Immobilization of Lipase from *Candida rugosa* on Hydrophobic and Hydrophilic Supports

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ABSTRACT

The aim of this study is to find the optimum conditions for the immobilization of *Candida rugosa* lipase and also to select the most suitable support maximizing the immobilized enzyme yield under these optimum conditions.

Prior to the immobilization studies, optimum working conditions of soluble *Candida rugosa* lipase were investigated. *Candida rugosa* lipase expressed maximum activity in pH 6.5 phosphate buffer with 1.0 M NaCl solution at 30 ^{0}C. Indeed, lipase was fully active between 4 and 37 ^{0}C. Under these conditions, activity of free lipase was 12.2 U/mg enzyme.

Immobilization studies were carried out under constant temperature and stirring rate. Immobilization of lipase on hydrophobic support, octyl-sepharose, was very rapid and the equilibrium was reached in 10 minutes. Immobilized enzyme ratio was maximized by the use of pH 7.0 phosphate buffer with 1.0 M NaCl at 37 ^{0}C. Also, optimum solid-liquid ratio was found to be 0.1 when 1 ml support was added to 9 ml of enzyme solution. Effect of enzyme loading on lipase immobilization on octyl-sepharose was investigated at two different temperatures. At 20 ^{0}C and in the specified range of enzyme concentration (0.25-75 mg/ml), the experimental data fitted well to the linear isotherms with a C value of 138.2. Also, at 4 ^{0}C, results were similar and the experimental data could be expressed with the linear isotherm with a C value of 209.1. Under these conditions, activity of immobilized enzyme was 410 U/ml hydrated support.

In the case of immobilization on hydrophilic support, chitosan, the reaction was much slower and the amount of the immobilized enzyme was maximized at the end of 24th hour. Optimum conditions of immobilization were determined as pH 6.5 and 20 ^{0}C. In this case, addition of salt decreased the immobilization and, therefore, low ionic strengths favored immobilization. Again, optimum solid-liquid ratio was found to be around 0.1 when the 1 ml support was added to 9 ml. of enzyme solution. The effect of enzyme loading was investigated at 20 ^{0}C and in the specified range of concentration, data fitted well to the Langmuir isotherm where the constants were calculated to be q_m=200 U adsorbed enzyme/ml hydrated support and K= 72.5 U/ml solution.

The storage stability of chitosan immobilized lipase was investigated at 4 ^{0}C under two different conditions. When immobilized lipase was kept dry for 25 days,

50 % of the initial activity remained. However, if the same lipase was kept in phosphate buffer of pH 6.5, activity loss was only 10 % after a period of 2 months.

Complementary work was also carried out to find the differences in the behavior of free and chitosan immobilized lipase against different substrates. The results showed that the presence of 33 % olive oil and 45.5 % corn oil in substrate yielded the maximum activity for free and chitosan immobilized lipases, respectively.

ÖZ

Bu çalışmada, *Candida rugosa*' dan elde edilmiş lipaz enziminin hidrofobik ve hidrofilik desteklere immobilizasyonu sırasında, en yüksek immobilize olan enzim oranının ve immobilize enzim aktivitesinin elde edilebileceği çalışma koşulları ve desteğin belirlenmesi amaçlanmıştır.

Bu amaçla, immobilizasyon denemelerinden önce serbest enzim için en uygun çalışma koşulları araştırılmıştır. *Candida rugosa*'dan elde edilen lipaz enziminin aktivitesinin pH değeri 6.5 olan ve 1.0 M NaCl içeren fosfat çözeltisi içinde ve 30 ^{0}C inkübasyon sıcaklığında en yüksek değere çıktığı görülmüştür. Ayrıca, 4 ile 37 ^{0}C arasında enzim aktivitesinde belirgin bir değişim olmadığı da gözlenmiştir. Belirtilen koşullarda serbest enzimin aktivitesinin 12.2 U/mg enzim olduğu ve enzimin belirtilen koşullarda aktivitesini 24 saat boyunca koruduğu saptanmıştır.

