β-GALACTOSIDASE OF *KLUYVEROMYCES LACTIS*: IMMOBILIZATION, CHARACTERIZATION AND HYDROLYSIS BEHAVIOR OF ENZYME

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İZMİR

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ABSTRACT

BETA-GALACTOSIDASE OF KLUYVEROMYCES LACTIS: IMMOBILIZATION, CHARACTERIZATION AND HYDROLYSIS BEHAVIOR OF ENZYME.

β-galactosidase (lactase) enzyme is of great industrial interest, since it can be used to solve problems associated with whey disposal and lactose crystallization in sweetened and frozen dairy products such as ice cream. All over the world, many people suffer from lactose intolerance and lactase preparations are used as supplements for these persons. β-galactosidase is also used to produce prebiotics since this enzyme hydrolyses lactose into galactooligosaccharides. Immobilized β-galactosidase preparations are preferred in many processes because they can be recycled and maintain their activities for a long time without loosing their chemical stability.

Novel cross-linked chitosan-hydroxyapatite composite support has been prepared, lactase from *Kluyveromyces lactis* was immobilized onto these beads. Lactase immobilization mechanism and effect of factors such as initial glutaraldehyde concentration, temperature, pH, initial lactase concentration, solid-liquid ratio on immobilzation mechanism were studied.

Optimum cross-linking was obtained at the glutaraldehyde concentration of 100 mg/L. The optimum values of temperature, pH and solid/liquid ratio on lactase/HA-Chitosan were found to be 20^0C, pH 7.5 and 0.3g/ml Vg/Vl, respectively. The pH and thermal stabilities of free and immobilized enzymes were also investigated and it was observed that the relative activity remained above 83.2% within pH 6-7.5 and maximum activity yield was obtained at 37^0C for free and all immobilized enzymes. The Michaelis constant K_m and V_{max} of immobilized and free enzyme on chitosan-hydroxyapatite composite beads were found to be 9.5 mM and V_m 454.5 μmol ONP min^{-1} mg^{-1} protein and 1.011 mM and 1098.9 μmol ONP min^{-1} mg^{-1} protein, respectively.

ÖZET

KLUYVEROMYCES LACTİS β-GALAKTOZİDAZ ENZİMİ: İMMOBİLİZASYONU, KARAKTERİZASYONU VE HİDROLİZ DAVRANIŞI.

β-galaktosidaz enzimi, atık peyniraltı suyu ve dondurma gibi tatlı ve dondurulmuş ürünlerde laktoz kristalizasyonundan kaynaklanan bazı problemlerin önlenmesi sağlanmak amacıyla kullanılabildiklerinde büyük bir ticari öneme sahiptirler. Tüm dünya üzerinde pek çok insan laktoz tahammülsüzlüğü sorunuyla karşı karşıyadır ve laktaz preparatları bu kişiler tarafından kullanılmaktadır. Beta-galaktozidaz enzimi aynı zamanda laktozu galaktooligosakkaritlere parçalamasından dolayı prebiyotiklerin üretiminde de kullanılmaktadır. İmmobilize β-galaktozidaz preparatları pek çok işlemde tercih edilmektedirler çünkü tekrar kullanılabilirler ve aktivitelerini kimyasal kararlılıklarını kaybetmeden uzun süre koruyabilirler.

Bu çalışmada kitosan-hidroksiapatit kompleks küreleri hazırlanmış ve ticari olarak sağlanan beta--galactosidaz enziminin (*Kluyveromyces lactis*) bu kürelere immobilizasyonu gerçekleştirilmiştir. İmmobilizasyon mekanizması ve etki eden parametrelerin (başlangıç glutaraldehit konsantrasyonu, sıcaklık, pH, başlangıç laktaz derişimi, katı matris-sıvı oranı) immobilizasyon mekanizmasına etkisi incelenmiştir.

Optimum glutaraldehit derişimi 0,1% olarak tespit edilmiştir. Sıcaklık, ortam asitliği ve katı-sıvı oranının laktaz/kitosan-hidroksiapatit sisteminin çalışması için optimum değerleri sırasıyla 20^0C, pH 7.5 and 0.3g/ml Vk/Vs olarak belirlenmiştir. Aynı zamanada immobilize enzimin ortam asitliği ve sıcaklık bakımından karakteristiği ele alınmış ve pH 6-7.5 aralığında relatif aktivitesinin 83.2% sini koruduğu ve optiumum sıcaklığın hem immobilize enzim hem de serbest enzim için 37^0C olarak kaldığı gözlenmiştir. The Michaelis sabitlerine bakıldığı zaman ise, serbest enzim için K_m and V_{max} değerlerinin sırasıyla 1.011 mM and 1098.9 μmol ONP min^{-1} mg^{-1} protein, immobilize enzim için ise sırasıyla 9.5 mM and V_m 454.5 μmol ONP min^{-1} mg^{-1} protein olarak bulunmuş ve sonuçta immobilizasyonla birlikte reaksiyon hızında bir düşüş gözlenmiştir.

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CHAPTER 1

INTRODUCTION

Enzymes are attractive catalysts which serve to accelerate the chemical reactions of living cells as they are effective 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 avoid 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. 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). Additionally, galactooligosaccharides can also be formed by transgalactosylation. 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 drawbacks of the free enzymes are their limited life time. Most enzymes are not stable when stored at high temperatures. They can be easily disturbed by external stresses such as high pressures and temperatures, extreme pH's, organic solvents, freezing. This affects the structures of proteins and causes a decrease in catalytic activity of an enzyme. Moreover, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Such drawback may be overcomed by using more stable and reusable immobilized enzyme biocatalysts that can be easily removed from the reaction medium, thus lowering the costs involved (Maio, et al. 2001, Tümtürk, et al. 2007).

Immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic.

It has been increasingly used in industrial applications as it facilitates the separation of biocatalysts from the effluents and, hence, the recovery and purification of the products. Moreover, solid biocatalysts offer the major advantage of being reusable. The large variety of matrices that can be used ranges from natural and synthetic

polymers to silica beads. Covalent immobilization often proceeds by the reaction of specific functionalities at the support surface with amino acid side chains that are readily available on the enzyme surface. The covalent coupling may induce drastic changes in the enzymatic kinetics especially when it occurs near the active site. Significant inactivation of the enzyme may result during the cross-linking step and is the major drawback of this method (Novick and Rozzell 2005). Another important effect is to reduce the enzyme flexibility. As the number of linkages between the enzyme and the support increases, so does the enzyme rigidity. By providing a maximum rigidity, multi-point covalent immobilization is likely to prevent enzyme unfolding upon heating or in the presence of a denaturant.

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). 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). *Kluyveromyces lactis* β galactosidase; with a neutral optimum pH, is one of the few yeasts that is well suited for the hydrolysis of lactose.

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.

With this perspective, in this research work the effects of various immobilization process parameters on the residual activity and the protein loading of the immobilized enzyme preparations were studied. Characterization of the immobilized enzyme in order to compare with free enzyme was done. Steps were also taken for the development of a suitable batch hydrolysis of lactose in order to run many reactions in parallel, which would aid in obtaining more information within short time.

CHAPTER 2

LACTOSE

Lactose, the main carbohydrate of milk, is a disaccharide with low relative solubility, which is hydrolyzed into its constituent molecules glucose and galactose. 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. 1995).

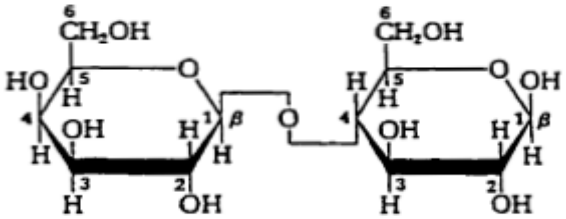


Figure 2.1.Structure of Lactose

Lactose in solid form can be crystalline or amorphous. Crystalline lactose can exist in one of two distinct forms: β-lactose and α-lactose monohydrate. Both forms can crystallize but the physico-chemical relationships between the different forms of lactose are very complex. Crystals of a-lactose can be formed by concentrating an aqueous lactose solution to supersaturation and allowing it to crystallize at temperatures below 93.5 ^{0}C. α-lactose is associated with one molecule of water; crystallizes as a monohydrate. These crystals are very hard and brittle. β-lactose; the anomer of a-lactose is formed when a highly concentrated solution of lactose is allowed to crystallized at temperatures above 93,5 ^{0}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 during and after spray drying, in powders such as whey powders which have high lactose content (Schuck and Dolivet 2002).

In milk, lactose is present in two isomeric forms called α- and β-lactose as well. 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 (Lactose 2008).

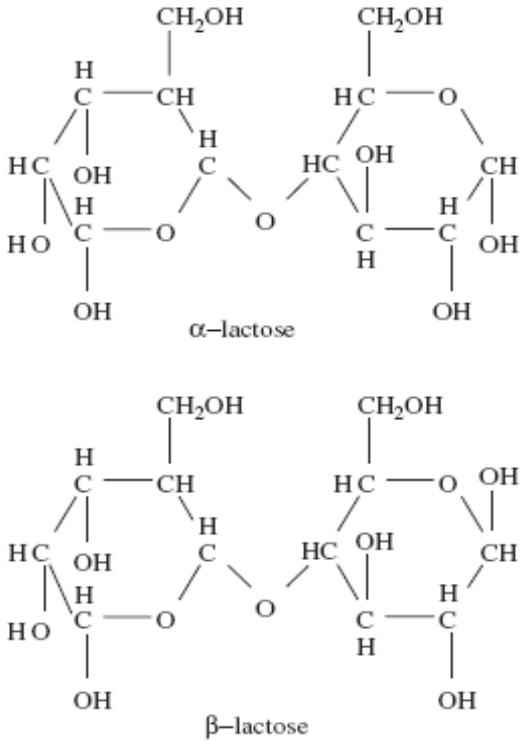


Figure 2.2. The structure of lactose molecules in α and β form.
(Source: Ganzle, et al. 2008)

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.

2. 1. Lactose Intolerance

Lactose intolerance is caused by the deficiency of the β-galactosidase enzyme (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. Such persons not only suffer from malabsorption but also suffer from general impairment of the normal digestion process.

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, Wilson 2005).

Lactose deficiency has mainly three types described as primary, secondary or congenital. 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 (Food Reactions 2008).

Table 2.1. Incidence of lactase non-persistent in different population groups around the world. (Source: 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

2.2. Hydrolysis

2.2.1. Acid 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).

2.2.2. Enzymatic Hydrolysis

Many research efforts have been focused on ways to reduce or remove the sugar in dairy products. Most common way to accomplish this is through the use of enzymes (Kocak 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 lactase obtained from Escherichia coli were described (Wallenfels and Malhotra 1961). In the reaction mechanism; the active site of β-galactosidase contains the cysteine and histidine residue which acts as proton donor and acceptor, respectively. Cysteine contains the sulphydryl group and histidine residues contains imidazole group acting as proton donor and as nucleophile site to facilitate splitting of the glycosidic bond respectively (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.

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).

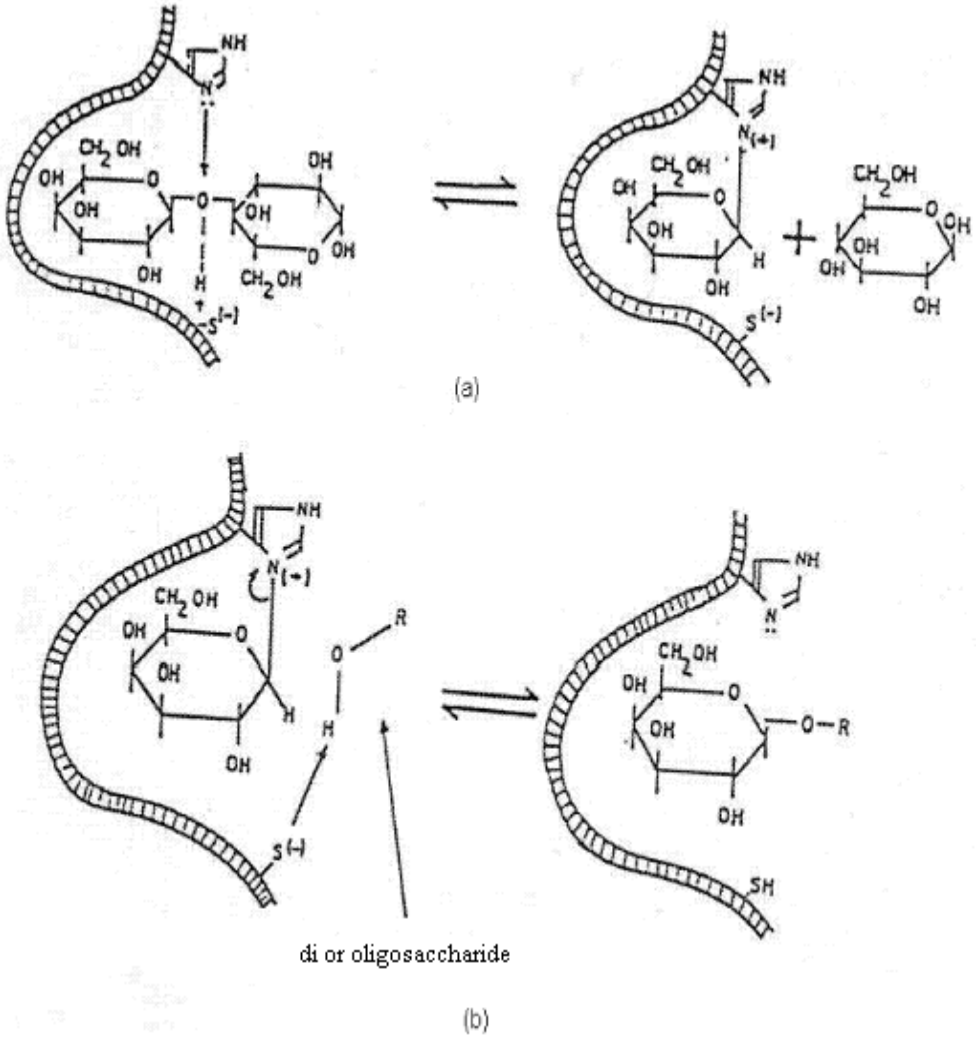


Figure 2.3. Schematic mechanism of lactose hydrolysis by β-galactosidase. a)enzyme-galactosyl complex formation and glucose liberation b)enzyme-galactosyl complex transferred to an acceptor containing hydroxyl group. (Source: Richmond, et al. 2002)

Oligosaccharides are polymeric carbohydrates consisting of two to ten monomer residues joined through glycosidic bonds. They can be obtained from different sources such as crops like onion and garlic(fructo-oligosaccharides) or lactose present in milk and whey (galacto-oligosaccharides) (Hsu, et al. 2007).

Galactooligosaccharides are $(galactosyl)_n$ lactose oligomers where n may vary from 2 to 4 and they are synthesized from lactose by a transgalactosylation reaction catalyzed by β-galactosidase. The reaction mechanism for formation of galacto-oligosaccharides was first proposed by Wallenfals and Malhotra who used as a growth factor for Bifidobacterium spp. In food owing to their roles in controlling pH of large intestine by promoting the growth of lactic acid and acetic which limits the growth of pathogens and putrefactive bacteria (Khalid and Beyong 1990, Rustom, et al. 1998).

The amount and composition of galacto-oligosaccharides vary with the source of enzyme, lactose concentration and the reaction conditions in process (Hsu, et al 2007). In case of having low concentration of lactose (high concentration of water) in reaction mixture, oligosaccharide production will be low. The yield of oligosaccharides formation can be raised by increasing initial concentration of lactose in reaction mixture (Mahoney, et al. 1998, Albayrak, et al. 2002).

