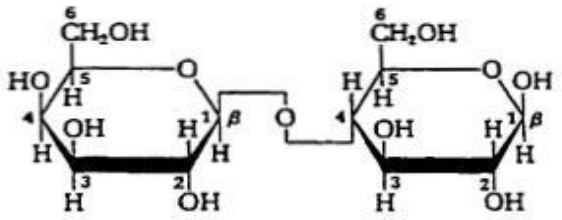

Figure 2.1. Structure of Lactose (Miller et al., 1993)

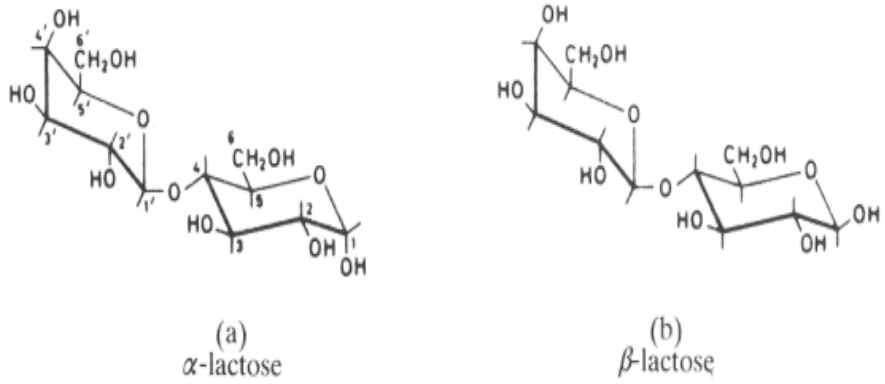

Figure 2.2. The structure of lactose molecules in α and β form (Anonym, 2008)

2.2. Lactose Intolerance

β-galactosidase enzyme deficiency in human instentine leads to lactose intolerance (Panesar et al., 2006). The prevalence of lactose intolerance is shown in Table 2.1. Unfortunately significant fraction of global population is lactose intolerant limiting the consumption of milk and dairy products. These people also have digestive system disorders as well as malabsorption. Lactose is hydrolyzed by an enzyme β-galactosidase known as lactase in the intestinal mucosa. As a result the lack of lactase, the hydrolysis of sugar lactose is incomplete. The undigested sugar pulls fluid into the intestine, moreover when the colonic bacteria acts on the undigested sugar lactic acid, hydrogen and other organic acids are produced.

At the end the combined osmotic effect results in the passage of acidic diarrheal stools. Lactose intolerance describes the presence of gastrointestinal symptoms such as abdominal pain, bloating, flatulence, nausea or diarrhea. In its most severe form, lactose intolerance leads to dehydration and failure to thrive (Panesar et al., 2006). Lactose deficiency has mainly three types described as primary, secondary or congenital.

Table 2.1. Incidence of lactose intolerance in different regions of the world. (Fernandes et al., 1987).

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

Primary lactase deficiency, also called Late Onset Lactase Deficiency, is the most common type of them all. Primary deficiency is caused by the decline lactose production from infancy into adulthood, in spite of a continuous intake of lactose. Secondary lactose deficiency results from small intestine resections and diseases damaging the intestinal epithelium. Lactose deficiency may also be a congenital occurrence when two ineffective genes from parents are inherited. This results in an inability of genes in the newborn for producing lactose. As lactose intolerant people have inability to digest milk and other diary products, the treatment of these products with β-galactosidase s required. However the industrial application of the process based on the hydrolysis of sugar lactose is limited due to the great expense of using soluble lactase (Çabuk, 2008).

2.3. Hydrolysis of lactose

2.3.1. Acidic hydrolysis

Acid catalysts for lactose hydrolysis can also be used in either of two forms: free acid, such as sulfuric acid, can be added to the solution or a solid acid, such as the acid form of a cationic exchange resin, can be used to treat the lactose solution.

Acid hydrolysis is a simple procedure which does not require expensive materials such as enzymes. However it is not preferable due to the process conditions including low pH and high temperature where protein denaturation occurs (Gekas and Lopez-Leiva, 1985).

2.3.2. Enzymatic hydrolysis

Many studies related to reducing or converting the milk sugar have been published. Most preferred method for this purpose is the use of enzymes (Koçak and Zadow, 1989). With improvements in processing techniques, hydrolyzing the lactose before packaging certain dairy products has become more prevalent. The hydrolyzing mechanism of lactose hydrolysis by using *Escherichia coli* lactase was described (Wallenfels and Malhotra, 1961). In the reaction mechanism; the cysteine and histidine residues are present in the active site of β-galactosidase. The sulphydryl group of cysteine acting as proton donor while imidazole group of histidine nucleophile site to facilitate cleavege of the glycosidic bond (Richmond et al., 1981; Mahoney, 1998; Zhou and Chen, 2001).

It has become clear that β-galactosidase from microbial sources has glutamic acid residues in two forma; Glu^{482}, Glu^{551} as proton donor and nucleophile/base at the same time in reaction. In other word glutamic acid residue was suggested as the active site. The reaction mechanism shown in Figure 2.3.

In the first step of the reaction enzyme-galactosyl complex is formed and glucose is released currently.

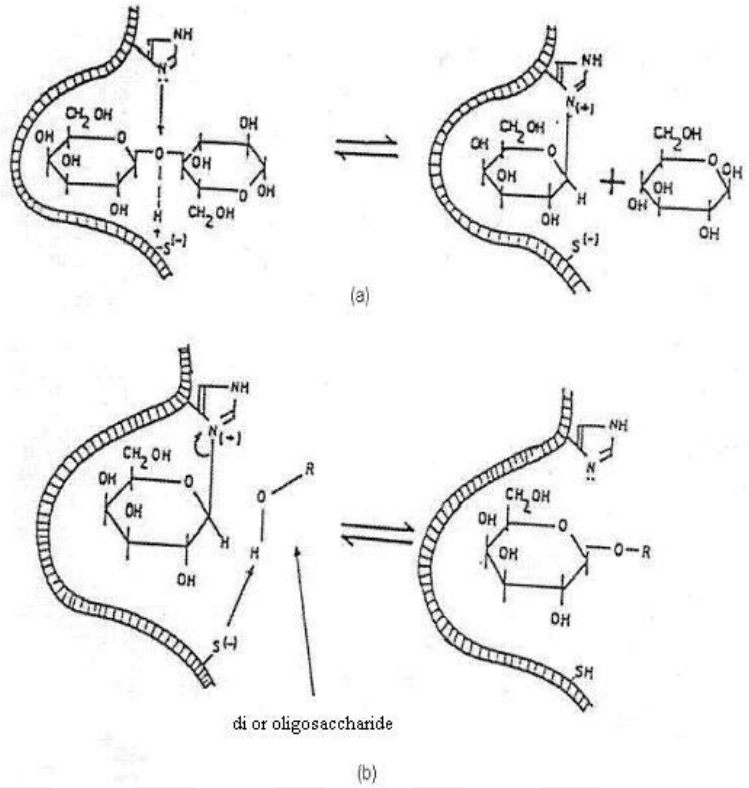

Figure 2.3. Schematic mechanism of lactose hydrolysis by β-galactosidase. a) enzyme-galactosyl complex formation and glucose liberation b) enzyme-galactosyl complex transferred to an acceptor containing hydroxyl group (Richmond et al., 1981).

In the second step; complex of enzyme-galactosyl is transferred to an acceptor which contains a hydroxyl group. In a diluted lactose solution, water is more competitive to be an acceptor; therefore galactose is formed and released from active site. In contrast to this, in a concentrated lactose solution, lactose is more competitive to be acceptor, binding with enzyme-galactose complex to form oligosaccharides (Zhou and Chen, 2001).

2.4. β-galactosidases

β-galactosidase (β-D-galactoside galactohydrolyze; E.C. 3.2.1.23) is an enzyme which catalyzes the hydrolysis of lactose into its constituent products glucose and galactose. This enzyme β-galactosidase also catalyzes the synthesis of oligosaccharides via the galactosyl transfer reaction. β-galactosidase has been isolated from a wide variety of sources such as microorganisms, plants and animals. Commercial lactases can be produced from both yeast such as *Saccharomyces lactis*, mould such as *Aspergillus oryzae* and bacteria such as *Bacillus stearothermophilus* (Seyis and Aksöz, 2004).