Tüm immobilizasyon denemeleri sabit sıcaklık ve karıştırma hızında gerçekleştirilmiştir. Lipaz enziminin hidrofobik yapıdaki oktil-sefaroz desteğine 10 dakika gibi kısa bir sürede, çok hızlı bir şekilde bağlandığı belirlenmiştir. İmmobilizasyon ortamında pH değeri 7.0 olan fosfat çözeltisi ile 1.0 M NaCl bulunduğunda ve ortam sıcaklığı 37 ^{0}C olduğunda immobilize olan enzim oranının en yüksek seviyeye çıktığı görülmüştür. Ayrıca, katı-sıvı oranının 0.1 olmasının yani 1 ml desteğe 9 ml enzim çözeltisi katılmasının en uygun sonucu verdiği belirlenmiştir. Enzim miktarının immobilizasyon üzerine etkisi iki farklı sıcaklıkta araştırılmıştır. 20 ^{0}C'de yapılan denemede, çalışılan konsantrasyon aralığında deney verilerinin lineer izoterme uyduğu görülmüş ve C sabiti 138.2 olarak hesaplanmıştır. 4 ^{0}C'de yapılan deneme de benzer sonuçlar vermiş ve lineer izoterme uyum sağlamıştır. Bu sıcaklıktaki C değeri ise 209.1 olarak saptanmıştır. Bu koşullar altında immobilize enzimin aktivitesi 410 U/ml destek olarak hesaplanmıştır.

Lipazın hidrofilik yapıda olan kitosana immobilizasyon sırasında ise, reaksiyonun çok daha geç dengeye geldiği görülmüştür. Bu durumda immobilizasyon işlemi 24 saat devam etmektedir. İmmobilizasyon ortamının pH değerinin 6.5 olması ve ortam sıcaklığının 37 ^{0}C olması durumunda immobilize olan enzim oranının en yüksek seviyeye çıktığı görülmüştür. Fakat, bu denemelerde ortamda tuz bulunmamasının immobilizasyon üzerinde olumlu bir etkisi olduğu gözlenmiştir. Ayrıca, hidrofobik destekte olduğu gibi, katı-sıvı oranının 0.1 olmasının yani 1 ml desteğe 9 ml enzim

çözeltisi katılmasının en uygun derişim olduğu tayin edilmiştir. Enzim miktarının immobilizasyon üzerindeki etkilerinin incelendiği denemelerde ise verilerin Langmuir izotermi'ne uyduğu görülmüş ve sabit değerler q_m=200 U immobilize enzim/ml destek ve K= 72.5 U /ml çözelti olarak saptanmıştır.

Kitosana immobilize olmuş lipazın buzdolabı sıcaklığında (4 ^{0}C) dayanımı iki farklı koşulda araştırılmıştır. İmmobilize enzimin kuru ortamda bekletilmesi durumunda, 25 günlük bir süreçte aktivitesini % 50 oranında kaybettiği görülmüştür. Fakat, aynı enzimin pH değeri 6.5 olan fosfat çözeltisi içinde bekletilmesi durumunda 2 aylık bir süreçte aktivite kaybının sadece % 10 oranında olduğu tespit edilmiştir.

Bunlara ilave olarak, serbest ve immobilize lipaz enziminin farklı substratlara karşı davranışını gözlemlemek için ek bir çalışma yapılmıştır. Sonuç olarak, hidroliz ortamında % 33 oranında zeytinyağının substrat olarak kullanılmasının serbest enzimin en yüksek aktiviteyi göstermesini sağladığı görülmüştür. Kitosan'a immobilize olmuş lipaz enzimi için ise hidroliz ortamında % 45.5 oranında mısır özü yağı bulunması gerektiği gözlenmiştir.

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Chapter 1

INTRODUCTION

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates (http:// www.iisc.ernet.in /~currsci/ july10/ articles18.htm). The estimated worldwide sales volume for industrial enzymes in 1995 is US $ 1 billion and this volume is foreseen to double until 2005. At least 75 % of all these enzymes are hydrolases, and 90 % of them are produced from microorganisms by fermentation. After the realization of the biocatalytic potential of microbial lipases in both aqueous and nonaqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance and following proteases and carbohydrates, lipases are considered to be the third largest group based on total sales volume (Jaeger et al., 1997).