CHAPTER 3

β-GALACTOSIDASE

β-galactosidase (B-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 Aksoz 2004).

3.1. Sources of β-galactosidase

β-galactosidase is distributed among microorganisms, plants and animals in which they fulfill a key role in hydrolysis of lactose (Table 3.1) (Gekas and Lopez-Leiva 1985).

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. 2006).

Lactase from several sources have been well characterized, especially the enzyme from *Escherichia coli,* which serves as a model for understanding of action of the enzyme. Although their use is not viable for products in food industry because of its

toxicity associated with coliform. (Jurado, et al. 2002). However enzyme characteristics including temperature-pH optimum and molecular weight can differ between sources.

Table 3.1. Sources of β-galactosidase
(Source: Gekas and Lopez-Leiva 1985)

Plants	peach, apricot, kefir, almond
Animals	small intestine, brain
Fungi	*Kluyveromyces lactis* *Kluyveromyces fragilis* *Candida pseudotropical* *Brettanomyces anomolus* *Wingea robersii*
Bacteria	*Escherichia coli* *Streptococcus thermophilus* *Bacillus circulans* *Bacillus steorotherrphilus* *Lactobacillus sporogenes*
Yeast	*Aspergillus niger* *Aspergillus oryzeae* *Mucur pucillus* *Alternaria palmi* *Curvularia inoegualli*

3.2. Properties of β-galactosidase

β-galactosidase can be found both in animals, plants and microorganisms. Despite, lactases from bacterial sources are more preferable due to their good stability higher enzymatic activity (Godfrey and West 1996). The commercially available 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. β-galactosidases from different microbial sources
(Sources: Gekas and Lopez-Leiva 1985, Mahoney 1985)

Sources	pH optimum	Temperature optimum	Activators	Inhibitors
A. niger	3.0-4.0	55-60	non needed	none
A. oryzae	5.0	50-55	non needed	none
K. fragilis	6.9-7.3	37	Mn^{+2}, K^{+2},Mg+2	Ca^{+2}, Na^{+2}
K. lactis	6.5-7.3	35	K^{+2}, Mg^{+2}	Ca+2, Na^{+2}
E. coli	7.2	40	Na^{+2}, K^{+}	
B. subtilis	6.5	50	no needed	
B.stearothermophilus	5.8-6.4	65	Mg^{+2}, K^{+}	Ca^{+2}
L. bulgaricus		42-45		

Ca^{+2} ions are known to be an inhibitor for β-galactosidase. Yet Ca^{+2} ions in milk is in the bound form for this reason, enzymatic activity is not effected. (Garman *et al*. 1996) Divalent cations such as magnesium and manganese may enhance the β-galactosidase activity, while monovalent cations may have a positive or negative effect (Garman, et al. 1996, Kraft and Jelen 2000).

All the mammals have this enzyme except Californian mammal. The enzyme β-galactosidase exists in three forms in human intestine;

Lactase 1; is found in the edge membrane of the epithelium of small intestine. Only lactase 1 is responsible for the hydrolysis of lactose

Lactase 2; is found in the lysosome of the epithelium cells of small intestine. Also called as acid β-galactosidase. Hetero-β-galactosidase; is found in the cytoplasm of the epithelium cells of small intestine.

3.3. Industrial Applications of β-galactosidase

β-galactosidase hydrolyzes lactose into glucose and galactose. Due to its hydrolyzing property, it has been used for milk and fermented milk products such as yoghurt and cheese. Low lactose milk and dairy products gives chance to lactose intolerants whose problem is generally related with lactase insufficiency for consumption of foods containing lactose. It is also used in production of fermented and alcohol containing beverages (Pivarnik, et al. 1995)

Using β-galactosidase to process milk products having high lactose content such as ice-cream, frozen milks, whey and condensed milk can reduce lactose concentrations to acceptable values to prevent lactose crystallization. As a result, it improves some technological and sensorial quality of dairy foods like increasing the digestibility, softness and creaminess.

In the cheese industry lactose is a waste, which causes several economical and environmental problems because lactose is associated with the high biochemical and chemical oxygen demand. Hydrolysis of lactose present in whey converts whey into very useful sweet syrup, which can be used in the dairy, baking and soft drinks industries (Grosová, et al. 2008).

Another aspect in use of β–galactosidase enzyme is oligosaccharide formation when modeling lactose hydrolysis. Galactooligosaccharides are a mixture of oligosaccharides consisting of D-glucose and D-galactose. They are produced from D-lactose via the action of the enzyme β-galactosidase. Galactooligosaccharides are not usually digested in the small intestine. They are fermented by colonic bacteria. This could lead to changes in the colonic ecosystem in favor of some bacteria, such as bifidobacteria, which may have health benefits, including protection against certain cancers and lowering of cholesterol levels (Chockchaisawasdee, at al. 2004).

Table 3.3. Structure of some oligosaccharides formed during β–galactosidase action on lactose

(Source: Mahoney 1998)

Disaccharides	β-D-Gal (1-6)-D-Glc	allactose
	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 (1-3)- β-D-Gal (1-4)-D-Glc	
	β-D-Gal (1-3)- β-D-Gal (1-6)- β-D-Gal (1-4)-D-Glc	
Pentasacchride	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

Gla, galactose; Glc, glucose

Table 3.4. Some uses of β–galactosidase enzyme in industry

(Source: Richmond, et al. 1981)

a) Low lactose milk processing
b) Low lactose dairy products
c) Low lactose yoghurt
d) Sweetened yoghurt
e) Low lactose concentrate for ice cream
f) Lactose processing of acid and sweet whey
g) Food syrups and sweetener manufacture
h) Lactase treatment during cheese making

CHAPTER 4

IMMOBILIZATION

Enzymes have been investigated intensively for possible industrial applications such as to modify the composition of foods but they have only recently become available for large scale use in industry. However progresses in genetics and in process technology enable the enzyme industry to offer products with improved properties and at reduced costs (Indian Academy of Science 2008) Immobilized enzymes can be considered to be heterogeneous catalysts which can be separated from the reaction mixture and can be stored and recycled. Immobilized enzymes may show improved stability to the effects of heat and chemical deactivation. Moreover, it should be possible to use immobilized enzymes to catalyze transformations in organic solvents in which the native enzymes could be insoluble. In some cases an increase in activity can be seen upon immobilization but generally a loss in activity is observed due to restricted mass transfer of reactants. 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 industrial application of the immobilized enzymes was reported by Chibata and co-workers in 1967, who developed the immobilization of Aspergillus oryzae aminoacylase for the resolution of synthetic racemic D-L-amino acids. Industrial production of sugars, amino acids and pharmaceuticals are other major applications of immobilized enzymes (Table 4.1.). Around 1970 two other immobilized systems are launched on a pilot scale which are immobilized penicillin acylase 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).

Table 4.1. Major products obtained using immobilized Enzymes
(Source: 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

4.1. 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.

4.2. Methods for 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. 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. The methods use for immobilization can also affect the kinetic parameters of the immobilized enzyme (Samoshina, et al. 1987). The most commonly used immobilization methods are shown in Figure 4.1 (Chibata 1966).

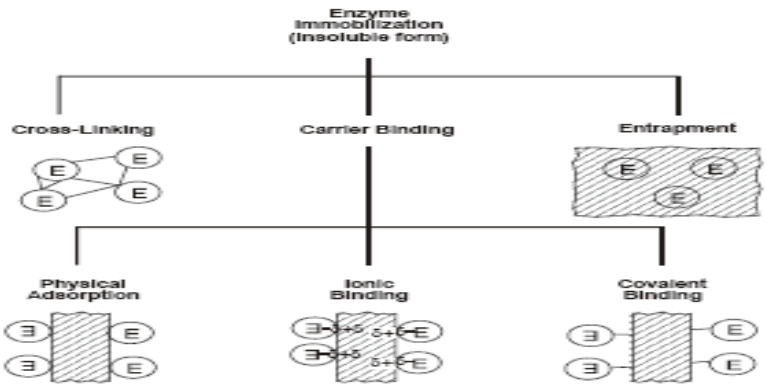


Figure 4.1.The schematic representation of the common immobilization techniques

4.2.1. Carrier Binding

Carrier binding method in which the amount of enzyme bound to the carrier and the activity after immobilization depends on the nature of the carrier. This method can be sub-classified into physical adsorption, covalent binding and ionic binding.

4.2.1.1. Physical Adsorption

The method relies on non specific physical interaction between enzyme protein and the surface of matrix. 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. (Kennedy and White 1985)

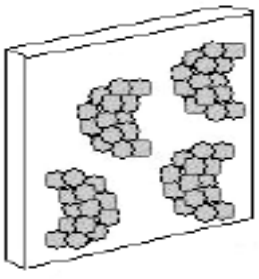


Figure 4.2. Binding of a catalyst to a carrier by adsorption
(Source: Costa, et al. 2004)

4.2.1.2. Covalent Binding

Covalent binding method which is widely used technique for immobilization of enzymes is based on the formation of covalent bonds. 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. (Mattiason and Kaul 1991, Kennedy and White 1980)

An advantage of this method is that because of 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. 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 and 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 and Rozzell 2007).

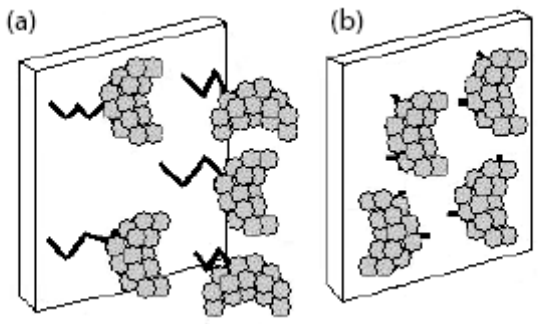


Figure 4.3. Biocatalysts bound to a carrier by ionic binding
(Source: Costa, et al. 2004)

4.2.1.3. Ionic Binding

Ionic binding is achieved by binding of the enzyme proteins to water-insoluble carrier containing ion-exchanger residues. 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. The carriers are classified as anion or cation exchangers depending on their ability to exchange anions (chloride or hydroxyl) or cations (hydrogen and sodium ions) of the carrier with anionic or cationic residues of the enzymes. (Brena and Batista 2008)

Ease of method and obtaining high activity preparations due to the mild conditions can be the advantages of ionic binding method. Hence, the ionic binding causes little changes in the conformation and the active site of enzyme. As the enzyme protein and carrier bind less strongly than in covalent binding, the leakage of enzyme may occur by changing the pH or the ionic strength.

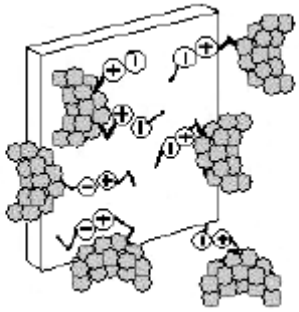


Figure 4.4. Covalent bond between the biocatalysts and a carrier
(Source: Costa, et al. 2004)

4.2.2. Entrapment

Entrapment which refers to the physical confinement 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).

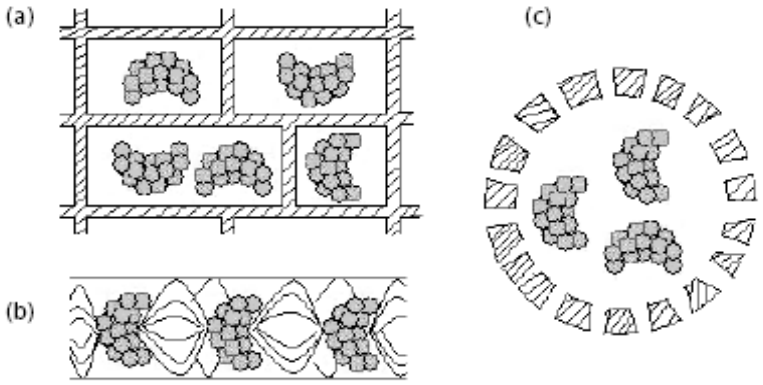


Figure 4.5. Enzyme encapsulation in a matrix (a), fiber (b), capsule (c)
(Source: Costa, et al. 2004)

4.2.3. 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 glutaraldehyde which establishes intermolecular cross-linking with amino groups on the enzyme. (Kim 2007)

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 that there may be loss of the enzyme activity due to the involvement of the active site in bound formation.

Table 4.2. Comparison of immobilization methods

(Source: Akşamoğlu 1997)

CHARACTERISTICS	CROSS LINKING	ADSORPTION	IONIC-BINDING	COVALENT BINDING	ENTRAPMENT
Preparation	Intermediate	Easy	Easy	Difficult	Difficult
Binding force	High	Low	Medium	High	Medium
Enzyme activity	Low	Medium	High	High	Low
Reusability	Impossible	Possible	Possible	Rare	Impossible
Cost	Intermediate	Low	Low	High	Intermediate
Stability	High	Low	Intermediate	High	High
Applicability	No	Yes	Yes	No	Yes

Table 4.3. Immobilization of β-galactosidase by different methods
(Source: Tanaka and Kawamoto 1999)

Source	Immobilization method	References
K. fragilis	covalent binding on corn grits	Siso *et al*. (1994)
	covalent binding on cellulose beads	Roy & Gupta (2003)
	covalent binding on porous silanised glass	Szczodrak (2000)
	entrapment in alginate-carrageenan gels	Mammarella & Rubiolo (2005)
	adsorption onto bone powder	Carpio et al. (2000)
K. lactis	covalent binding onto glutaraldehyde-agarose	Giacomini et al. (2001)
	covalent binding onto thiosulfinate-agarose	Giacomini et al. (2001)
	covalent binding on graphite surface	Zhou & Chen (2001)
K. marxianus	covalent binding on oxides supports: alumina, silica, silicated alumina	Di Serio, et al. (2003)
E. coli	covalent binding onto glutaraldehyde-agarose	Giacomini, et al. (2001)
	covalent binding onto thiolsulfinate-agarose	Rodriguez-Nogales & Delgadillo-Lopez (2006)
	entrapment in liposomes	Rodriguez-Nogales & Delgadillo-Lopez (2006)
	covalent binding onto gelatin cross-linking with chromium (III) acetate	Sungur & Akbulut (1994)
A. oryzae	entrapment in spongy polyvinyl alcohol cryogel	Rossi, et al. (1999)
	carbodiimide coupling to alginate beads	Dominguez, et al. (1988)
	microencapsulation into gelatin and cross-linking with transglutaminase	Fuchsbauer, et al. (1996)
	adsorption on celite	Gaur, et al. (2006)
	covalent binding to chitosan	Gaur, et al. (2006)
	cross-linking with glutaraldehyde	Tanrıseven & Dogan (2002)
	adsorption on polyvinylchloride (PVC)	Bakken, at al. (1990)
A.niger	adsorption on porous ceramic monolith	Papayannakos& Markas (1993)

4.3. Supports Used for Enzyme Immobilization

The support material utilized for immobilizing enzymes are classified as organic and inorganic matrices according to their chemical composition, with organics being sub-classified into natural and synthetic polymers. For industrial applications in the food industry, the carrier material must be non-toxic, readily available and affordable.

For the immobilization a suitable support should meet the requirements listed below (Kennedy and White 1985);

- Resistance towards microbial attack
- Stability under the conditions to be used
- Insolubility in process solutions
- Availability at moderate cost
- Compatibility with the enzyme, substrate and process solution

The classification of the carriers into organic and inorganic supports is not wholly adequate for full description of the carrier. Pore diameter, surface area, mechanical strength will also affect the performance of the immobilized systems and will determine the type of the reactor used under technical conditions. Particularly, pore parameters and particle size determine the total surface area hence; affect the loading of the enzymes. Therefore, a further classification based on morphology can be considered into non-porous and porous carriers.