2.4.1. Sources of β-galactosidase

β-galactosidase is distributed among microorganisms, plants and animals in which they fulfill a key role in hydrolysis of lactose (Panesar et al., 2010). Animal β-galactosidase was extracted from rabbit and rat testis and rat brain. Second type of β-galactosidase source is plants such as peach or apricot. As microorganisms have a number of advantages such as ease of handling, higher production and multiplication rate, they are the most common source of lactase. Due to high commercial interest in β-galactosidase, large numbers of microorganisms have been assessed as potential sources of lactase.

Yeast like *Kluyveromyces lactis*, *Kluyveromyces fragilis* and Can*dida pseudotropicalis*, fungi like *Aspergillus niger* and *Aspergillus oryzae* and bacteria like *Bacillus stearothermophilus* are commonly used and commercially safe sources for lactase production (Panesar et al., 2010). Lactase from several sources have been well characterized, especially the enzyme from *Escherichia coli,* which serves as a model its toxicity associated with coliform. (Jurado et al., 2002). However enzyme characteristics including temperature-pH optimum and molecular weight can differ between sources.

2.5. Enzyme immobilization

Enzymes have been investigated intensively for possible industrial applications such as to change the combination of nutritions and they have been entered in the industry for decadays. Moreover, by advences in genetics and in process technology enzymes could be used for improving products quality and decreased the product costs. Immobilized enzymes are heterogenous catalysts which can be removal from the reaction medium and can be used in the repated processes.

Immobilized enzymes can exhibit increased stability against to higher temperatures, extreme pHs and chemical denaturants and the severe operational conditions. Moreover, enzymes can be used in immobilized form in organic solvents in which they natually insoluble. Furthermore, immobilized enzymes can show higher activity than soluble counterparts but immobilization of enzymes leads to lower activity than soluble counterparts due to limited subbstrate transfer.

Whether the interest in producing lactose free milk and milk products are based

on nutritional problems such as lactose deficiency or technological interest such as sweetness, solubility and functionality of lactose, the lactose hydrolysis process must be economically feasible. As free enzyme technology costs great expense, immobilized enzymes in food industry were in focus and the methods of immobilization with various enzyme systems including β-galactosidase were discussed.

The first immobilized enzyme used in the food industry was *Aspergillus oryzae* aminoacylase for resolution of synthetic racemic D-L-amino acids. Immobilized enzymes have been used in the food, pharmaceutical and other industries nowadays (Table 2.2).

Around 1970, immobilized penicillin acylase other immobilized systems are launched on a pilot scale which are used to prepare 6-amino penicillanic acid from penicillin G or V and immobilized glucose isomerase used to convert glucose into fructose (Katchalski-Katzir and Kreamer, 2000).

2.5.1. Properties of immobilized enzymes

As a result of immobilization, changes in some properties of the enzyme such as catalytic activity and thermal stability have been observed by the fact that the interaction between the immobilized enzyme and the substrate takes place in a microenvironment that is different from the bulk solution. The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix.

The main problem observed with the immobilized enzymes is loss of the catalytic activity when acting especially on high molecular weight substrates due to the accessibility of the substrate to the active site of enzyme is less easily achieved (Brena et al., 2013).

Table 2.2. Major products obtained using immobilized Enzymes (Katchalski-Katzir, 1993)

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
β-galactosidase	Lactose-free milk

After immobilization is achieved onto a support, enzyme is converted from homogeneous to heterogeneous catalysts, which results in the appearance of some novel features. a diffusional layer is created around the enzyme-support system, as a consequence substrates has to be transported from bulk of the solution across this layer to reach the active site of the enzyme.

The pH profile of the enzyme of interest in immobilized form is one of the properties investigated since the change in pH can influence the stability. The pH at which immobilized enzymes operate best may be different from the native enzyme due to micro-environmental effects caused by charged support materials (Weetall ve Pitcher, 1986).

The most important cause of enzyme inactivation in reactors is heat, as the mechanisms of thermal inactivation of enzymes cause considerable conformational changes in the protein molecules. Generally the structure of immobilized enzyme molecule is much more rigid than that of free enzyme; thus the attached enzyme molecule unfolds and is inactivated much less easily, in other words immobilization procedure enhances their resistance to thermal inactivation (Klibanov, 1983).

2.5.2. Advantages and disadvantages of immobilization

Enzymes are generally soluble. Thus, in the absence of costly separation process, enzymes can be used only once in free solutions. The main technical advantage of immobilized enzymes is that they allow heterogeneous catalysis of enzymatic reactions. Some other advantages are listed below:

- ✓ Possibility of enzyme recycling.
- ✓ Low downstream processing cost.
- ✓ Easier reactor operation.
- ✓ No contamination due to added enzyme.
- ✓ Wider choice of reactor design.
- ✓ Generally greater pH and thermal stability.
- ✓ Easier product separation.
- ✓ Processes can be operated continuously.

In spite of the advantages, the immobilization process has some disadvantages, such as drop of the enzyme activity after immobilization or leakage of the enzyme from

the matrix.

The drop the enzymatic activity depends on the method of the immobilization and the source of the enzyme. Thus industrial applications are limited due to some disadvantages and these disadvantages are shortly listed below:

- ✓ Loss of enzyme activity due to immobilization process
- ✓ Mass transfer limitations (substrate)
- ✓ Investment needed to introduce new equipment to already implanted process.

2.5.3. Methods for enzyme immobilization

The selection of the immobilization technique is based on the process specifications for the enzyme catalyst used. These immobilization techniques are classified in to two as chemical and physical methods. Chemical methods involve the formation of covalent bonds between the functional groups on the support material and the functional group on the enzyme. Physical methods do not involve covalent bonding with the enzyme (Seçkin, 2009). Chemical and physical properties of the support material including porosity, functional group on the surface, particle size, and morphology have to be considered when making a choice for the immobilization technique (Adlercreutz, 2006). The methods use for immobilization can also affect the kinetic parameters of the immobilized enzyme (Seçkin, 2009). The most commonly used immobilization methods (Tosa et al., 1966) are shown in Figure 2.4.

2.5.3.1. Cross-linking

In this method immobilization is performed by the formation of intermolecular cross-linkages between enzyme molecules on an insoluble matrix. Bi- or multi-functional compounds serve as reagents for intermolecular cross-linking of enzymes, creating insoluble aggregates that are effective heterogeneous catalysts.

Reagents generally have two identical functional groups reacting with specific amino acid residues. Most widely used method employs glutardialdehyde which establishes intermolecular cross-linking with amino groups on the enzyme. (Kim, 2004)

Many of the active enzyme molecules are bound within the particles formed by cross linkage. Especially when high-molecular substrates are employed, access of the substrate to the innermost catalytic sites is limited by diffusion. Other disadvantages are

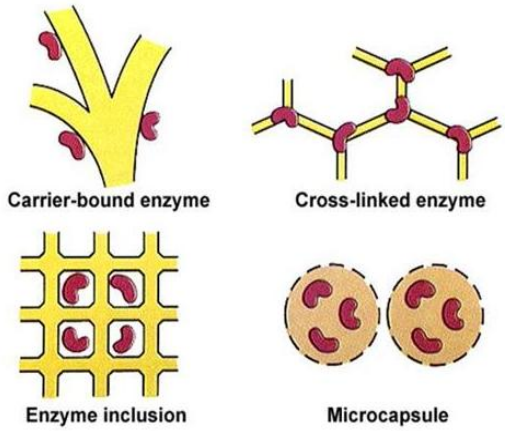

Figure 2.4. The schematic representation of the common immobilization techniques

that there may be loss of the enzyme activity due to the involvement of the active site in bound formation.A lot of cross-linking reagents such as bis-oxirane, carbodiimide, chromium (iii) acetate, glutardialdehyde, polyethyleneimine, sulfate-dextran, tris (hydroxymethyl) phosphinehave been used in the enzyme immobilization by cross-linking method (Panesar et al., 2010).