Non-porous supports reveal less diffusional limitations, however low loading capacity is observed. Thus, porous supports are generally preferred since the high surface area allows for a greater enzyme loading and the immobilized enzyme will have higher protection form environment.

Table 4.4. Examples of Carriers Used for Enzyme Immobilization (Source: Kennedy and White 1985, Swaisgood 1985, Scouten 1987).

Organic Carriers	Inorganic Carriers
Natural polymers	Glass beads (porous and nonporous)
Activated carbon	Metal oxides (e.g., ZrO_2, $Ti0_2$, Al_2O_3)
Agarose (Sepharose)	Sand
Cellulose	Silica gel
Collagen	
De'ctran (Sephadex)	
Gelatin	
Starch	
Synthetic polymers	
Acryl amide-based polymers	
Maleic anhydride copolymers	
Nylon	
Styrene-based polymers	

4.4. Chitosan and Hydroxyapatite

Chitosan is a linear polysaccharide, obtained by deacetylation to a varying extent that is characterized by the degree of N-deacetylation. Its structure is basically constituted of D-glucosamine units with contents of N-acetyl-D-glucosamine in the range of 0-50%. Chitin can be isolated from a variety of sources such as the shells of several crustaceans, krill, but it also forms part of the exoskeleton of insects and is present in the cell walls of fungi. Since it is inexpensive, non-toxic and possesses potentially reactive amino functional groups, chitosan has been shown to be of potential use in many different fields, including as an antifungal compound in agriculture, in wastewater treatment (removal of heavy metal ions, flocculation/coagulation of dyes and proteins, membrane purification processes), the food industry (anticholesterol and fat binding, preservative, packaging material, animal feed additive) and alimentary industries, hydrating agent in cosmetics, in pulp and paper industry (surface treatment, photographic paper), and, more recently, in biomedicine and several pharmaceutical preparations (Krajewska 2004, Chang and Juang 2007).

Chitosan is known as an ideal support for enzyme immobilization because of its many characteristics such as hydrophilicity, biocompatibility, biodegradability, anti-bacterial property, the presence of hydroxyl and amino groups. It exhibits high affinity towards proteins. Chitosan can be easily cross-linked by the reagent such as glutaraldehyde to form rigid aquagels (Arguelles-Monal, et al. 1998). However, the deformation could not be easily avoided upon drying the chitosan carriers. This can be improved in conjunction with other solid powders to increase its density and strengthen its physical properties, and thus to expand its applications.

Hydroxyapatite was also known as a biocompatible, inert, biodegradable and safe material. Hydroxyapatite and chitosan complexes are used as a biomaterial and known to form rigid and mechanically strong composites. Thus, in this study we have chosen to use these two materials to form composite beads with these expectations.

4.5. 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 and Batsita-Viera 2008). 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 and 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).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

For this study as a support material chitosan and hydroxyapatite were used to achieve the immobilization of β-galactosidase of *Kluyveromyces lactis* (Sigma G-3664, 3000 $U.mL^{-1}$).

5.1.2. Chemicals

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

5.2. Methods

5.2.1. Assay of β-galactosidase Activity

The activity of the enzyme using ONPG was determined as described by Food Chemicals Codex-National Academy of Sciences. The chromogenic substrate onitrophenol-β-D-galactopyranoside (ONPG) (8.3 x 10-3 M) dissolved in 0.02 M potassium phosphate buffer (pH 6.5) containing $MgCl_2$ was used. The amount of substrate and enzymes used were 4 ml and 1 ml, respectively. At time zero, 1ml of enzyme solution was added to the ONPG solution and incubated for 15 minutes for the reaction. The reaction was stopped by the addition of 1 ml of reaction mixture to the tubes containing 1 ml of 10% sodium carbonate and the addition of 8 ml deionized H_2O. The procedure was followed by reading the absorbance at 420 nm. One unit was defined as that quantity of enzyme that would liberate 1 μM of o-nitrophenol per minute under the conditions of assay. Units were calculated using the equation 4.1.

$$\text{FCCLacU}=\frac{\Delta A \times 5 \times 10}{\varepsilon \times 15} \quad (4.1)$$

Where;

A: average absorbance of the reaction sample

ε: extinction coefficient (determined from the 0-nitrophenol standard curve)

For the activity of immobilized enzyme the procedure is same as the free enzyme except in order to add 1 ml enzyme solution an amount of immobilized beads were used and 1 ml buffer was added to reaction mixture to obtain the same dilution.

The specific enzyme activity was defined as the ratio of enzyme activity [U/ml] to the mg of protein per ml of the specific buffer solution [mg/ml]. Therefore,

$$\text{Specific enzyme activity} = \frac{\text{Enzyme actvity}}{\text{mg of protein}}$$

5.2.2. Protein Assay

Protein amount was estimated using Bradford method with Bovine Serum Albumin (BSA) as a standard (Bradford 1976).

5.2.3. Characterization of Soluble and Immobilized β-galactosidase

In order to find the optimum working conditions which will maximize the enzyme activity, effect of some parameters were investigated.

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

Effect of temperature on soluble enzyme activity was investigated by measuring the activity using standard assay procedure at different temperatures in phosphate buffer. The reaction temperatures range from 20-55^{0}C. The relative activities were

expressed as the ratio of the enzyme activity obtained at certain temperature to the maximum activity obtained at the given temperature range and given as a percentage.

Temperature stability was investigated by incubating the β-galactosidase in phosphate buffer at various temperatures ranging from 20-55^{0}C for 30 minutes, and then measuring the remaining (residual) activity.

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

The optimal pH was determined by measuring the activities at different pH values at 37^{0}C using the following buffer systems; sodium acetate (4.0, 5.0), potassium phosphate (pH 6.0, 6.5, 7.0, 7.5) and Tris-HCl (pH 8.0, 9.0). The relative activities were expressed as the ratio of the enzyme activity obtained at certain pH to the maximum activity obtained at the given pH range and expressed as a percentage.

pH stability of β-galactosidase was determined by incubating the β-galactosidase at different pH values for 30 min. at 37°C, and then the residual activity was measured (%) with respect to control, under standard assay conditions.

5.2.3.3. Kinetic Studies of β-galactosidase

Kinetic constants, K_m and V_{max}, of the β-galactosidase were determined by changing the ONPG substrate concentration. ONPG substrate concentrations in the assay medium range from 0,5-12.5mM (in 0.02 M phosphate buffer at pH 6,5 with $MgCl_2$). Lineweaver –Burk double reciprocal (1/V versus 1/S) plot was constructed to calculate the K_m and V_{max}. With a slope of K_m / V_{max} and the intercept of 1/ V_{max} estimated K_m and V_{max} values were calculated as given in double-reciprocal plot.

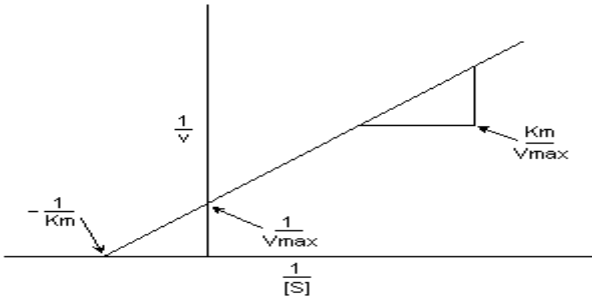


Figure 5.1. Double Reciprocal Plot of 1/v versus 1/[S]

5.2.4. Immobilization of β-galactosidase

Chitosan (1.5 g) and hydroxyapatite (1.5 g) were dissolved in (100 cm3) on aqueous solution of acetic acid (1M). This solution was added drop wise to gently stirred (1.5% (w/v) sodium tripolyphosphate solution (150 cm^3) using needles of. Before immobilization, they were washed until neutrality with dH_2O and suspended in 0.025 M KH_2P0_4 and 0.025 M Na_2HP0_4 buffer of pH 6.86.

In a standard experiment, after the activation with glutaraldehyde, matrices were thoroughly washed with the same buffer. β-galactosidase dilution of 5 ml volume with buffer (pH 6.86**)** added to an appropriate volume of support (1.5 g). The preparations were left at 20^0C with intermittent mixing (150 rpm). The preparations obtained were washed with the same buffer (pH 6.86**)** used for the activation until no enzyme remained in the washing solution, the yield of lactase immobilization was estimated on the basis of the difference between protein amount added to the beads and that recovered in the pooled supernatant and washing fractions.

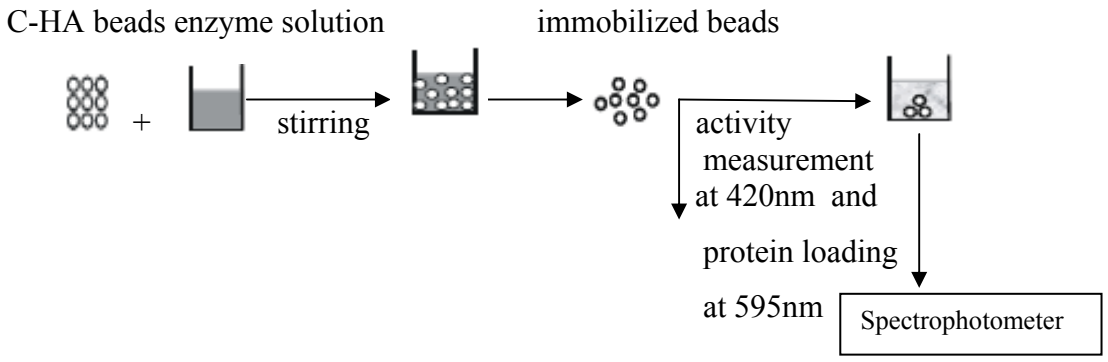


Figure 5.2. Schematic diagram of enzyme immobilization process

5.2.4.1. Determination of protein

The protein content of the enzyme solution and washed solution were determined by the Bradford's method at 596 nm using Bovine Serum Albumin (BSA) as the Standard. The immobilized protein was estimated as the difference between the protein amount of enzyme solution and the protein amount of washes and supernatants after immobilization.

5.2.4.2. Effect of Degree of Cross-linking with Glutaraldehyde on Immobilization Capacity

The chitosan-hydroxyapatite beads were prepared according to the method described earlier. Certain amount of beads were treated with glutaraldehyde solution (25%) with different concentrations ranging from 0.1% to 0.7% per gram bead under constant stirring at 150 rpm for 24 hours and the excess glutaraldehyde was washed off with KH_2P0_4- Na_2HP0_4 buffer (pH 6.86), then the immobilization procedure was applied. For the determination of the effect on immobilization, activity of immobilized enzyme and protein loading were determined.

5.2.4.3. Effect of Temperature

In order to determine the optimum immobilization temperature, lactase solution (5ml) in concentration $0.46x10^3$ mg/g bead with KH_2P0_4 and 0.025 M Na_2HP0_4 buffer, pH 6.86 was added to 1.5 g. chitosan-hydroxyapatite complex media. The temperatures investigated were 10, 15, 20, 25, 30 and 35^0C. Stirring rate was 150 rpm for 24 hours.

5.2.4.4. Effect of pH

Enzyme solutions (5 ml) in concentration $0.46x10^3$ mg/g bead prepared using following buffer systems; sodium acetate (4.0, 5.0), KH_2P0_4- Na_2HP0_4 (pH 6.0- 7.5) and Tris-HCl (pH 8.0, 9.0) were added to 1.5 g. chitosan-hydroxyapatite complex media. Analyzes were performed for 24 hours under constant temperature (20^0C) and constant stirring at 150 rpm.

5.2.4.5. Effect of Enzyme Loading

Different amounts of enzyme loading on beads were examined by incorporating various concentrations of β-galactosidase in order to find the optimum conditions for the immobilization. β-galactosidase solution (5ml) which were prepared using in 0.025

M KH_2P0_4 and 0.025 M Na_2HP0_4 buffer, pH 7.5 with concentrations 0.23- 0.32-0.46-1.16-1.86x10^3 mg enzyme/g bead were applied on 1.5 g. of chitosan-hydroxyapatite complex beads at 20^0C under constant stirring 150 rpm for 24 hours. For different concentrations of β-galactosidase, immobilized enzyme activity and protein loading were determined.

5.2.4.6. Effect of Solid-Liquid Ratio

In addition to the factors stated before, solid-liquid ratio also affects the efficiency of the coupling yield. The experiments were conducted keeping the volume of the support and the enzyme solution concentration constant (0.23x10^3 mg/g bead), yet varying the volume of the enzyme solution in each experiment. Analyzes were performed for 24 hours under constant temperature (20^0C), pH (7.5) and constant stirring at 150 rpm.

5.2.4.7. Effect of Salt Concentration

Effect of salt concentration on immobilization is another important factor in terms of hydrophobic interactions. In order to find the optimum salt concentration for immobilization, phosphate buffers, pH 7.5 within the concentrations of 0.25-0.5-0.75-1-1.5M of NaCl were used in immobilization process at 20^0C under constant stirring.

5.2.4.8. Effect of Contact Time on Immobilization

In order to determine the time course for immobilization, lactase solution (5ml) in concentration 0.23x10^3 mg/g bead with KH_2P0_4 and 0.025 M Na_2HP0_4 buffer, pH 7.5 was added to 1.5 g. chitosan-hydroxyapatite complex media. . Samples were collected in determined time intervals. These analyses were carried out under constant stirring 150 rpm at 20^0C, ph 7.5 for 24 hours.

5.2.4.9. Reusability of the Immobilized Enzyme

One of the limitations associated with the industrial application of enzymes is their non-reusability. Long enzyme lifespan in enzymatic reactions will significantly decrease the cost of the process, which will accelerate industrial applications of enzyme technology. Moreover the immobilization stability of an enzyme can be observed from the number of cycles of reuse. In this section immobilized enzyme activity was determined with the same method described in section 5.2.1. The same immobilized enzyme was reused after it was thoroughly washed with buffer 5 times.

5.2.4.10. Storage Stability of the Immobilized Enzyme

Enzymes are generally not stable during storage in solutions and their activities decrease gradually by time. The ability to be stored for a period of time at a certain temperature is one of the key factors to be considered when using immobilized enzymes.

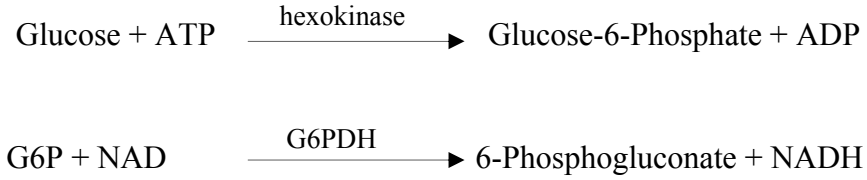
In this section to determine the storage effect on the activity of the immobilized β-galactosidase immobilized enzyme was stored at 4^0C for 15 days and residual activities was measured in certain times.

5.2.5. Lactose Hydrolysis with Immobilized Enzyme

The lactose hydrolysis was conducted in solution containing 100 ml of 5% of lactose and the flasks were incubated for 5h at 37^0C in a water bath shaker and agitated at 150 rpm. In the case of immobilized lactase, the lactose solution pH 6.0.containing 5% of lactose was again incubated for 5 h at 37^0C in a water bath shaker agitated at 150 rpm. Samples were withdrawn periodically every hour. The samples were boiled for 10 minutes for enzyme inactivation. The glucose formed in the reaction was determined using Glucose Assay Kit (GAHK-Sigma).

To determine the lactose hydrolysis using enzymatic method, glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose- 6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the

presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.



CHAPTER 6

RESULTS AND DISCUSSION

6.1. Immobilization Of β-Galactosidase

In this chapter, β-galactosidase enzyme was covalently bound onto chitosan-hydroxyapatite microspheres. Determination of the optimum immobilization conditions such as effect of temperature, pH, ionic strength, enzyme concentration, solid-liquid ratio and glutaraldehyde concentration. Besides immobilization kinetics and isotherms were presented.

Firstly glutaraldehyde treatment of immobilization support was described

6.1.1. Effect of Glutaraldehyde Concentration

Under basic conditions glutaraldehyde undergoes aldol condensation to form α,β-unsaturated multimeric aldehydes, which can react with proteins or carriers to form a stabilized Schiff bases. The concentration of glutaraldehyde can affect the activity of the immobilized enzymes directly as the glutaraldehyde is not only the cross-linking reagent, but also the denaturing reagent. The equilibrium adsorption of β-galactosidase at 20^0C onto chitosan-hydroxyapatite beads with different degrees of cross-linking is shown in Figure 6.1.

As is shown in Figure 6.1, when the initial concentration of glutaraldehyde was increased, the activity of immobilized β-galactosidase was decreased. The reason for this is that extensive cross-linking may result in a distortion of the enzyme structure. With the distortion of the enzyme conformation, the accessibility of the substrate may be reduced, thus affecting the retention of biological activity (Chui and Wan 1997).

In this study, when the glutaraldehyde concentration was increased from 0.1 % to 0.3% immobilized enzyme activity was decreased by about %40 firstly but then increased when the concentration is further increased (Figure 6.2) This can be explained by the change in active site of the enzyme accessible for enzyme immobilization.

As a result of this study it appears that the lowest glutaraldehyde concentration (0.1%) is the optimal value for the immobilization of β-galactosidase.

The efficiency of immobilization was calculated in terms of enzyme coupling yields. The enzyme coupling yield, Yİ (%) and was calculated using the equation 6.1.

$$Yİ\ (\%) = \frac{P_1}{P_0} \times 100 \qquad (6.1)$$

Where;

P_1: the amount of immobilized lactase

P_0: the initial amount of lactase

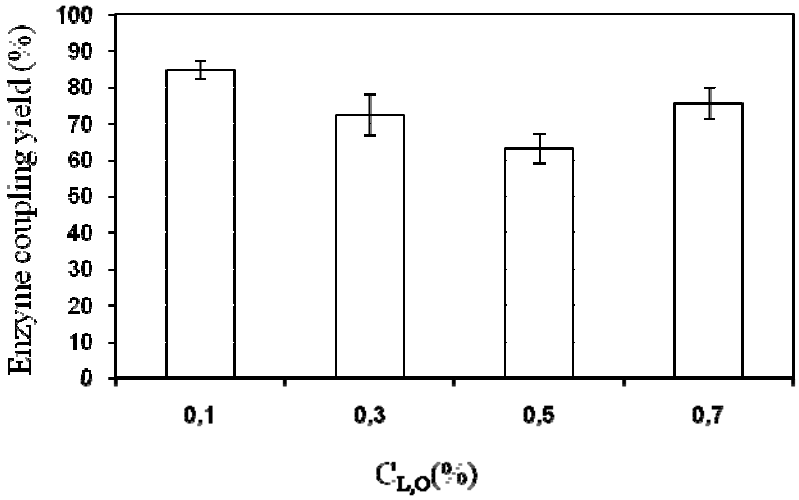


Figure 6.1. The effect of glutaraldehyde concentration on immobilization efficiency of immobilized β-galactosidase. Conditions: 1.5 g of chitosan-hydroxyapatite beads, $0.46x10^3$ mg/g bead enzyme in 5 ml of phosphate-buffer (pH 6.86), 150±5-rpm stirring speed

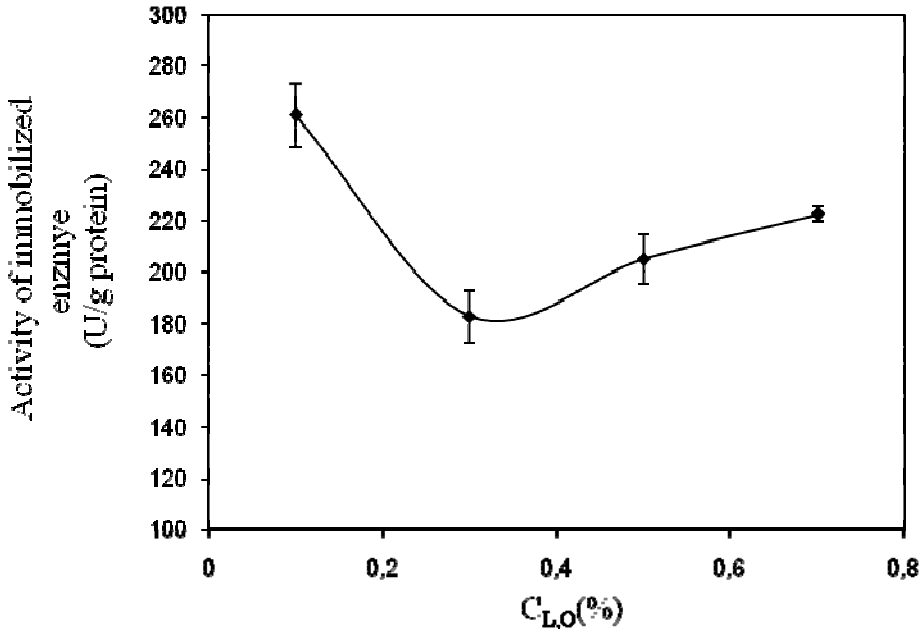


Figure 6.2. The effect of glutaraldehyde concentration on activity of immobilized β-galactosidase. Conditions: $0.46x10^3$ mg/g bead of β-galactosidase in 5 ml of phosphate-buffer (pH 6.86), 1.5 g of chitosan-hydroxyapatite beads, 150 0±5-rpm stirring speed, 20^0C

In a study conducted by Zhang et al. (2008), they observed a decline of the activity of immobilized enzyme when the concentration of the glutaraldehyde was more than 0.8%. According to Synowiecki et al. (2000), similar activity losses at high glutaraldehyde concentration were monitored.

6.1.2. Effect of Medium Temperature

In order to effectively facilitate the immobilization reaction and prevent enzyme deactivation at higher or lower reaction temperatures, it is very important to choose the optimum immobilization temperature. In most chemical reactions it is observed that the increase in temperature resulted with an increase of the reaction rate. Usually when the reactive temperature of reaction rises, the amount of bound enzyme will increase, which leads to relatively high catalytic activity.

In this study, the influence of temperature on immobilization system was investigated. The data reported in Figure 6.3 shows that the amount of enzyme

immobilized increased as the process temperature increased from 10-30^{0}C. As seen in the Figure 6.3, %50 of the enzyme was immobilized at 10^{0}C while this ratio was increased up to %81 at 30^{0}C. Immobilization at 15^{0}C to 30^{0}C changed the amount of immobilized enzyme amount significantly.

However another experiment was conducted to check the temperature effect on bounded lactase activity (Figure 6.4), it was observed that the residual activities of the immobilized beads decrease above 20^{0}C. This may be explained by the thermal deactivation of the enzyme during the immobilization. Therefore, 20^{0}C was found to be less detrimental to the enzyme during immobilization.

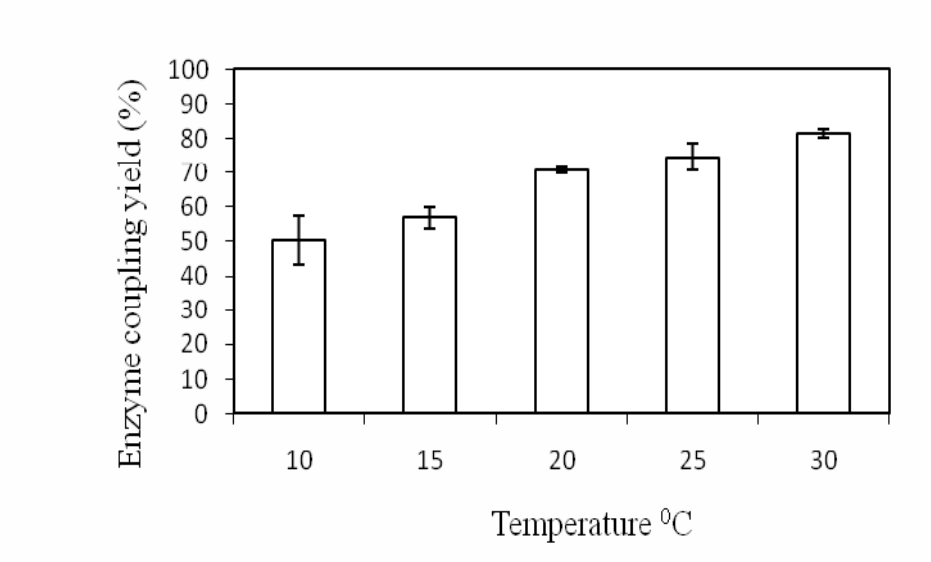


Figure 6.3. The effect of stirring temperature on immobilization efficiency of immobilized β-galactosidase. Conditions: 1.5 g of chitosan-hydroxyapatite beads, 0.46x10^3 mg/g bead of β-galactosidase in 5 ml of phosphate-buffer (pH 6.86), 150±5-rpm stirring speed

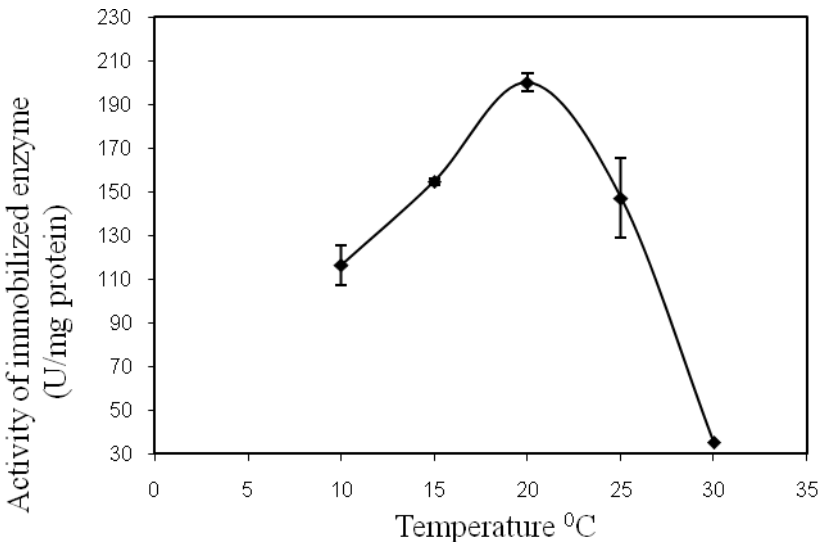


Figure 6.4. Effect of stirring temperature on activity of immobilized β-galactosidase. Conditions: $0.46x10^3$ mg/g of β-galactosidase, 1.5 g of chitosan-hydroxyapatite beads, $0.46x10^3$ mg/g bead of β-galactosidase in 5 ml of phosphate-buffer (pH 6.86), 150±5-rpm stirring speed.

6.1.3. Effect of Medium pH

In the present study, the effect of pH was investigated in the range of 4.0–9.0 using different buffer solutions. Figure 6.5 shows the effect of medium pH on immobilization of β-galactosidase on chitosan-hydroxyapatite support. It is also expected that the enzyme, immobilized at the optimum pH level; should show the highest activity. This Figure indicates that the immobilization system is affected by pH. The highest activity and amount of immobilization was obtained at pH 7.5. Therefore, pH 7.5 was established as the optimal pH of the immobilization system. This value corresponds to the %81 of immobilized enzyme by this support.

The loss in enzymatic activity might be contributed to the alterations in the properties of the enzyme such as changes in conformation resulting in inactivation of the enzyme activity. These kinds of changes may occur in cases where β-galactosidase enzyme shows the lowest activity below pH 6 and above pH 7.5. Furthermore near isoelectric point, enzyme tends to form less active aggregates. Thus, at about pH 4 and 5 immobilized β-galactosidase showed activity below 50 U/mg protein (Fig. 6.6).

The net charge of chitosan and β-galactosidase changes with pH. At a pH below their *pI,* proteins carry a net positive charge. Above their *pI* they carry a net negative

charge. The charge on the protein may improve the coupling which is maximal where repulsive charges are minimal. However at basic pH chitosan is negatively charged so if electrostatic interactions are the forces in binding the immobilization yield must have decreased by the increase in pH of the medium.

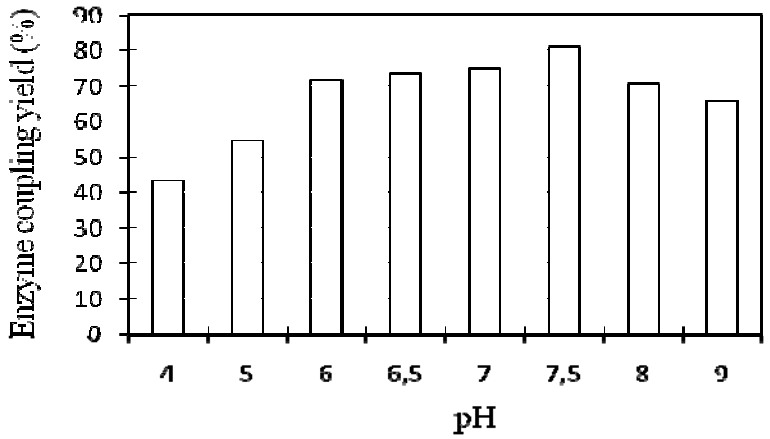


Figure 6.5. Effect of medium pH on immobilization efficiency of β-galactosidase. Conditions: $0.46x10^3$ mg/g bead of β-galactosidase in 5ml of phosphate buffer, 1.5 g of chitosan-hydroxyapatite beads, 20^0C, 150±5-rpm stirring speed

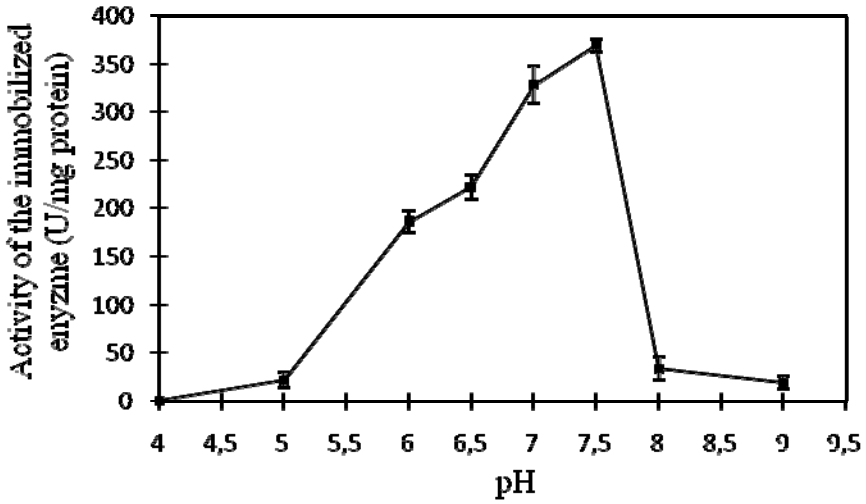


Figure 6.6. The effect of medium pH on the activity of immobilized β-galactosidase. Conditions: $0.46x10^3$ mg/g bead of β-galactosidase in 5ml of phosphate buffer, 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C

6.1.4 Effect of Contact Time

In this study Figure 6.7 shows the effect of stirring time on immobilization efficiency. It can be easily seen that most of the protein bound to chitosan-hydroxyapatite support in about one hour which corresponds to 85% of immobilization. Nearly all protein was bound to the immobilization support within nine hours and very little immobilization was observed up to 24 hours.