2.5.3.2. Physical adsorption

The method relies on non specific physical interaction between enzyme protein and the surface of matrix (Figure 2.5). Major advantage of adsorption as a general method of insoluble enzymes is that usually no reagents and only a minimum of activation step is required.

The interactions formed between the enzyme and the support material will be dependent on the existing surface chemistry of the support and on the type of amino acids exposed at the surface of the enzyme molecule. Adsorption involves, normally, weak interactions between the support and the enzyme such as ionic or hydrophobic interactions, hydrogen bonding, and van der Waals forces.

As the weak bonds are involved in this method, desorption of enzymes resulting from pH, temperature, presence of substrate is often observed. The other disadvantage of adsorption is the non-specific binding of other substances. This may cause the alteration of the properties of immobilized enzyme or the rate will probably decrease depending on the surface mobility of enzyme (Brena et al., 2013).

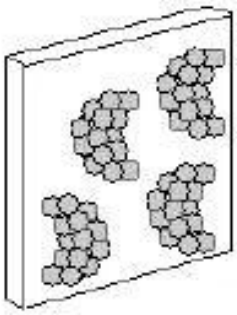

Figure 2.5. Binding of a catalyst to a carrier by adsorption (Costa et al., 2004)

2.5.3.3. Ionic binding

Ionic binding is achieved by binding of the enzyme proteins to water-insoluble carrier containing ion-exchanger residues (Figure 2.6). Polysaccharides and synthetic polymers having ion-exchange centers are usually used for carriers. The resultant binding forces are than those in the case of physical adsorption.

2.5.3.4. Entrapment

Entrapment which refers to the physical confinement (Figure 2.7) of enzyme in an environment where substrate is able to penetrate whereas enzyme can not escape is extremely popular for the immobilization of whole cells, because the transport of the nutrients and metabolites to and from the cell is possible through the gel (O'Driscoll, 1976). This method differs from the covalent binding and cross-linking in that enzyme itself does not bind to the gel matrix or membrane, so has a wide applicability.

The major limitation of entrapment for the enzyme immobilization is the possible leakage during repeated use due to having small molecular size compared to the cells. Next disadvantage of the method is diffusion limitations. Entrapment method is classified into five major types: lattice, microcapsule, liposome, membrane, and reverse micelle (Grosová et al., 2008). For β-galactosidase immobilization, the lattice method which enzyme is entrapped in the matrix of the various synthetic or natural polymers is the most widely used method. The microcapsule type involves an entrapment to a semi-permeable polymer.

The liposome type employs entrapment within an amphiphatic liquid-surfactant membrane prepared from lipid (usually phospholipids). In the reversed micelle type, β-galactosidase is entrapped within the reversed micelles, which are formed by mixing a surfactant with an organic solvent, for example aerosol OT/isooctane reverse micelles (Chen and Ou-Yang, 2004).

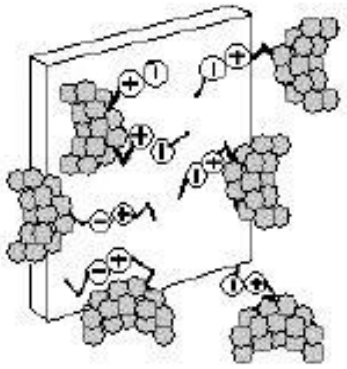

Figure 2.6. Ionic binding between the biocatalysts and a carrier (Costa et al., 2004)

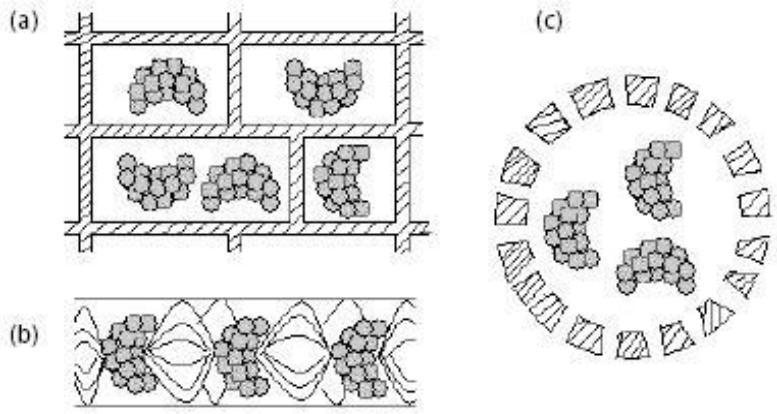

Figure 2.7. Enzyme encapsulation in a matrix (a), fiber (b), capsule (c) (Costa et al., 2004)

2.5.3.5. Covalent binding

Covalent binding method which is widely used technique for immobilization of enzymes is based on the formation of covalent bonds (Figure 2.8). Enzymes are covalently linked to water-insoluble matrix through the functional groups in the enzyme such as amino, hydroxyl, carboxyl, imidazol or phenolic groups which are not essential for the catalytic activity (Brena et al., 2013). An advantage of this method is that because of

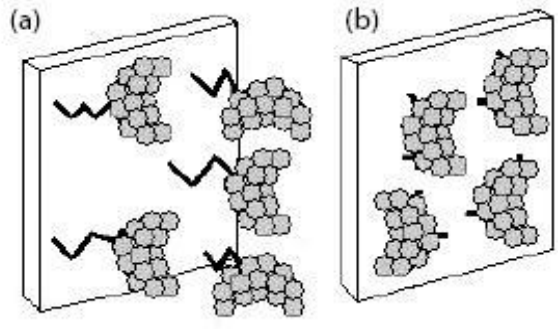

Figure 2.8. Biocatalysts bound to a carrier by covalent binding (Costa et al., 2004)

stable nature of bonds formed between enzymes and matrix, the enzyme is not released into the solution upon use. The wide range of choices is possible by selecting carrier materials and binding method (Husain, 2010).

This allows great flexibility in designing an immobilized enzyme with specific physical and chemical properties such as charge distribution, portioning capabilities. Furthermore, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). A frequently stated disadvantage of covalent binding is that it places great stress on the enzymes (Carrara and Rubilio, 1994; Albayrak ve Yang, 2002).

Different matrixes and techniques have been used for the covalent immobilization of β-galactosidase. They can be either natural polymers, such as modified cellulose, starch, dextran, agal polysaccharides, collagen, and gelatin; or they can be synthetic, such as polystyrene, polyacrylamide, polyacrylates, hydroxyalkyl methacrylates, and polyamides. Inorganic supports can also be used, such as porous glass, metal oxides, metals, sand, charcoal, and porous ceramics (Panesar et al., 2006; Novick ve Rozzell, 2005).

2.5.4. Enzyme immobilization with Eupergite CM

Eupergit C, the matrix having reactive epoxy groups and are commonly used for the immobilization of biomolecules. The matrice Eupergit C consisting of porous beads formed by co-polymerization of methacrylamide, allylglycidyl ether and N, N'-methylene-bis-acrylamide. Eupergite C is chemically and mechanically stable at pH 1-12. It is also very resistant against microbial degradation. It can be used in all reactors (Katchalski-Katzir and Kraemer, 2000).

Epoxy groups can form covalent bounds between enzyme molecules and Eupergit via amino, sulfhydryl and carboxyl groups that present on the side chain of amino acids residues in enzyme molecules at acidic, neutral and basic pHs. Figure 2.9 shows the covalent bonding between the epoxy group of Eupergit and amino grou of enzyme molecule. Eupergit C plurality of increasing the pH and thermal stability of the enzyme which binds and retains activity for a long time (Katchalski-Katzir and Kraemer, 2000).