Even though there was no significant increase regarding to enzyme binding in between 9 to 24 hours period, immobilized enzyme kept its activity without any loss for 24 hours at 20^0C.

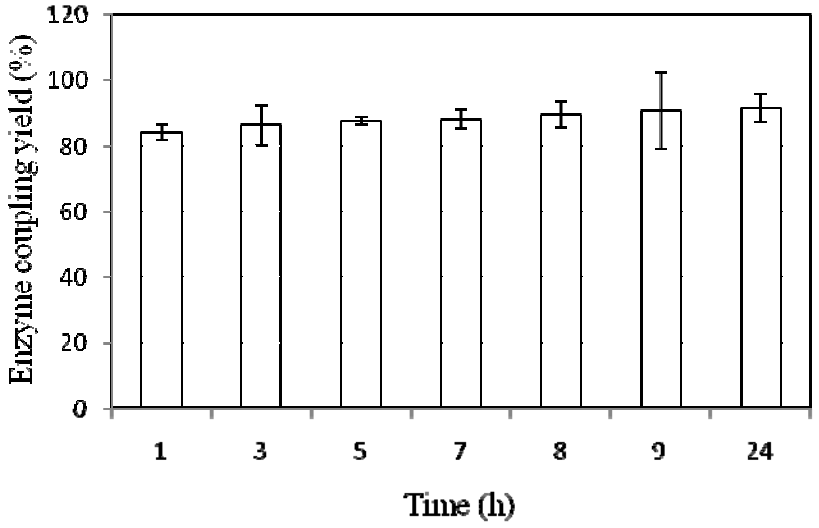


Figure 6.7. The effect of stirring time on immobilization efficiency of immobilized β-galactosidase. Conditions: $0.23x10^{-3}$ mg/ g β-galactosidase in 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C

In Figure 6.8 the effect of time on the activity of immobilized enzyme is shown and these results are in similar way with the immobilization efficiency.

The loss of the enzyme activity during 24 hours immobilized enzyme activity is expected to decrease due to the conformational changes but in contrast immobilization is observed to keep the enzyme activity at 20^0C for 24 hours.

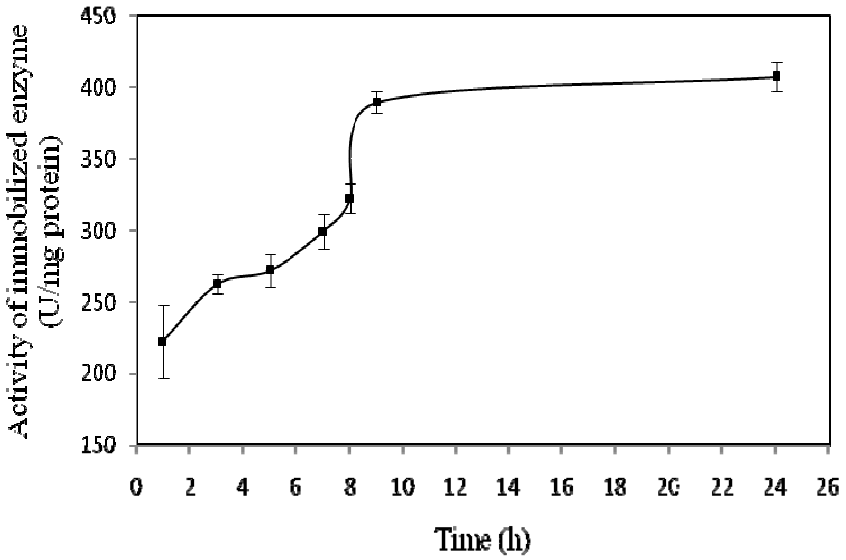


Figure 6.8. The effect of stirring time on activity of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g β-galactosidase in 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C

Similarly in a study conducted by Zhang et al. (2008), increase in enzyme binding was with time, after 12 hours a decrease in immobilized enzyme activity was recorded which was attributed to the to the conformational changes. In contrast to the study conducted by Zhang et al. (2008), our results showed that the immobilized enzyme maintained its activity with the increasing time (Figure 6.8).

6.1.5. Effect of Enzyme Concentration

An attempt was made to achieve binding of high levels of enzyme with high retention of initial activity. Thus, β-galactosidase concentration in the enzyme solution in the range of $0.23\text{-}2.56x10^3$ mg enzyme/ g. wet bead was changed; enzyme coupling yield and activity of the immobilized enzyme were calculated. The results are shown in Figure 6.9. In each experiment, 1.5 g of wet beads was immersed in 5 ml of enzyme solution.

It can be concluded that the increase of the initial enzyme concentration in the enzyme solution results in an increase of the enzyme loading on support (Fig. 6.9). But when the binding efficiency (ratio of immobilized protein to the protein at initial concentration) is considered, the efficiency decreases. In other words the immobilization is not increasing in a linear ratio with the increasing enzyme concentration.

Therefore, the maximum enzyme loading under described immobilization conditionswere approximately 1.082 g enzyme /g of the wet support which was equivalent to 45.2% enzyme coupling yield. In other words to explain clearly, the bound protein was increased by the increased enzyme concentration up to 23 mg of protein /g of support (1.082 g enzyme / g support). Yet, when the yield of coupling (the ratio to bound enzyme to unbound enzyme) is calculated, the percentage of unbound enzyme in the supernatant solution was observed to increase (Figure 6.9).

Moreover, the activity of the immobilized lactase increases rapidly with enzyme loading on supports up to about $2.57x10^3$ mg g^{-1} support (Fig. 6.11). However it seems that the specific activity is not correlated with the amount of enzyme bound. Because, as the enzyme concentration in the immobilization solution increased from $0.23x10^3$ to $2.56x10^3$ mg/g support, the specific activity decreased from 365 U/mg protein to 168 U/mg protein. The maximum specific activity of 365 U/mg protein, can be achieved working at low enzyme loading ($0.23x10^3$ mg/g) (Figure 6.10).

The possible reason for the lower activity yields at high enzyme loadings may be close packing of the enzyme molecules on the support surface, which could limit the access of substrates needed during the hydrolysis reaction. As more enzymes go into the interior of the carrier after the active sites of the carrier are saturated, enzymes could not efficiently perform the catalysis to limit substrate diffusion. Additionally, enzyme will be hidden and some active sites damage with high immobilization enzyme systems.

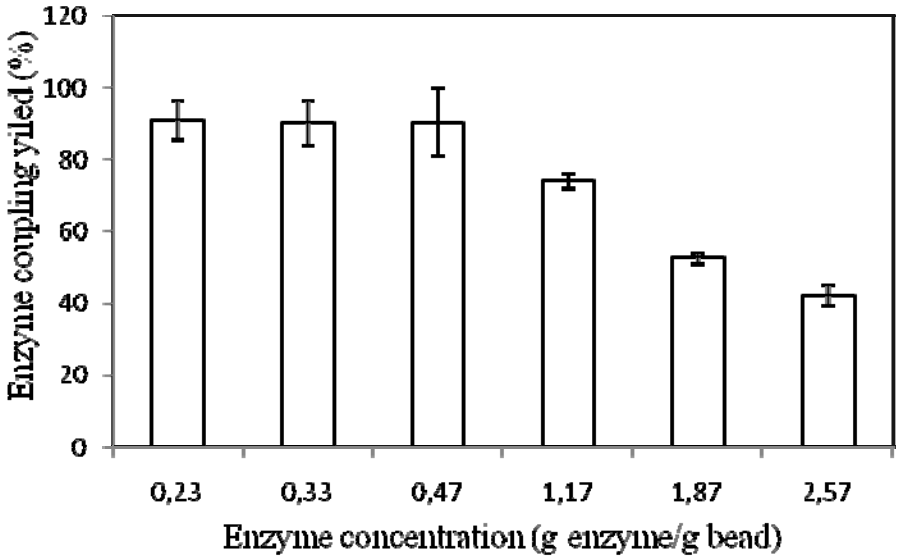


Figure 6.9. The effect of enzyme concentration on immobilization efficiency of immobilized β-galactosidase. Conditions: 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h.

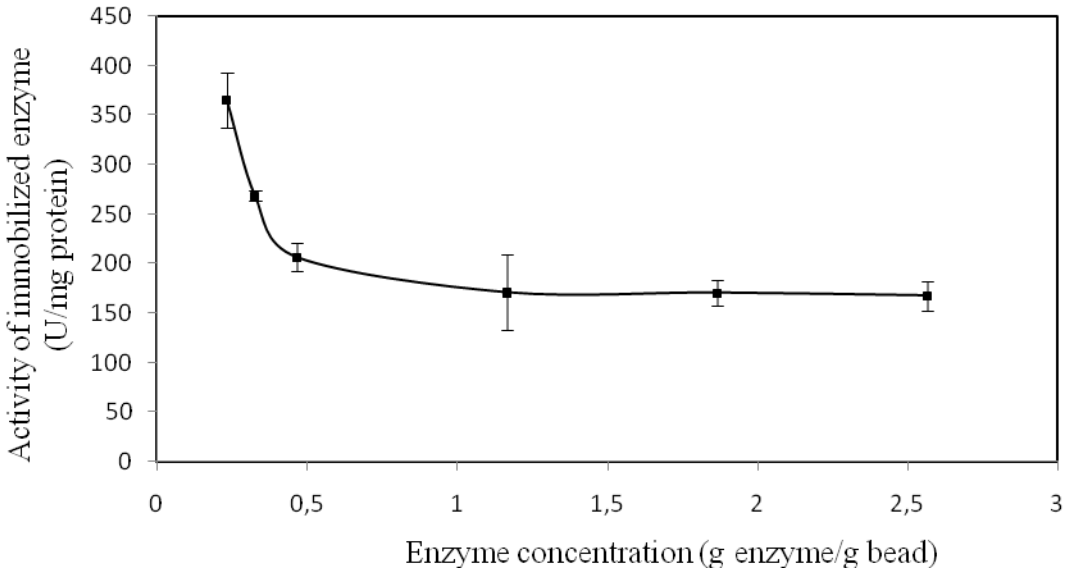


Figure 6.10. The effect of enzyme concentration on activity of immobilized β-galactosidase. Conditions: 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h

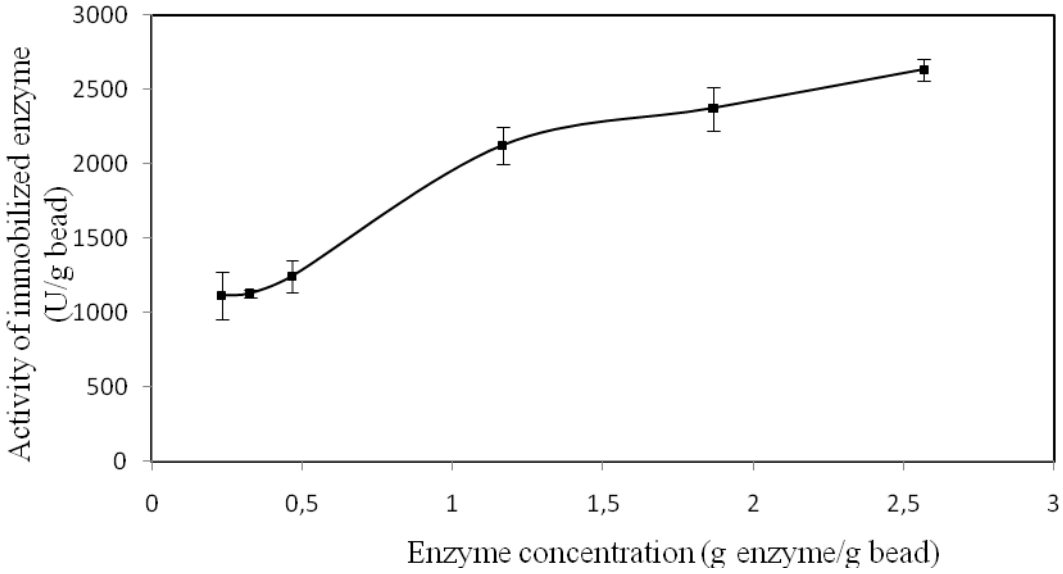


Figure 6.11. The effect of enzyme concentration on activity of immobilized β-galactosidase. Conditions: 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h

The equilibrium adsorption of β-galactosidase onto chitosan-hydroxyapatite beads was determined in batch experiments. Immobilization capacity was calculated by using the following relationship;

$$Q = \frac{Q_m \cdot C^*}{k_d + C^*}$$

$$\frac{1}{Q^*} - \frac{1}{Q_m} + \left(\frac{k_d}{Q_m}\right).\left(\frac{1}{C^*}\right)$$

Where;

Q^*: is the amount of lactase immobilized per unit weight of beads at equilibrium (mg g^{-1})

Q_m: is the maximum amount of lactase immobilized per unit weight

C_o: is the concentration of the lactase in the initial solution

C^*: is the concentration of lactase in the equilibrium

k_d: is the kinetic constant

As seen in Figure 6.12 and Figure 6.13, data obtained for the lactase/chitosan-hydroxyapatite system fitted well to the Langmuir isotherm where K_d = 28,. mg /ml solution and Q_m= 1176.47 mg/g wet bead. The constants were summarized in Table 6.1.