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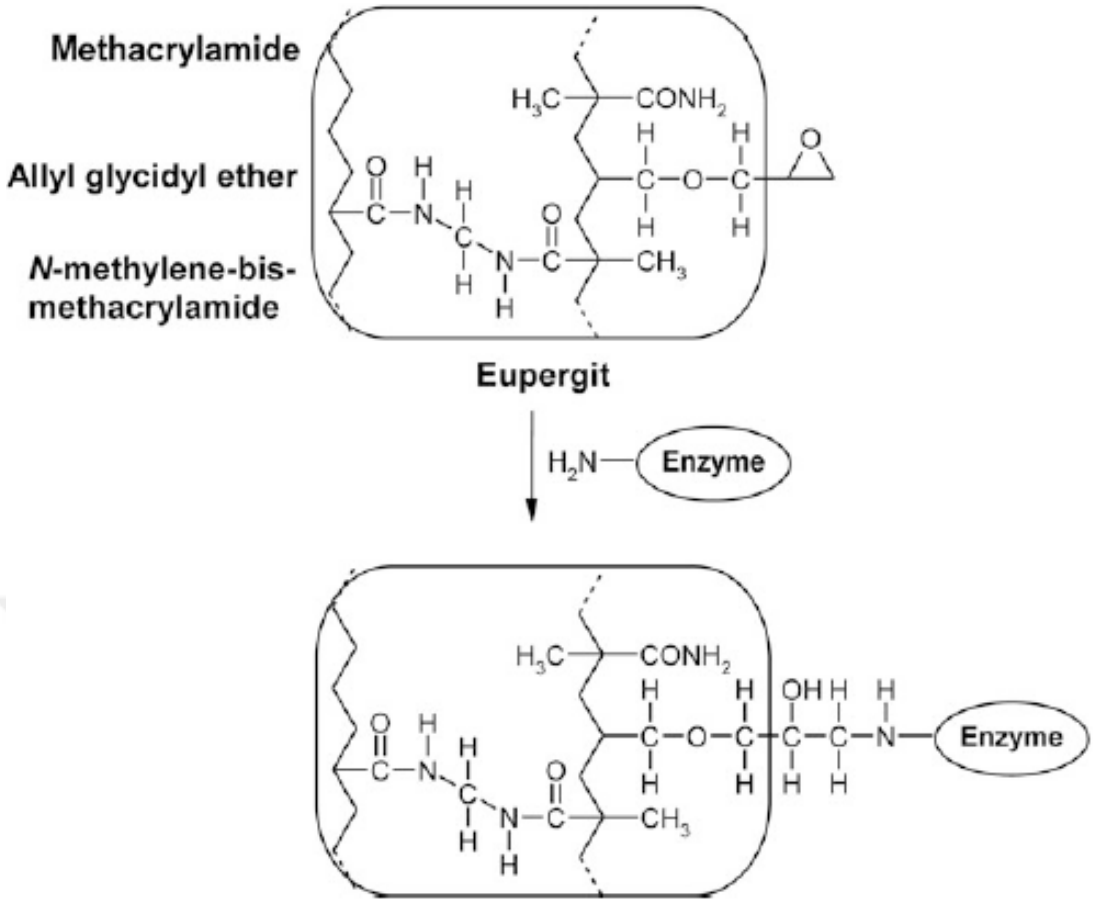

Figure 2.9. Enzyme immobilization with Eupergit (Knežević-Jugović et al., 2017)

Eupergit optimum pH and temperature of the enzyme may vary as a result of immobilization C kinetic constants (Katchalski-Katzir and Kraemer, 2000).

Eupergit C and the amount of enzyme, pH of the immobilization solution, molarity of the immobilization solution and binding time, binding efficiency and activity efficiency are significantly affected (Katchalski-Katzir and Kraemer, 2000).

In an immobilization study, these conditions must be optimized. Numerous enzymes with industrial potency have been covalently immobilized onto Eupergit C, usually above 80% efficiency (Katchalski-Katzir and Kraemer, 2000).

In some immobilization studies with matrixes bearing epoxy groups, significantincreases in enzyme activity were obtained after immobilization. In a study, Streptomyces levandulea ATCC 13664 was immobilized on penicillin V acylase Eupergit C with 144% activity (Torres-Bacete et al., 2000).

In another study, Aspergillus aculeatus β-Galactosidase was immobilized on

Eupergite C at a yield of 124% (Aslan and Tanrıseven, 2007). In another study, lipase was immobilized on Eupergite CM, which has a chemical structure similar to Eupergit C, with a yield of 170% activity (Aslan et al., 2014).

2.5.5. Immobilization of *Aspergillus oryzae* β-galactosidase

There are numerous studies in the literature concerning the immobilization of *Aspergillus oryzae* β-galactosidase for lactose hydrolysis. These are summarized below.

The immobilization of Aspergillus oryzae β-Galactosidase onto teflon membranes was carried out by reacting the functional groups of different acrylic monomers (2-hydroxyethyl methacrylate and methacrylic acid) grafted using Ɣ-radiation with two different crosslinking agents (glutaraldehyde or cyanuric chloride). 1,6-hexamethylenediamine was used as an intermediate and four different catalytic membranes were obtained with the same matrix. The highest activity yield obtained under optimum conditions was determined as 100% (Mohy Eldin et al., 1999).

In another study, El-Masry et al., (2000) immobilized *Aspergillus oryzae* β-Galactosidase with styrene and methyl methacrylate on a nylon plate with a high activity yield (95%) by covalent binding method.

In another study, Haider and Husain (2007) used insoluble Concavalin A obtained from bean extracts in the immobilization of *Aspergillus oryzae* β-Galactosidase after cross-linking with glutaraldehyde and obtained 92% activity yield.

In another study, *Aspergillus oryzae* β-Galactosidase was immobilized on calcium alginate-starch beads coated with concavalin A by adsorption and cross-linking methods and achieved 76% and 71% activity yield, respectively. The initial activity decreased by 61% when the immobilized enzyme was stored at +4 ^{o}C for 2 months (Haider and Husain, 2009).

Wu et al. (2010), immobilized *Aspergillus oryzae* β-galactosidase onto tetraethylorthosilicate and P123 triblock copolymer formed by molecular imprinting technique and they obtained 94% binding efficiency by encapsulating. Immobilized enzyme protects basal activity throughout nine repeated uses.

Guidini et al. (2010), immobilized *Aspergillus oryzae* β-galactosidase onto ionic exchange resin Duolite A568 by using ionic bonding method, and obtained 40.7% binding efficiency by applying the crosslinking method using glutaraldehyde. The activity of the enzyme crosslinked with glutaraldehyde down to 90% after thirty times usage.

In another study, *Aspergillus oryzae* β-galactosidase was immobilized in alginate-gelatin beads with 30% efficiency by entrapment and glutaraldehyde cross-linking methods (Freitas et al., 2011).

In another study, *Aspergillus oryzae* β-Galactosidase was immobilized with 85% efficiency by physical adsorption on zinc oxide nanoparticles, and the activity of immobilized enzyme gradually decreased to 75% after seven days (Husain et al., 2011).

Ansari et al. (2012) immobilized *Aspergillus oryzae* β-galactosidase, on the silver nanoparticles modified with glutaraldehyde and they have obtained 93% activity yield. While maintaining 88% of the immobilized enzyme activity at the end of six repeated use, and retained 93% of its initial activity at the end of sixty days.

Ansari and Husain (2012) immobilized the Aspergillus oryzae β-Galactosidase with 71% activity yield on the A-Celite 545 particles conjugated with glutaraldehyde and ethanolamine after adsorption . Immobilized enzyme activity is decreased to 71% after seven repeated use is decreased to 90% after one month when stored at +4 ° C.

In another study, Aspergillus oryzae β-galactosidase was immobilized with 97% activity yield onto ion exchange resin Duolite A568 by ionic adsorption followed cross-linking by glutaraldehyde. The activity obtained retained for thirty days (Fischer et al., 2013).

Aspergillus oryzae β-galactosidase was immobilized with 95% activity yield on the superdispersed particles that its surface modified by using glutaraldehyde and the activity obtained retained for two months (Ansari et al., 2015).

Aspergillus oryzae β-galactosidase covalently immobilized onto polyethylenimine and glutaraldehyde-treated calcium pectinate gel bead and the activity obtained decreased to 79.34% after repeated fourteen use (Wahba, 2016).

The increased activity after immobilization of the enzyme with epoxy activated supports have been reported in some studies (Torres-Bacete et al., 2000; Aslan and Tanriseven, 2007; Zarcula et al., 2009; Aslan et al., 2013; Aslan et al., 2014). Therefore, in the present thesis study, *Aspergillus oryzae* β-galactosidase will be trried to immobilize covalently onto Eupergit CM with highest immobilization and activity yields as possible as.