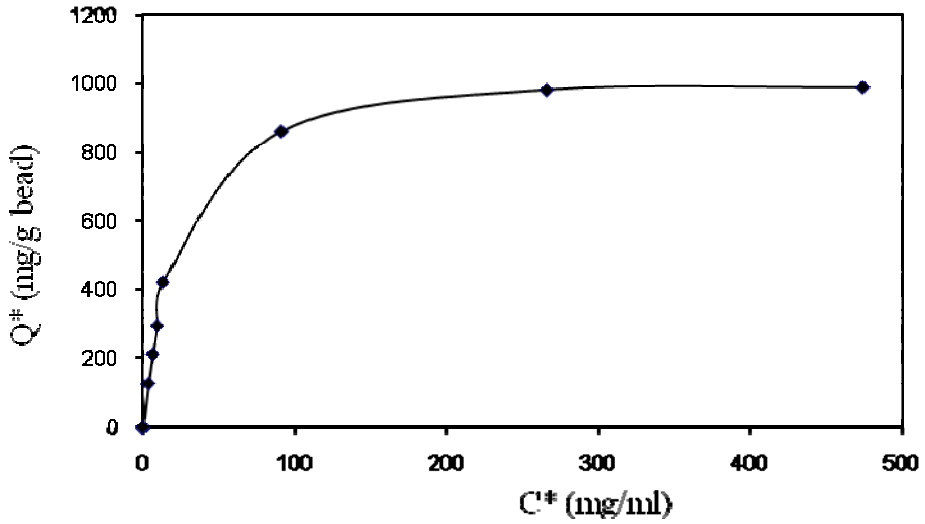


Figure 6.12. Sorption isotherm of β-galactosidase immobilized onto chitosan-hydroxyapatite beads

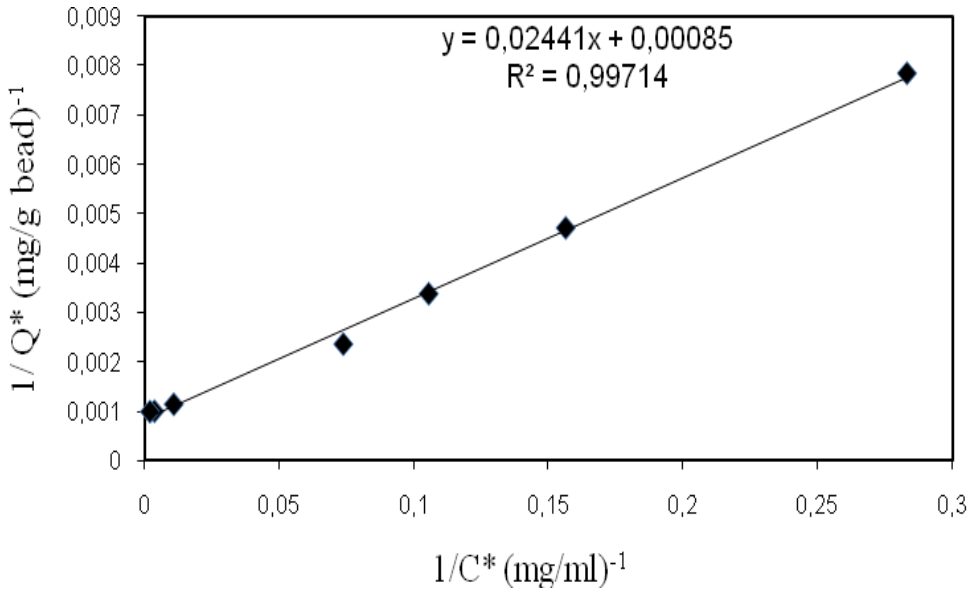


Figure 6.13. The reciprocal of the Langmuir isotherm for β–galactosidase immobilized onto chitosan-hydroxyapatite composite beads

Table 6.1. Isotherm constants for lactase/chitosan-hydroxyapatite system

Isotherm type	**R^2 value**	**Q_M (mg/g wet bead)**	**K_d (mg/g wet bead)**
Langmuir	0.99714	1176.470588	28.71765

Similar study was conducted by Zhang et al. (2008) on the stability of β-galactosidase immobilized on Artemisia seed gum and chitosan. In that study it was observed that up to 1ml/g support of enzyme concentration, activity of immobilized enzyme increases and then a decline occurs above this concentration. These results can be due to the change of the substrate specificity. In a study conducted by Finocchiaro, et al. (1980), they observed a decline in binding efficiency of β–galactosidase when enzyme concentration was increased and the most efficient use of enzyme was found to be at the lowest protein concentration (1mg/ml).

6.1.6. Effect of Ionic Strength

In Figure 6.14 and 6.15, the effect of ionic strength on immobilization and activity of β-galactosidase were presented. A significant decrease of the immobilization efficiency was observed when the ionic strength of the immobilization medium was increased from 0 to 1.5 M in the reaction mixture.

Immobilization efficiency was negatively affected by increasing ionic strength that can be attributed to two major reasons. Firstly, by an increase in the ionic strength, the competition for the charged groups on the support increases. Decreasing the number of ionic groups available for binding in the immobilization solution increases the binding of the enzyme. Secondly, the increment of salt concentration reduces the polarity of the solution and decreases the surrounding water structure on the surface of the absorbents and protein. The decrease in amounts of water structure on the protein surface reduce the stability of the protein structure, contributing to the exposure

of the hydrophobic regions of the inner protein core in the higher salt solution, and providing stronger ineractions with the hydrophobic sorbent whereas lowering the binding efficiency of protein onto hydrophilic supports such as chitosan. (Chen, at al. 2003)

In a study performed by Pessela et al. (2006), the highest β-galactosidase activity was obtained when the ionic strength of the buffer solution was lower. This shows that β-galactosidase immobilization is mainly affected by the amount of salt concentration of the enzyme solution. In order to optimize the immobilization conditions the activity of the immobilized β-galactosidase was also determined and found that increase in ionic strength decreases the activity as observed in immobilization efficiency (Figure 6.15).

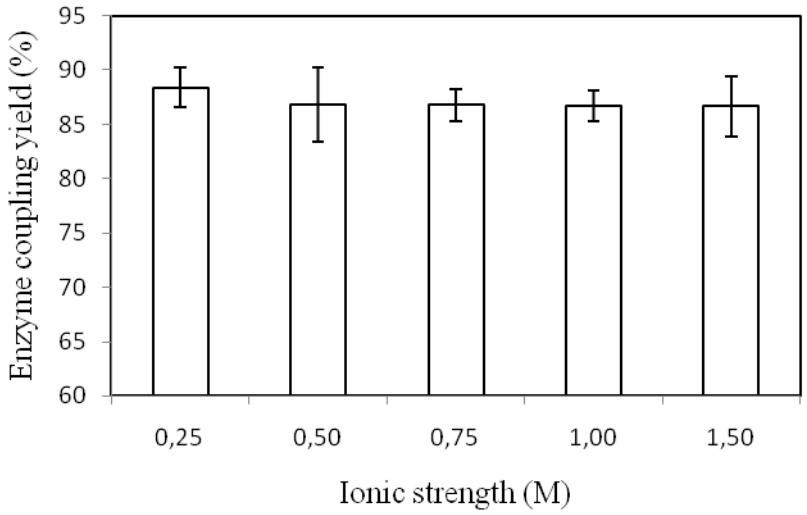


Figure 6.14. The effect of ionic strength on immobilization efficiency of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g bead of β-galactosidase in 0.46 10^3 mg/g bead of β-galactosidase in 5 ml of phosphate buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h

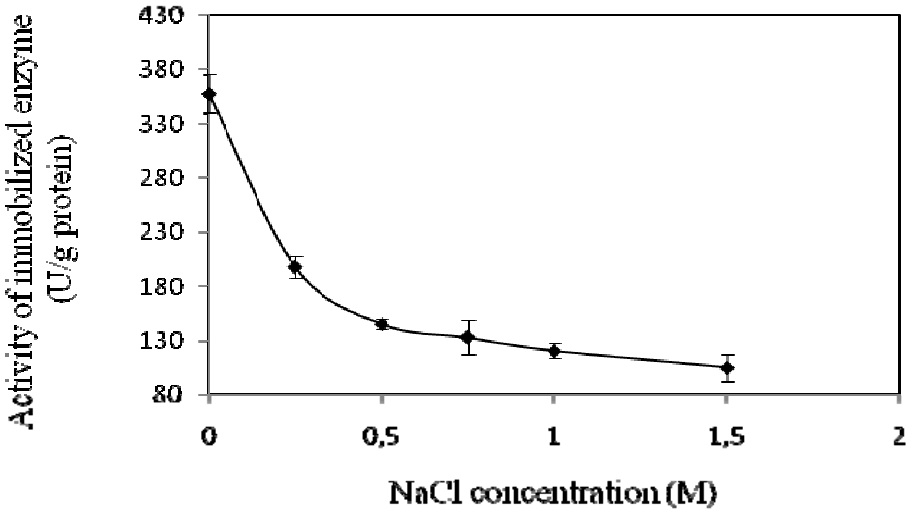


Figure 6.15. The effect of NaCl concentration on activity of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g bead of β-galactosidase in 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h

6.1.7. Effect of Solid-Liquid Ratio

The increase in solid (V_S: volume of support) to liquid ($V_{L:}$ volume of enzyme solution) ratio was seemed not to change enzyme coupling yield. Figure 6.16 shows that the immobilization reached 98% of coupling yield when the solid to liquid ratio was 0.1 g/cm^3. However the activity of the immobilized enzyme declined slowly with the increase of the enzyme solution quantity. It can be observed from the Figure 6.17, that specific activity increases with the decrease in the ratio of enzyme solution to solid support up to 0.3 g/cm^3. This result also can be explained by the same way explained in Chapter 6.1.5. As the liquid enzyme solution ratio increased, the amount of enzyme molecules in the immobilization solution increased. So As the enzyme amount increased, the enzyme molecules may be close packed on the support surface, which could limit the access of substrates needed during the hydrolysis reaction.

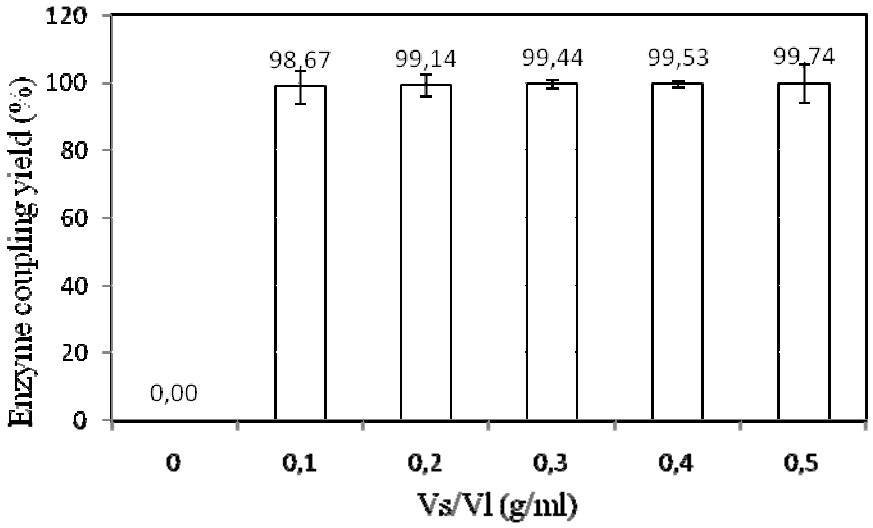


Figure 6.16. The effect of solid-liquid ratio (V_S/V_L) on immobilization efficiency of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g in 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h.

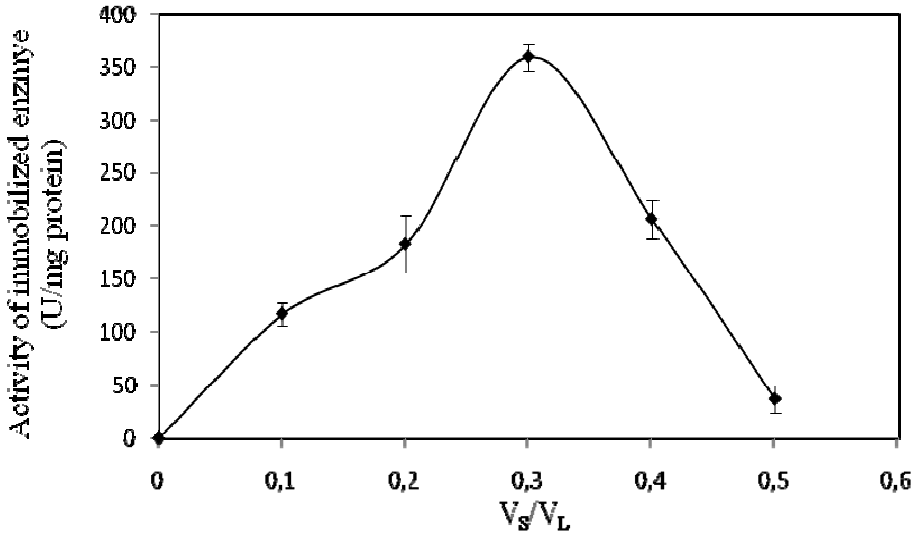


Figure 6.17. The effect of solid-liquid ratio on activity (U/mg protein) of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g β-galactosidase is 5 ml of phosphate-buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h.

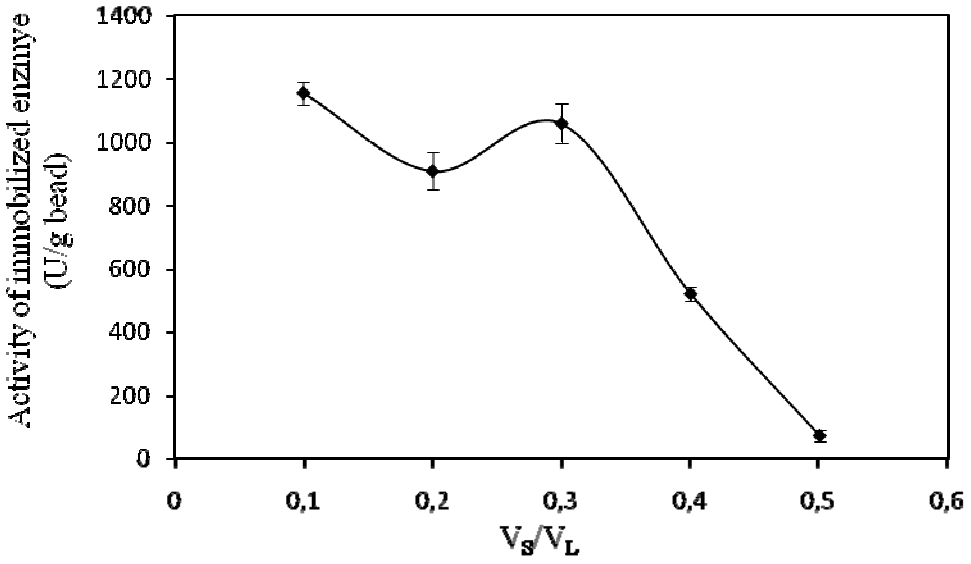


Figure 6.17. The effect of solid-liquid ratio on activity (U/g bead) of immobilized β-galactosidase. Conditions: $0.23x10^3$ mg/ g β-galactosidase is 5 ml of phosphate buffer (pH 7.5), 1.5 g of chitosan-hydroxyapatite beads, 150±5-rpm stirring speed, 20^0C, 24 h.

6.2. Characterization of Free and Immobilized β-Galactosidase

6.2.1. Effect of Temperature on Activity and Stability of Immobilized β-galactosidase

The temperature dependency of enzyme activity is shown in Figure 6.18. At low temperatures, the enzyme activity of both free and immobilized lactase increased with an increasing temperature. β-galactosidase from *Kluyveromyces lactis* was found to have an optimum temperature of 37°C. The enzyme retained %97 of its activity at 35^0C. This optimum temperature was in agreement with the studies of Cavaille and Combes (1995). They found that the optimum temperature of β-galactosidase from *Kluyveromyces lactis* as 37^0C. At high temperature level, the enzyme activity of both the free and immobilized β-galactosidase decreased sharply with an increasing temperature. Possible explanation is that the enzyme molecules lost their biocatalytic activity with the increase of temperature

The results in Figure 6.18 indicate that maximum enzymatic activities for soluble and immobilized lactase were at 37^0C. Immobilization did not shift or alter the optimum temperature of the enzyme. However, bound enzyme exhibited a slightly broader temperature profile, suggesting a slightly improved temperature-activity profile. This may be due to the fact that immobilization protects the enzyme against inactivation caused by heat exchange.

Similar to this study conducted by Roy and Gupta (2003), the optimal temperature of the immobilized enzyme stayed unchanged. In another work reported by Zhou and Chen (2001), effect of temperature on the activity of immobilized β-galactosidase on graphite surface was investigated. The optimal temperature for maximum activity of the immobilized enzyme was observed to increase. Such a result can be explained by the diffusional effects. The increase in temperature of the bulk solution makes the contact of the immobilized enzyme to substrate easier.