3. MATERIALS AND METHODS

3.1 Materials

Aspergillus oryzae β-galactosidase (Fungal Lactase) which have 7.70 IU/g activity, is a commerical powdered enzyme preparation, was provided as a gift by Bio-Cat (Troy, USA). Eupergit CM and was the gifts by degussa (Darmstadt, Germany). UV-VIS Spectrometer (UV-6300PC) was purchased from VWR (Radnor, USA). pH meter (Hanna HI 2020 edge), was purchased from Hanna Instruments Ltd. (Bedfordshire, UK). Magnetic stirrer (Heidolph MR Hei-Standard) was purchased from Heidolph UK-Radleys (Shire Hill, UK). Pure water appliance (Mini Pure 1, MDM-0170) was purchased from MDM Co. Ltd. (Suwon-si, South Korea). Precision scale (Shimadzu-ATX224) was purchased from Shimadzu Corporation (Kyoto, Japan). Orbital shaking heated incubator (Mipro-MCI) was purchased from Protek Lab Group, professional laboratory solutions company (Ankara, Turkey). Vacuum pump (Biobase, GM-0.50A) was purchased from Biobase Biodustry Co., Ltd. (Shandong, China). Bovine Serum Albumin (BSA), sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, lactose, sodium sulphite, phenol and D-glucose were purchased from Sigma-Aldrich (Taufkirchen, Germany). 3,5-dinitrosalicilic acid (DNS) was purchased from Alfa Aesar (Kandel, Germany). Sodium potasium tartrate (Roechelle salt) was purchased from VWR Prolabo Chemicals (Leuven Belgium). Sodium azide was purchased from Merck Millipore (Darmstadt, Germany). The semi-skimmed UHT cow's milk purchased from a local market. Semi-skimmed UHT cow milk was purchased from a local market.

3.2. Methods

3.2.1. Determination of protein

The amounts of proteins present in the immobilization buffer before and after immobilization were determined were determined by using Bradford Protein Assay Method (1976). Accordingly, the 0.1 mL samples were added to 3000 mL Bradford reagents in 10 mL vials and incubated 45 minutes at room temperature for the completing the formations of colour and after then the absorbances were measured at 595 nm by using UV spectrophotometer. The amount of immobilized enzyme protein

was assayed from the difference between the amount of protein used for immobilization minus that recovered into the supernatant plus washings (Manjon et al., 1995).

3.2.2. Determination of β-galactosidase activity

Firstly, 1 % (w/v) of lactose solutions prepared 5 mL 25 mM sodium di hydrogen phosphate buffer (pH 4.5 for free enzyme and 5.5 for immobilized enzyme) were reacted with 200 µL of free or 0.317 g immobilized enzymes having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively at 55 ^{o}C for 60 min in an incubator with gently shaking. 200 µL of aliquots from reaction mixtures were added to 1800 µL of distilled waters and boiled for 10 min to inactivate the enzymes. The amount of D-glucose formed was determined by measuring its absorbance using a UV spectrophotometer at 575 nm, according to method of Miller (1959). One IU *Aspergillus oryzae* β-galactosidase activity was defined as the amount of enzyme forming 1 µmol D-Glucose from lactose per minute, under optimum activity assay conditions.

3.2.3. Immobilization Procedure

Immobilization of *Aspergillus oryzae* β-galactosidase was performed by reacting 100 mg of Eupergit CM in 5 mL of sodium phosphate buffer (0.5 M, pH 5.5) at 25 ◦C for 24 hours in an orbital shaking incubator with gentle shaked at 150 rpm. After immobilization, the beads were filtered and washed with 15 mL of sodium phosphate buffer (0.1 M, pH 5.5) and 15 mL of distilled water as three aliquots respectively, on a sintered glass filter by suction under vacuum. After then, the beads have been stored in 5 mL of sodium phosphate buffer (0.1 M, pH 5.5) containing sodium azid at 0.02 % concentration in a refrigerator at +4 ^{o}C until use.

3.2.4. Optimization of Immobilization conditions

The amount of matrix used, the pH and solution Molarity immobilization with immobilization period affects the efficiency of the activity and immobilization (Katchalski-Katzir and Kraemer, 2000), the impact of each of these factors in turn can be determined by examining the immobilization conditions have been optimized. Optimum conditions for immobilization were determined by changing individually the conditions, (amount of Eupergit CM from 100 mg to 600 mg; pH from 3.5 to 5.5;

buffer concentration from 0.5 M to 2.0 M; and duration of immobilization from 24 h to 120 h).

3.2.5. Characterization of free and immobilized *Aspergillus oryzae* β-Galactosidase

3.2.5.1. Optimum pH

The optimum pHs for free and immobilized enzymes were investigated by performing the activity assay method with 1% (w/v) lactose solutions, at different pHs, at 55 ^{o}C.

3.2.5.2. Optimum temperature

The optimum temperatures of free and immobilized enzyme was found by conducting the activity assay method with 1% (w/v) of lactose solutions (pHs are 4.5 and 5.5 for the free and the immobilized enzymes respectively) at different temperatures.

3.2.5.3. pH Stability

Firstly, 200 μL of free or 0.317 g immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, were incubated with 2.5 ml of 25 mM sodium phosphate buffers with different pHs in an incubator at 55 ^{o}C) for 1 hour. Lastly, the retained activities were determined according to standard activity assay method by adding 2.5 mL of 2% (w/v) buffered lactose solutions (pH 4.5 for free enzyme and 5.5 for immobilized enzyme).

3.2.5.4. Thermal stability

Firstly, 200 μL of free or 0.317 g immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively were incubated with 2.5 ml of 25 mM sodium phosphate buffers at different pHs in an incubator at different temperatures (30-80 ^{o}C) for 1 hour. After then all free and immobilized enzymes were cooled in a ice bath for ten minutes for stoppinh the temperature effect. Lastly, the retained activities were determined according to standard activity assay method by adding 2.5 mL of 2% (w/v) buffered lactose solutions (pH 4.5 for free enzyme and 5.5 for immobilized enzyme).

3.2.5.5. Kinetic constants

Initial velocities for kinetic parameters were determined by performing the reactions between 200 μL of free or 0.317 g of immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively and lactose solutions at different concentrations (10 g/L to 200 g/L) for 15 min. K_m and V_{max} were calculated from Lineweaver–Burk plots.

3.2.5.6. Operational and storage stabilities

The operational and the storage stabilities of the immobilized enzyme were determined by performing the standard activity assay method in 20 repeated batch experiments and every two days during storage, respectively. The immobilized enzymes used for the determination of storage stability were stored in 0.1 M sodium phosphate buffers (pH 5.5) containing 0.02% (w/v) of sodium azide, in a refrigerator at +4 ^{o}C until next use. Before each use, the immobilized enzymes were filtered and washed with 15 mL of sodium phosphate buffer (0.1 M, pH 5.5) and 15 mL of distilled water on a sintered glass filter by suction under vacuum.

3.2.7 Hydrolysis of milk lactose

0.317 g of immobilized enzyme having 0,099 IU β-Galactosidase activity was reacted with 5 mL of semi-skimmed UHT cow's milk for 7 hours and the concentration changes were determined by using UV-VIS spectrophotometer at 30 minutes intervals.

3.2.8. Statistical Analysis

Each value represents the mean for three independent experiments performed in triplicates. All graphics were plotted by using OriginPro 8.0 software. Data was analyzed by using Microsoft Windows Excell

4. RESULTS AND DISCUSSION

4.1. Determination of protein

The BSA standard graph obtained according to Bradford (1976) method is shown in Figure 4.1. The coefficient of determination (R^2) for the correct equation (Equation 4.1) obtained from the graph is 0,99424.

$$Y = 0.29958X \tag{4.1}$$

The enzyme concentrations in the solutions are calculated by using Equations (4.1). Accordingly, *Aspergillus oryzae* β-Galactosidase enzyme concentration in 200 µL (77.2 IU) of enzyme solution used for immobilization was 9.58 mg/mL. The enzyme concentration of powder *Aspergillus oryzae* β-Galactosidase preparation was also as 958.3 mg / g. This result is consistent with the declaration (GA content is between 80 – 97%) of the manufacturer company (Murphy, 2017).

4.2. Determination of β-Galactosidase activity

The amount of D-glucose formed at the end of the reaction of 200 µL of free *Aspergillus oryzae* β-Galactosidase at optimum activity assay conditions, was found to be 7965.3 µg by using Equation 4 obtained from Figure 4.2. Free *Aspergillus oryzae* β-Galactosidase activity was calculated as 0.077 IU/mg by using Equation 5. Furtheremore, the activity of powder *Aspergillus oryzae* β-Galactosidase was also calculated to be 7.70 IU/g for lactose as substrate. The amount of free *Aspergillus oryzae* β-Galactosidase having 1 IU activity was also calculated as 124.4 mg.

$$Y = 0.00104X \tag{4}$$

$$IU / mg\ Enzyme = \frac{Released\ D\text{-}Glucose\ (\mu mol)}{Enzyme\ used\ (mg)\ x\ Duration\ of\ reaction\ (min)} \tag{5}$$

4.3. Optimization of immobilization conditions

4.3.1. Effect of buffer's pH on immobilization efficiency

Table 4.1 shows the influence of pH on immobilization. The highest immobilization yield (100.00%) and activity yield (129.82%) were obtained at optimum pH (4.5) for free enzyme. The epoxy groups on Eupergit C can react with various reactive groups of enzymes in a wide pH range (0–12) . But, the highest activity yield is

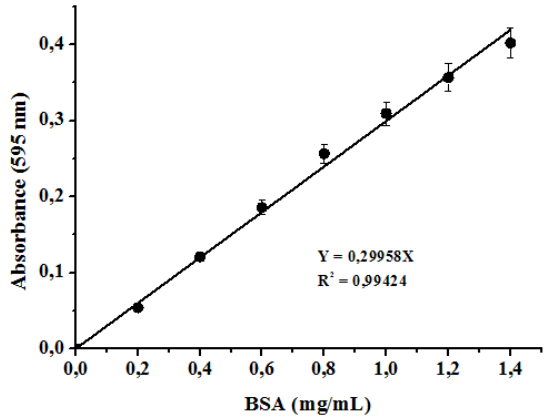

Figure 4.1. BSA standard graph

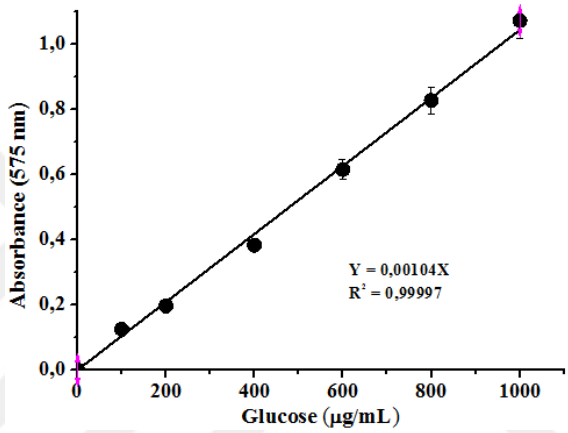

Figure 4.2. D-Glucose standard graph.

Table 4.1. Effect of immobilization buffer's pH on immobilization efficiency

Immobilization Buffer's pH	Immobilization Yield* (%)	Activity Yield **(%)
3.5	97.43 ± 0,05	78.79 ± 0,03
4.0	96.92 ± 0,03	86.32 ± 0,02
4.5	79.34 ± 0,02	92.16 ± 0,04
5.0	97.84 ± 0,04	79.41 ± 0,05
5.5	98.10 ± 0,03	73.51 ± 0,04

* 200 µL of enzyme solutions having 0.077 IU β-Galactosidases activity were incubated with 100 mg of Eupergit CM in 5mL of sodium phosphate buffers (0.5 M) at different pHs and room temperature with shaking at 150 rpm in an incubator for 24 hours.

** 200 µL of free or 0.317 g of immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively were reacted with 5 mL of 1% (w/v) buffered lactose solutions (pH 4.5 for the free enzyme and 5.5 for the immobilized enzyme) at 150 rpm and at 55 ^{o}C in an incubator for 60 minutes.

usually achieved at the optimum pH range in the immobilization of enzymes with Eupergit C (Katchalski-Katzir and Kraemer, 2000).

4.3.2. Effect of buffer molarity on immobilization efficiency

Table 4.2 shows that the maximum immobilization yield (100.00%) and activity yield (129.82%) were obtained with 0.5 M phosphate buffer. According to Table 2, when the molarity of immobilization buffer increased, both of immobilizetion and activity yields have been decreased. Binding and activity yields in covalent enzyme immobilization on synthetic carriers such as epoxy carriers were often affected by the properties of the salts and their concentration (Smalla et al., 1988). The higher salt concentrations can make unfavorable changes in the three dimensional structures of enzyme molecules. The decrease in the immobilization and in the activity yields can be explained with this general information.

4.3.2. Effect of Eupergit CM amount on immobilization efficiency

Different amounts of Eupergit CM (100–400 mg) for *Aspergillus oryzae* β-Galactosidase (200 μL) were tested. Table 4.3 shows that highest immobilization yield (100.00%) and activity yield (129.82%) were obtained for 100 mg of Eupergit CM. The usage of the higher amounts of Eupergit CM has been resulted in the low activity yield. This is possibly the result of the deterioration of the three dimensional structure of enzyme molecules upon multipoint attachment of enzyme molecules to the support. Furthermore the reaction of epoxy groups on support with amino acid residues associated with active site of enzyme molecules can lead to decreasing the activity.

4.3.4. Effect of immobilization duration on immobilization efficiency

The duration of immobilization is of importance: maximum immobilization yield (100.00%) and activity yield (129.82%) were achieved for 96 hours (Table 4.4). As seen in Table 4.4, activity yield has decreased by increasing the duration of immobilization after 96 hours. This probably is due to changing the three dimentional structure of enzyme, upon multipoint attachement of enzyme to the support. By optimizing the immobilization conditions 100.00% of immobilization yield and 129.82% of activity yield were achieved. Similar results are frequently encountered in the literature (Torres-Bacete et al., 2000; Aslan and Tanrıseven, 2007; Zarcula et al., 2009; Aslan et al., 2013; Aslan et al., 2014). This result is the best in the literature related to immobilization of *Aspergillus oryzae* β-Galactosidase. The maximum activity yield obtained in previous studies is 97 % (Fischer et al., 2013).

Table 4.2. Effect of immobilization buffer's molarity on ımmobilization efficiency

Buffer's Molarity (M)	Immobilization Yield* (%)	Activity Yield** (%)
0.5	79.34 ± 0,02	92.16 ± 0,04
1.0	90.54 ± 0,03	77.59 ± 0,03
1.5	92.70 ± 0,04	66.73 ± 0,05
2.0	94.17 ± 0,05	53.42 ± 0,02

* 200 µL of enzyme solutions having 0.077 IU β-Galactosidases activity were incubated with 100 mg of Eupergit CM in 5 mL of sodium phosphate buffers (pH 5.5) at different concentraions at room temperature by shaking in an incubator at 150 rpm for 24 hours.

** 200 µL of free or 0.317 g of immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively were reacted with 5 mL of 1% (w/v) buffered lactose solutions (pH 4.5 for the free enzyme and 5.5 for the immobilized enzyme) at 150 rpm and at 55 ^{o}C in an incubator for 60 minutes.

Table 4.3. Effect of Eupergit CM amount on immobilization efficiency

Eupergit CM (mg)	Immobilization Yield (%)	Activity Yield (%)
100	79.34 ± 0,02	92.16 ± 0,04
200	87.88 ± 0,04	80.73 ± 0,05
300	89.96 ± 0,05	73.95 ± 0,02
400	97.03 ± 0,02	64.22 ± 0,03

* 200 µL enzyme solutions having 0.077 IU β-Galactosidases activity were incubated with different amounts of Eupergit CM in 5mL of sodium phosphate buffers (0.5 M, pH 5.5) and room temperature with shaking at150 rpm in an incubator for 24 hours.