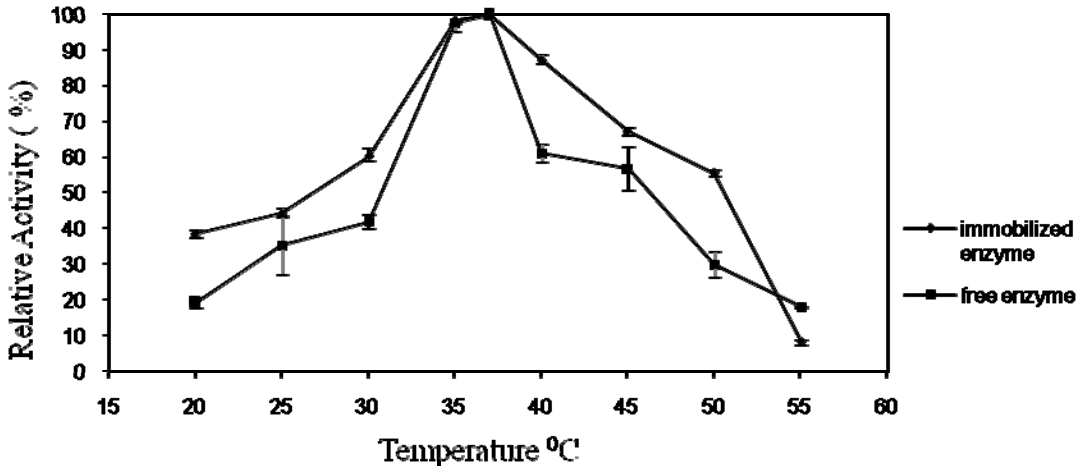


Figure 6.18. Effect of temperature on activity of free and immobilized β-galactosidase

Thermostability is the ability of an enzyme to resist against thermal unfolding in the absence of substrates (Bhatti, et al. 2006). The thermostability of free and immobilized β-galactosidase was determined by measuring the residual activity of enzyme after incubation at various temperatures ranging from 20 to 55ºC for 30 minutes. As it is shown in Figure 6.19, after 30 minutes of incubation the free enzyme is stable at 25 to 37^0C with the residual activity ranging from 91% to 86%. The immobilized enzyme shows improved stability compared to free enzyme.

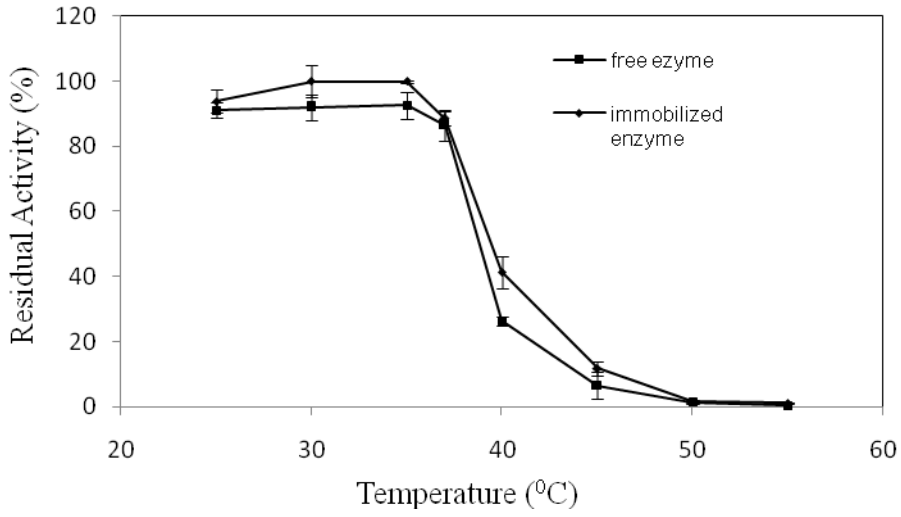


Figure 6.19. Effect of temperature on stability of free and immobilized β-galactosidase

6.2.2. Effect of pH on Activity and Stability of Immobilized β-galactosidase

The effect of pH on the activity of the free and immobilized preparations was examined in the pH range from 4.0 to 9.0 at 37^{0}C and the results are presented in Figure 6.20. The optimal pH ranges of the free enzyme are in the region of 6–7.5. The enzyme has very low activity at the pH of around 5. This is because; the *iso*-electric point (p*I*) of the enzyme is 5.42.

Figure 6.20 points out that the optimum pH of the enzyme changed upon immobilization. The optimum pH of the immobilized enzyme was shifted (by 0.5 pH unit) from the alkaline region (pH 7.5) compared to the free enzyme. As milk has a neutral pH, it was important that immobilization did not change the pH optima of bound enzyme to the acidic values.

Generally immobilization of an enzyme on a support is known to cause significant changes in the catalytic behavior of the enzyme. The optimum value of pH of an immobilized enzyme may shift to a higher or lower pH, which depends on surface charges of the supports.

The pH of the microenvironment may differ from the pH of the bulk solution if hydrogen ions are partitioned into or out of the immobilized enzyme matrix. The activity of the immobilized enzyme both depend on the local micro-environmental pH which reflects the pH of the bulk solution. This causes shifts in the behavior of the kinetic constants with respect to the solution pH (London South Bank University 2004). Thus, in this study the amino groups on the surface of the support may prevent the uniform distribution of hydrogen ions between the surface and the bulk solution and may lead to the shift from alkaline pH to neutral pH.

The result of this study was in agreement with that of Goddard *et al*. (2007), who found the optimal pH value for the immobilized enzyme pH 7. However, in contrast to our study below the optimal pH, the activity of the immobilized enzyme was nearly the same as its free counterpart.Also, Bayramaoğlu et al. (2006) had immobilized β-galactosidase from *E.coli* onto magnetic poly (GMA–MMA) beads and the optimal pH for the immobilized β-galactosidase was found to have shifted to pH 7.0 from pH 7.5. On the other hand, according to Sungur and Akbulut (1994), results showed no optimal pH shift after the enzyme was immobilized on chromium (III) acetate.

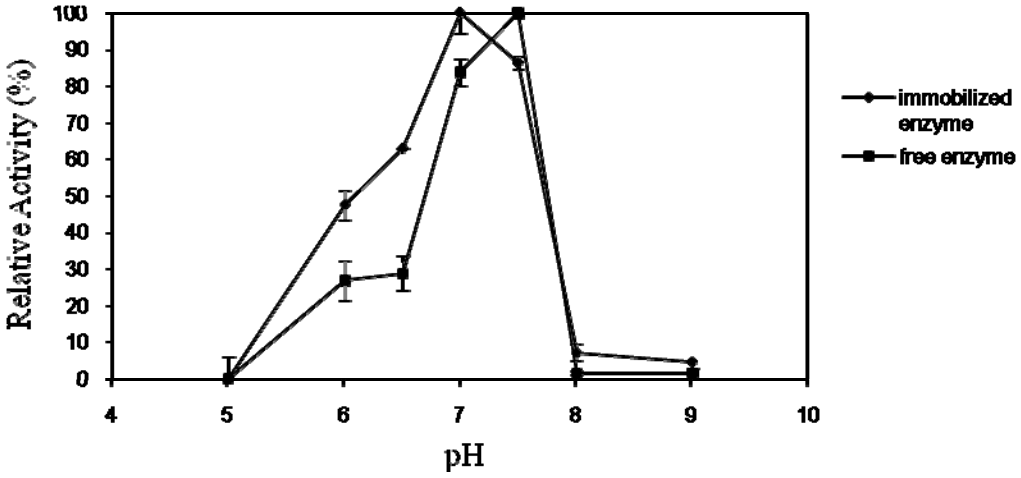


Figure 6.20. Effect of pH on the activity of free and immobilized β-galactosidase

In addition, it can be observed that the pH of the free enzyme is more stable on both sides of the optimum pH value, in comparison to that of the immobilized lactase (Figure 6.21). This pH profile of the immobilized lactase is a result of the change in the microenvironment of the enzyme.

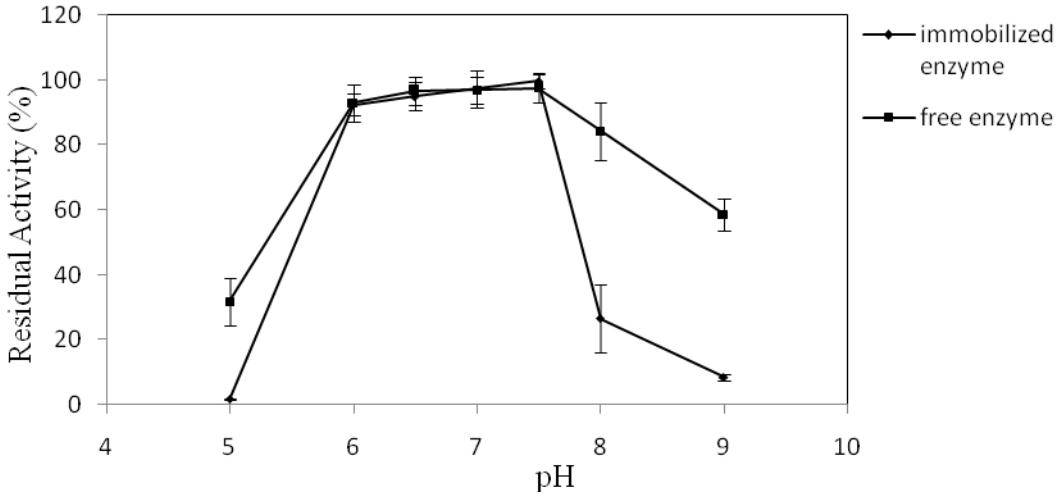


Figure 6.21. Effect of pH on the stability of free and immobilized β-galactosidase

6.2.3. Calculation of Kinetic Parameters of Immobilized β-galactosidase

Kinetic parameters for β-galactosidase derivatives were determined for hydrolysis towards ONPG as substrate and the data were processed using a typical double reciprocal Lineweaver Burk plot. The Michaelis–Menten constants K_m and V_m for the free enzyme have been found to be about 1.011 mM and 1098.9 μmol ONP min^{-1} mg^{-1} protein, respectively (Figure 6.22). In that study, the estimated K_m value which indicates the affinity of the enzyme towards the substrate was increased to 9.5 mM and V_m was markedly decreased to 454.5 μmol ONP min^{-1} mg^{-1} protein after the enzyme was immobilized onto the chitosan-hydroxyapatite (Figure 6.23). This reduced catalytic activity indicates that inactivation of the enzyme was occurring during the immobilization procedure reducing the reaction rate. This can be attributed to several factors, such as protein conformational changes induced by the support, steric hindrances and diffusional effects. Moreover it can be deduced from the increase in K_m that the immobilized enzyme is not accessible to the substrate as much as free enzyme and higher amounts of substrate is needed.

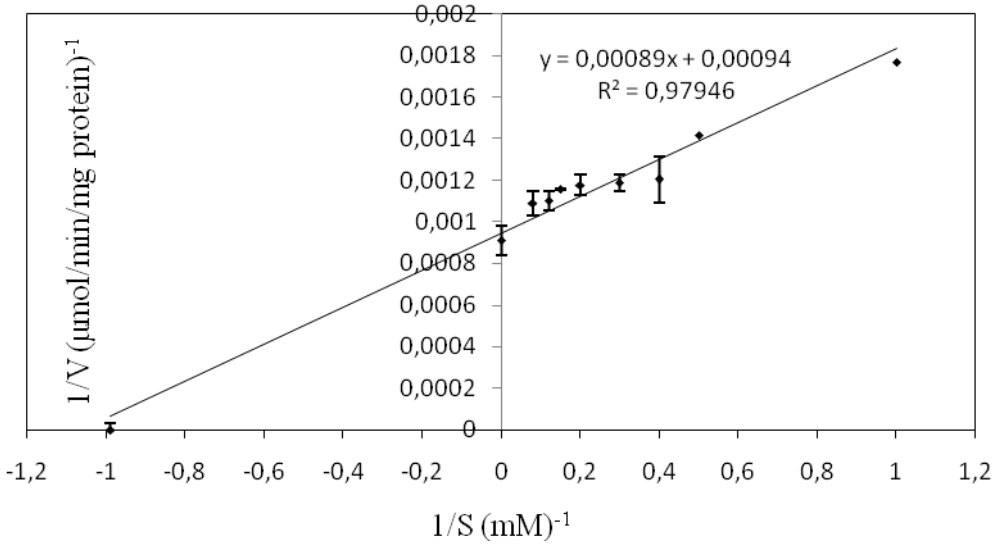


Figure 6.22. Double reciprocal plots to determine constants for ONPG hydrolysis by free β-galactosidase

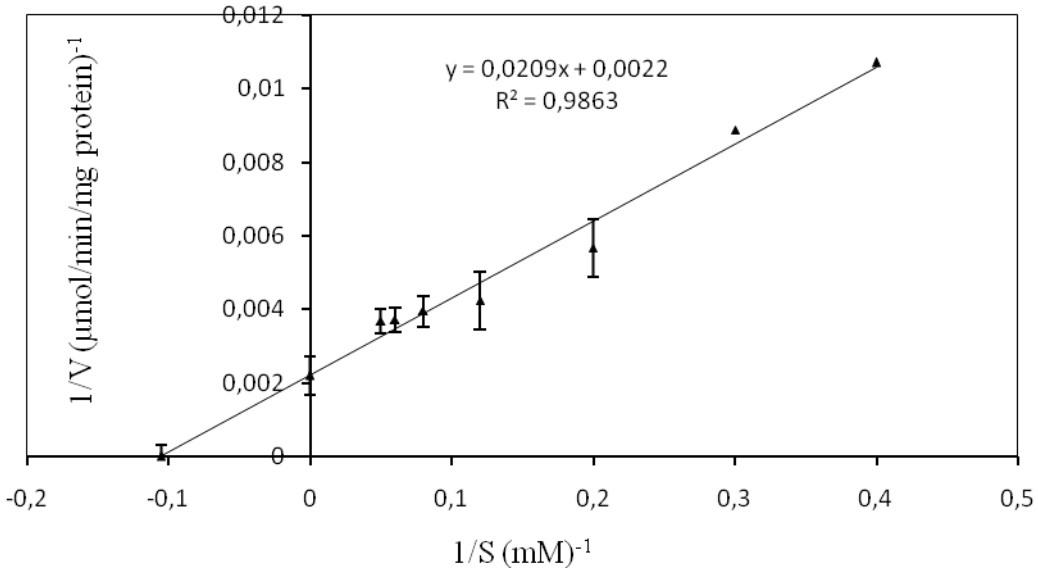


Figure 6.23. Double reciprocal plots to determine constants for ONPG hydrolysis by immobilized β-galactosidase

Higher K_m values after β-galactosidase immobilization compared to those calculated for the soluble enzymes have been reported by other authors. In a study on the immobilization of β-galactosidase in chitosan (El-Masry, et al. 2001), Km values had increased approximately 4-fold.However in another study on about the adsorption

on poly(2-hydroxyethylmethacrylate) membranes, an insignificant change of K_m value with respect to that of the free enzyme was observed (Baran, et al. 1997).

6.2.4. Reuse of the Immobilized β-galactosidase

The reuse of the immobilized enzyme is very important from the point of view of reducing the cost of the enzyme, which is an important factor while considering its suitability for commercial application.

Residual activity of the immobilized enzyme on reuse is shown in Figure 6.24. The activity of the first batch was taken as 100%. The immobilized lactase retained 81% of its activity after the reaction repeated eight times. The results confirmed that the stability of immobilized enzyme for repeated uses was effective and could be used more than eight times effectively for the further hydrolysis studies.

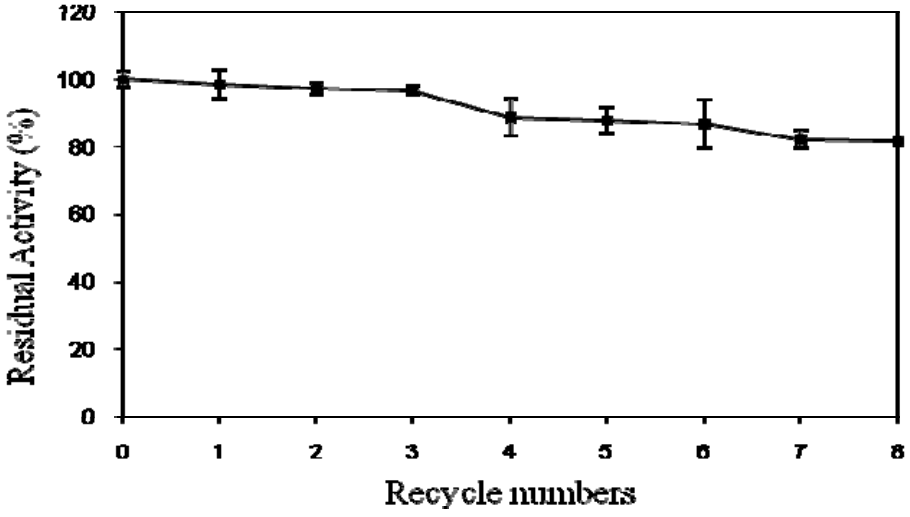


Figure 6.24. Batchwise reuse of chitosan-hydroxyapatite immobilized β-galactosidase with ONPG

Gaur et al. 2006 investigated the galactooligosaccharide synthesis by immobilization of lactase. After immobilization, there was no significant loss of original enzymatic activity after four subsequent cycles. In another study on immobilization onto composite microspheres of Artemisia seed gum and chitosan (Zhang, et al. 2008), immobilized enzyme retained 53% residual activity after eight times and deactivation of

enzyme,occured and relative activity of the immobilized enzyme decreased gradually when it was reused more than five times.

6.2.5. Storage Stability of Immobilized β-galactosidase

Enzyme stability are always of high significance in the production of a stable and reproducible biosensor.The immobilization of enzymes especially by covalent attachment is sometimes detrimental to the stability of the enzyme (Brena, et al. 2003).

In order to investigate the industrial feasibility of the immobilized enzyme process, the loss of enzymatic activity during storage must be considered. The immobilized enzyme was stored for 15 days at 4°C, the retention of the activity of free and immobilized β-galactosidase were determined using ONPG as the substrate in batch operation as described in activity assay. As seen in Figure 6.25, after 15 days storage, nearly 82% of its original activity was maintained.

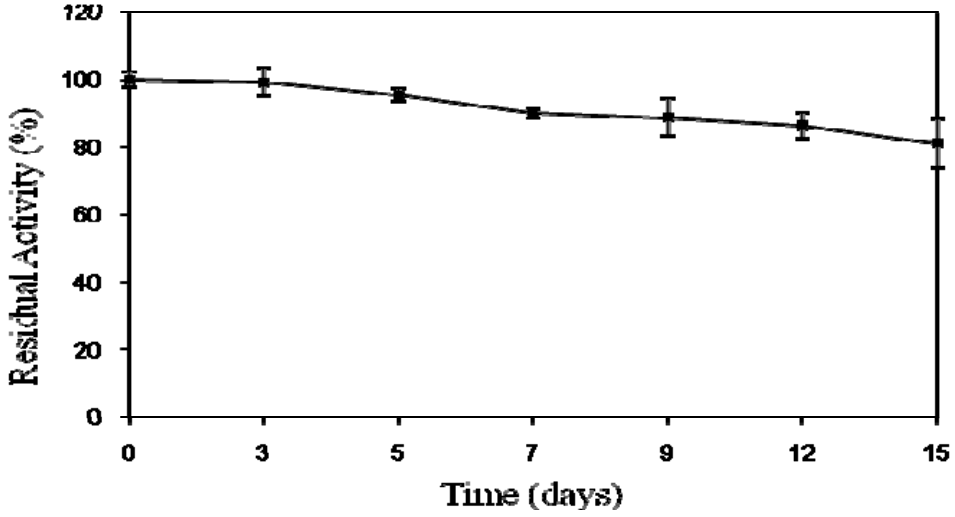


Figure 6.25. Effect of storage conditions on the activity of Immobilized β-galactosidase
Reaction conditions: 4^0C, potassium phosphate buffer (pH 6.5), 15 days

The storage stability of commercial β-galactosidase from *Kluyveromyces lactis* immobilized on the tolylene-2, 4-diisocyanate-activated alumina was investigated by Finocchiaro et al. (1980). Enzyme was stored at 4°C, in phosphate buffer (pH 7.5) and finally, similar to our study immobilized enzyme was found to conserve approximately

%80 of its original activity after 20 days. In another study on immobilization of β-galactosidase on graphite surface (Zhou and Chen, 2000), immobilized enzyme has explained to loose %86 of its original activity after 30 days storage.

6.3. Hydrolysis of Lactose with Immobilized β-Galactosidase

To monitor the lactose hydrolysis behavior, immobilized and free lactase preparations were used to hydrolyze the lactose in 5% (w/v) buffered (pH 6.5) solution. In batch experiments, the percentage of lactose conversion up to 180 minute of incubation for immobilized lactase was 64%. In terms of free enzyme 89% hydrolysis of lactose solution was achieved (Figure 6.26)

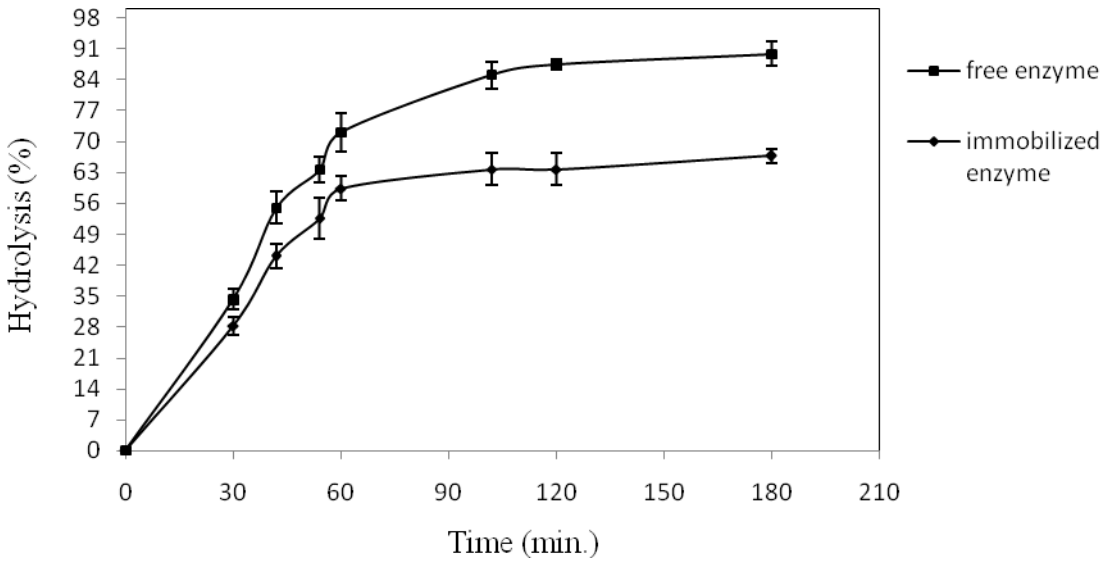


Figure 6.26. Hydrolysis of lactose with free and immobilized β-galactosidase Reaction conditions: 37^0C, potassium phosphate buffer (pH 6.5,$MgCl_2$)

Different researches have reported different time courses for lactose hydrolysis. In a study conducted by Batsolova et al. (1987), 75% hydrolysis in whey was achieved after six hours with the immobilized β-galactosidase. On the other hand, 85% hydrolysis in milk was revealed in a study carried out with thiosulfonate/thiosulfinate immobilized lactase derivatives (Ovsejevi, et al.1998).

Mateo et al. (2004) studied the effect of immobilization on the inhibiton promoted by glucose and found that when using the soluble enzyme or the Eupergit C-boronate preparation, the reaction stopped at about 90% conversion. 100% hydrolysis was obtained when glutaraldehyde or glyoxyl preparations were used within 100-150 minutes.

CHAPTER 7

CONCLUSION

In this study, commercially available β-galactosidase enzyme was immobilized onto chitosan and hydroxyapatite composite beads using the optimal conditions determined.

Equal weights of chitosan and hydroxyapatite were used for the preparation of the composite beads. The enzymatic activity of β-galactosidase in the immobilized system was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Chitosan-hydroxyapatite beads are routinely cross-linked with glutaraldehyde during enzyme immobilization, which increases the binding capacity of chitosan through covalent bonding to the enzyme. The selection of glutaraldehyde concentration appeared to be much more important for the behavior of composite beads. The enzymatic activity seemed to need a minimum of glutaraldehyde concentration.

Another factors that affect the enzymatic activity is temperature and pH of the medium. As it is known by the increase in temperature reactions may accelerate however enzymatic activity is affected by the temperature, so maximum immobilization temperature was chosen as 20^0C. Maximum yield was obtained at pH 7.5, where free enzyme has an optimum pH of 7.5. Also it is known that above isoelectric point proteins have negative charges and when support and enzyme has similar charges, repulsion may ocur and yield will decrease. Thus, electrostatic interactions are not an effective factor for our system.

Ionic strength of the solution is another important factor fort he immobilization. In this study it was observed that the increase in NaCl concentration of the buffer, lead to sharp decrease in binding efficiency. This is the result of the hydrophilic support (chitosan). Moreover increase in the total number of ions present in the solution decreases the strength of the binding efficiency by increasing the competition for the binding sites.

Ratio of the amount of the enzyme solution to support resulted that when solid-liquid ratio is 0.3, immobilized enzyme reached to maximum.

Characterization studies revealed that the immobilized β-galactosidase retained much of their activity in wider ranges of temperature and pH, compared to free enzyme. The optimal temperature of the immobilized enzyme was 37°C same as its free counterpart. However, a shift was observed for the maximal activity in terms of pH 7. Km value of the immobilized β-galactosidase was (9.5 mM) higher than that of the free lactase (1.011 mM). Additionally, the immobilized enzyme exhibited improved stability property in terms of temperature.

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APPENDIX A

CHEMICALS USED

Table A.1. Chemicals Used

NO	CHEMICAL	CODE
1	Acetic acid	Panreac 141008.1612
2	A-lactose monohydrate	Sigma L 3625
3	Beta-galactosidase from *Kluyveromyces lactis*	Sigma G3665
4	Citric acid	Riedel 33114
5	Coomassie (R) brilliant blue G 250	Fluka 27815
6	Ethanol	Merck 1.11727
7	Glukocose assay kit	Sigma GAHK20
8	Glutaraldehyde grade II, %25	Sigma G6257
9	Hydrochloric acid	Riedel 07102
10	Magnesium chloride	Fluka 63063
11	O-Nitrophenyl B-D-galactopyranoside	Sigma N 1127
12	ortho-phosphoric acid 85 %	Riedel 04107
13	Potassium phasphate monobasic	Fluka 60218
14	Potassium phosphate dibasic	Sigma P 5504
15	Protein standard	Sigma P 5619
16	Sodium acetate trihydrate	Merck-1.06267
17	Sodium carbonate	Riedel 13418
18	Sodium chloride	Riedel 31434
19	Sodium hydroxide	Riedel 06203
20	Sodium phosphate dibasic	Riedel 04270
21	Sodium phosphate monobasic	Sigma S 8282
22	Trizma base	Sigma T8404
23	Chitosan	sigma C3646
24	Hydroxyapatite	Sigma H0252
25	Sodium tripolyphosphate pentabasic	Sigma T5883

APPENDIX B

STANDARD CALIBRATION CURVE FOR ACTIVITY

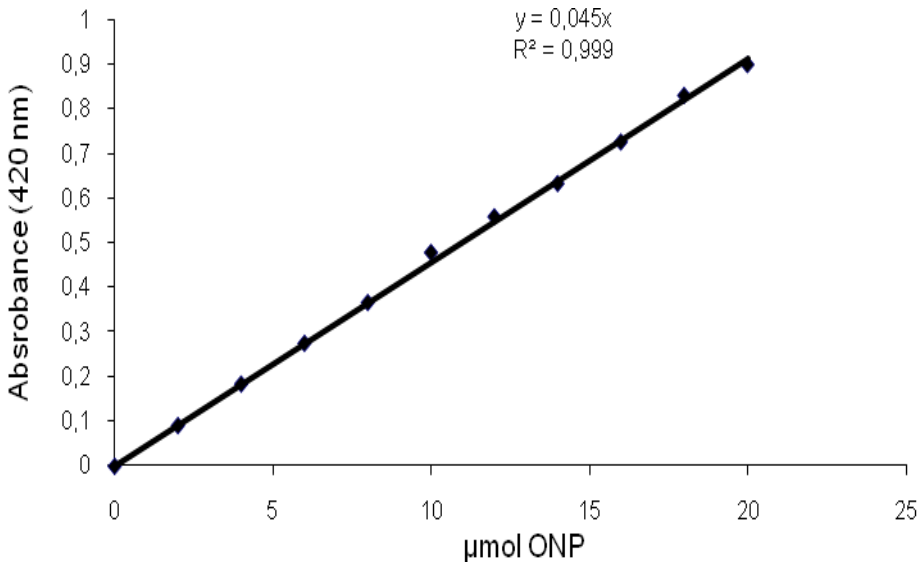

Figure B.1. Standard calibration curve for activity

APPENDIX C

STANDARD CALIBRATION CURVE FOR TOTAL PROTEIN

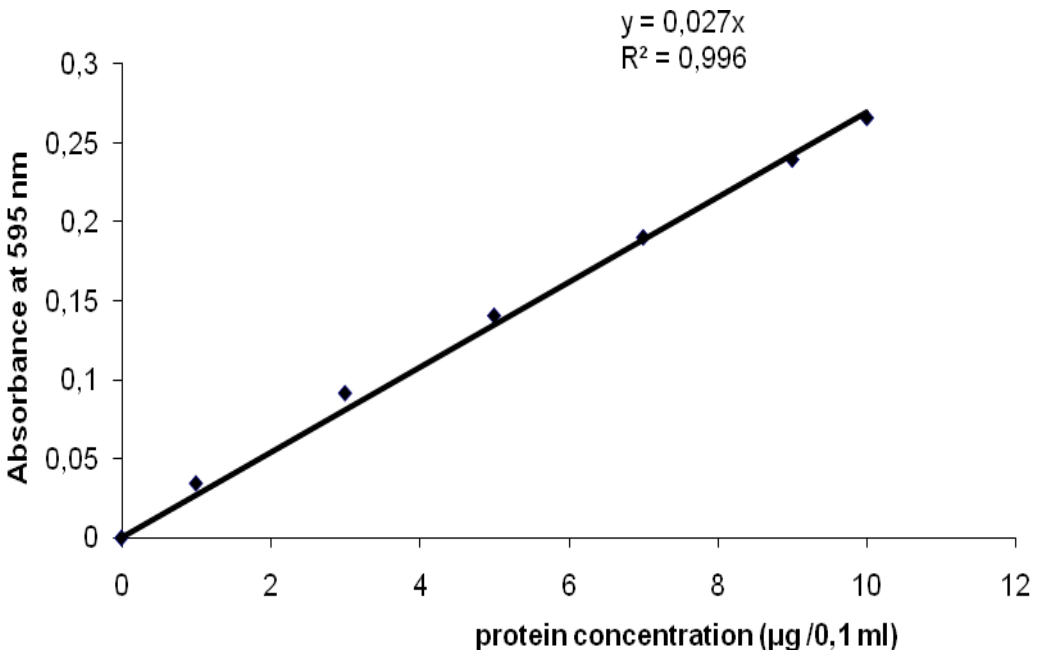

Figure C.1. Standard calibration curve for total protein