** 200 µL free or 0.317 g immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively were reacted with 5 mL of 1 % (w/v) buffered lactose solutions (pH 4.5 for the free enzyme and 5.5 for the immobilized enzyme) at 150 rpm and at 55 oC in an incubator for 60 minutes.

Table 4.4. Effect of Immobilization Duration on Immobilization efficiency

Duration of Immobilization (hours)	Immobilization Yield* (%)	Activity Yield** (%)
24	79.34 ± 0,02	92.16 ± 0,04
48	84.02 ± 0,04	104.52 ± 0,03
72	93.39 ± 0,05	118.02 ± 0,05
96	100.00 ± 0,02	129.82 ± 0,04
120	100.00 ± 0,03	112.56 ± 0,02

* 200 µL of enzyme solutions having 0.077 IU β-Galactosidases activity were incubated with 100 mg of Eupergit CM in 5 mL of sodium phosphate buffers (0.5 M, pH 5.5) at room temperature with shaking in an incubator at 150 rpm for different durations.

** 200 µL of free or 0.317 g of immobilized enzyme having 0.077 IU and 0.099 IU β-Galactosidases activities, respectively were reacted with 5 mL of 1% (w/v) buffered lactose solutions (pH 4.5 for the free enzyme and 5.5 for the immobilized enzyme) at 150 rpm and at 55 ^{o}C in an incubator for 60 minutes.

4.4. Characterization of free and immobilized *Aspergillus oryzae* β-Galactosidase

4.4.1. Optimum pH

According to Fig 4.3, the optimum pH of *Aspergillus oryzae* β-Galactosidase was shifted from 4.5 to 5.5 by immobilization. There are a lot of studies in the literature that report the changed optimum pH after immobilization. For example, the optimum pH of *Aspergillus oryzae* β-Galactosidase had shifted from 4.0 to 4.5 and 5.0 after

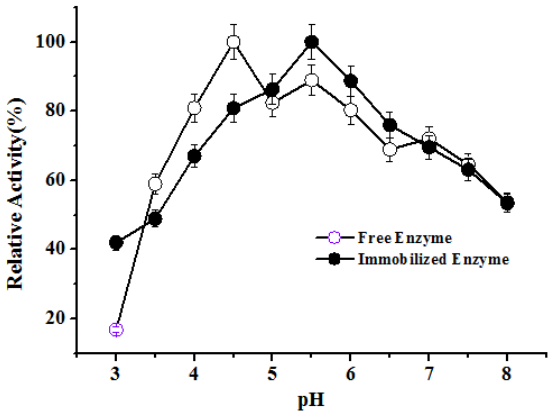

Figure 4.3. Optimum pH of free and immobilized *Aspergillus oryzae* β-Galactosidase.

immobilization with low-pressure plasma-modified cellulose acetate (CA) membrane (Güleç et al. 2010). The optimum pH of free and immobilized *Aspergillus oryzae* β-Galactosidase was found as 4.5 (Husain et al., 2011). The optimum pH of *Aspergillus oryzae* β-Galactosidase had shifted from 4.0 to 4.5 and 5.0 after immobilization with different membranes (Mohy Eldin et al., 1999). In another study, after immobilization with nylon Hydrolon membrane, the optimum pH of *Aspergillus oryzae* β-Galactosidase had shifted from 4.0 to 4.5 (El-Masry et al., 2000). It is known that optimum pH of enzymes can be changed negatively or positively around of optimum pH due to hydrophilic or hydrophobic interactions between supports and enzymes. This effect is known as partitioning effect. On the other hand, immobilized enzyme is more active than free enzyme in the pH ranges tested (Wang, 2012). This result agree with previous studies.

4.4.2. Optimum temperature

Figure 4.4 shows that the optimum temperature gange (55-60 °C) was not affected by immobilization. This result agree with previous studies. For example, Husain et al. (2011) determined the optimum temperature 50-60 ^{o}C for *Aspergillus oryzae* β-Galactosidase. It is also clear that the immobilized enzyme exhibits higher activity than the free enzyme at the entire temperature range tested. Further, we can see in the Figure 4.4 that the immobilized enzyme exhibit more activity than free enzyme at the tested temperatures range. Generally, at high temperatures, immobilized enzymes exhibit higher activity than free enzymes thanks to immobilization increases the thermal stability of the enzyme and the optimum temperature may not changed (Torres-Bacete et al., 2000).

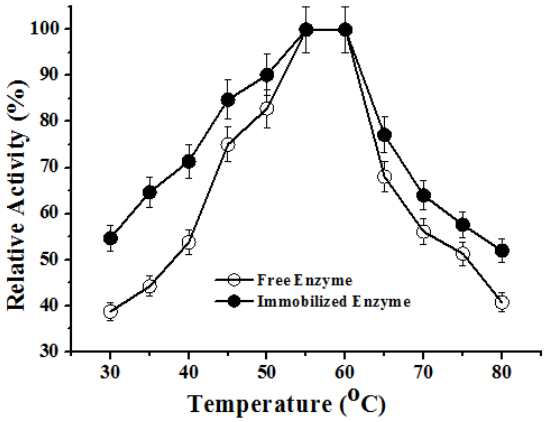

Figure 4.4. Optimum temperature of free and immobilized Aspergillus oryzae β-Galactosidase.

4.4.3. pH stability

In Figure 4.5, the stability of immobilized enzyme appears that is higher than free enzyme. Accordingly, it is also seen that the immobilized enzyme is more stable than the free enzyme at all tested pH values. Probably this is a result of strengthening the three-dimensional structure of the enzyme, after binding of the enzyme molecules to matrix via covalent bond that is strongest bond.

4.4.4. Thermal stability

Figure 4.6 shows the effect of temperature on the stabilities of free and immobilized enzyme. It can be seen in the figure that immobilization increased the thermal stability of the enzyme at the higher temperature range tested. Free enzyme lost approximately 60 % of its activity at 80 ^{o}C while the immobilized enzyme retained more than 85 % of its activity at same temperature. *Aspergillus oryzae* β-Galactosidase has previously immobilized on Eupergit C (Huzjak et al., 1994). But their immobilized enzyme has lost 60 % of its activity at 65 ^{o}C. It is generally known that immobilization improves the thermal stability of enzymes (Torres-Bacete et al., 2000). Increased thermal stability is important for the using the immobilized enzymes in higher temperatures.

4.4.5. Kinetic constants

The Michaelis-Menten constants of free and immobilized enzyme were calculated using the Lineweaver-Burk plot (Figure 4.7). After immobilization, V_{max} value has been decreased from 0.366 g/L. min to 0.415 g D- Glucose/L.min, while K_m value has decreased from 307.7 g/L to 234.2 g lactose /L. while K_m value decreased from K_m

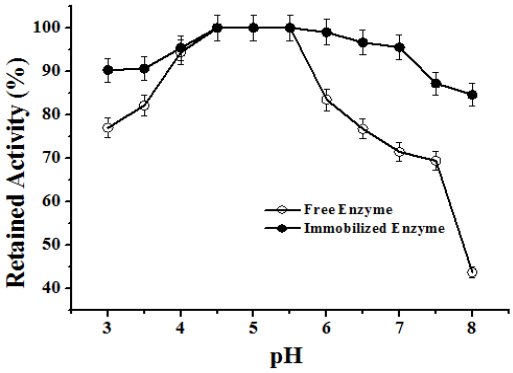

Figure 4.5. pH stability of free and immobilized *Aspergillus oryzae* β-Galactosidase.

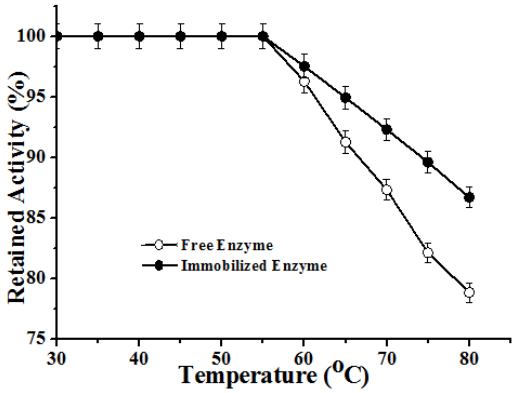

Figure 4.6. Thermal stability of free and immobilized *Aspergillus oryzae* β-Galactosidase.

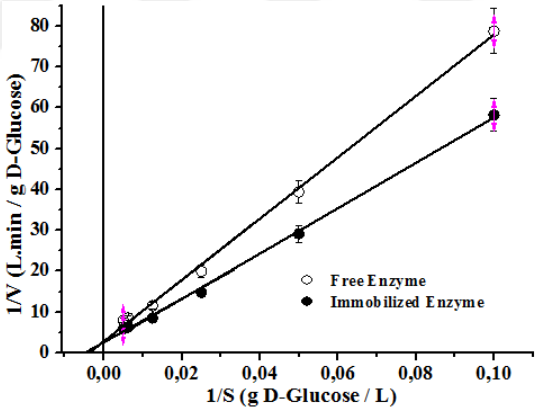

Figure 4.7. Lineweaver–Burk plots of free and immobilized *Aspergillus oryzae* β-Galactosidase.

value indicates the affinity of an enzyme to a substrate (Torres-Bacete et al., 2000). It is well known that when the K_m value decreases, the affinity of the enzyme to substrate increases. The lower K_m value of immobilized *Aspergillus oryzae* β-Galactosidase shows that its affinity to lactose has increased. Furtheremore, the higher V_{max} value of immobilized *Aspergillus oryzae* β-Galactosidase shows that substrate molecules can easily reach than free enzyme to the active site due to conformational changes. Therefore, it can be said that these results explain the reason of increased activity after immobilization.

4.4.6. Operational and storage stabilities of immobilized enzyme

Operational and Storage stabilities of immobilized enzyme are shown in Figure 4.8 and Figure 4.9, respectively. The immobilized enzyme retained its initial activity during repeated 20 consequtive batch experiments and during the storage at optimum conditions (in 5 mL of 0.1 M sodium phosphate buffer containing 0.02% (w/v) sodium azide, pH 5.5, at +4 ^{o}C in a refrigerator). Therefore, it can be said that immobilized enzyme developed in this study have high operational and storage stabilities.

4.5. Hydrolysis of milk lactose

0.317 g of immobilized enzyme having 0.099 IU β-Galactosidase activity was reacted with 5 mL of 5 semi-skimmed UHT cow's milk at 55 ◦C for 7 hours and concentrations of lactose and glucose were determined with 30 minutes intervals by using UV spectrophotometer (Figure 4.10). As seen in the figure, the milk lactose was completely hydrolyzed in four hours. Therefore, it can be said that the immobilized *Aspergillus oryzae* β-Galactosidase obtained in this thesis study can be succesfully used for the industrial production of lactose-free milk.

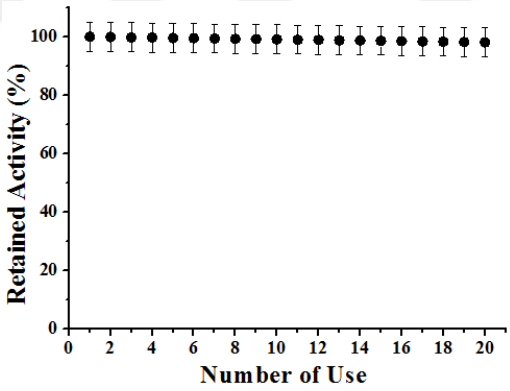

Figure 4.8. Operational stability of immobilized *Aspergillus oryzae* β-Galactosidase.

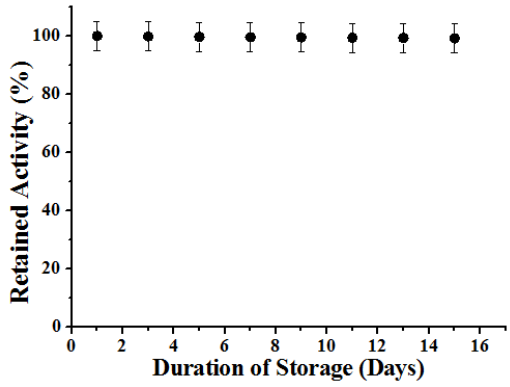

Figure 4.9. Storage stability of immobilized *Aspergillus oryzae* β-Galactosidase.

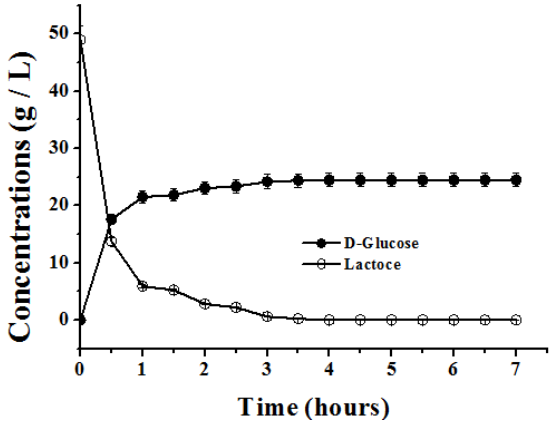

Figure 4.10. Hydrolysis of milk lactose by using immobilized *Aspergillus oryzae* β-Galactosidase.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusions

Aspergillus oryzae β-Galactosidase was covalently immobilized onto Eupergit CM with 100 % immobilization yield and 129.8% activity yield, by optimizing the immobilization conditions. The optimum pH was shifted from 4.5 to 5.5 while the optimum temperature (55 °C) was not changed after immobilization. The Km value decreased from 307.7 g/L to 234.2 g/L while the Vmax value increased from 0.366 g D-glucose/L.min to 0.415 g D-glucose/L.min after immobilization.

The immobilized enzyme has not lost its activity during twenty repeated uses and during fifteen days of storage under optimum conditions (in 5 ml of 0.1 M sodium phosphate buffer at + 4 °C in a refrigerator).

The semi-skimmed UHT cow's milk lactose was completely hydrolyzed within four hours. As a result, we can say that the immobilized *Aspergillus oryzae* β-Galactosidase obtained in this thesis study can be used in the industrial production of lactose free milk.

5.2. Recommendations

Firstly, the results achieved in this thesis can be published in any SCI indexed journal.

Secondly, a patent application can be made for the production of lactose-free cow's milk using the immobilization method developed in this thesis for *Aspergillus oryzae* β-Galactosidase.

Finally, we can work with the large dairy companies in our country to make industrial production of lactose-free cow's milk for those peoples have lactose intolerance.

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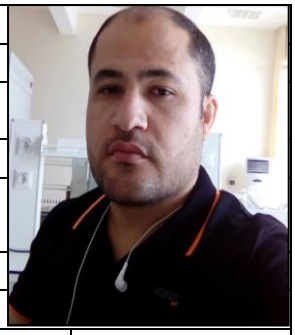
Name and Surname	Alan Yaseen Taher	
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Bachelor of Science	Salahaddin University, College of Agriculture, Department of Food Science & Technology. Erbil (IRAQ)	2002 – 2006
Master of Science	Siirt University Faculty of Engineering and Architecture Food Engineering Department Siirt (TURKEY)	2016 – 2018
WORK EXPERIENCE		
Organization	**Position and Responsibilities**	**Time Interval**
Massafi Water Factory Rasty cross-section, Erbil (IRAQ)	Arrangement all managerial orders, warehouse arrangement materials and other works.	February 2017 Present
Kar Oil Group Maghmur, Khurmala Pipeline, Erbil (IRAQ)	**Administrative** - Documentation & Planning. - Controlling Document, Managerial Orders, - Coordinating with the head office in Erbil - Supplying them with daily and Monthly progress reports	September 2009 – July 2015
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PERSONEL SKILS		
Languages	Kurdish (Mother Language)	
	Arabic	
	English	
Computer skills	MS word, MS excel, MS Publisher, MS PowerPoint and MS access (Database), MS Note One	
Publications	Announcement Representative of food organization, Erbil. www.facebook.com/rekxrawy.xorak www.kenanaonline.com/alanblbas	
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