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoglycerols and glycerol. Besides this, they are also efficient in various reactions such as esterification, transesterification and aminolysis in organic solvents. Therefore, those enzymes are nowadays extensively studied for their potential industrial applications. Lipases can be obtained from animal, plant and also many natural or genetically engineered microorganisms both in endogenous and exogenous forms. Among lipases of plant, animal and microbial origin, it is the microbial lipases that find immense application. This is because microbes can be easily cultivated and their lipases can catalyze a wide variety of hydrolytic and synthetic reactions. The mechanisms of lipase-catalyzed reactions resemble closely the natural metabolic pathways; hence, lipase-based processes may be viewed as more environment-friendly than some bulk chemical synthesis. Owing to their chemical- and stereo-selectivity, lipases can produce high-added value products. Because of low activation energies,

lipase-mediated processes require mild temperature and pH, thus energy consumption is small and there is little damage to reactants and products. Also, they are stable in organic solvents do not require cofactors.

Lipases find use in a variety of biotechnological fields such as food and dairy, pharmaceutical, agrochemical, oleochemical, cosmetic industries and detergents. Thus, they are today the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists. Also, they can be further exploited in many newer areas where they can serve as potential biocatalysts.

In spite of its usefulness, application of lipases in industrial level is still limited because of drawbacks of the extensive use of lipases and biocatalysts in general compared to classical chemical catalysts can be found in the relatively low stability of enzyme in their native state as well as their prohibitive cost. Consequently, there is a great interest in methods trying to develop competitive biocatalysts for industrial applications by improvement of their catalytic properties such as activity, stability (pH or temperature range) or recycling capacity. Such improvement can be carried out by chemical, physical or genetical modifications of the native enzyme. At this point, enzyme immobilization makes sense because of enabling reusability, operational flexibility and ease of product recovery from the enzyme. Among all immobilization types, adsorption by hydrophobic interaction is an efficient method for lipase immobilization due to peculiar physicochemical character of the enzyme.

This dissertation focuses on the immobilization of lipase enzyme from a microbial source, *Candida rugosa*, on a hydrophobic and a hydrophilic support and addresses our attempts to find the optimum conditions for immobilization. Attempts were done to increase the immobilized enzyme ratio and yield by investigating the effects of some factors, such as temperature, pH, salt concentration, etc. Time course of immobilization, effect of enzyme loading and immobilization isotherms are also investigated. Moreover, effects of different substrates on hydrolysis efficiency and storage stability of immobilized enzyme are considered in this study.

Chapter 2

LIPASES

Lipases (EC.3.1.1.3, triacylglycerol acylhydrolyse) are enzymes that catalyze the reversible hydrolysis of animal fats and vegetable oils under natural conditions. Annual sales of lipases in 1989 account for only $ 20 million, which corresponds to less than 4 % of the worldwide enzyme market estimated at $ 600 million (Paiva et al., 2000; Balcao and Malcata, 1998). The apparent misconception of the economic significance of lipases can be caused by several factors. This may be because of, first, the most detailed studies involving lipases are recent and the usual time lag prior to full commercial exploitation has not yet elapsed, and the second is the relatively prohibitive cost of native enzyme. Moreover, the low stability, low activity or selectivity encountered occasionally with a number of these enzymes have been the chief obstacle hindering more rapid expansion of industrial lipase technology on a large scale. Therefore, customization of lipases by chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis involving aqueous and non-aqueous solvents. In addition, the cost of enzyme can be reduced by the application of molecular biological tools, such as recombinant DNA technology and protein engineering, which may allow the production of lipases in large quantities and with genetically enhanced properties (Villeneuve et al., 2000).

The interest in lipase research is increasing over the past decades. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, and chemo-, regio- and enantioselectivity. These properties allow them to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure. Secondly, they are widely distributed among the animals, plants and microorganisms and their activity is greatest against water-insoluble substrates and enhanced at the substrate (oil)-water interface; that is, they exhibit "interfacial activation". Thus, maximum activities are obtained in emulsion systems where high surface areas of the substrate can be obtained. Lipases are active not only in normal phase emulsions where the substrate is emulsified into an aqueous system (oil-in-water), but they are also active, often more active, in invert (water-in-oil) emulsions and in

reverse micelle systems containing an organic solvent solution of the substrate. Thus, it is not surprising that lipases, for many years, served as models for studying the regulation of interfacial, enzyme-catalyzed reactions. Final and the most important reason is linked to the enzyme's medical relevance, particularly to atherosclerosis and hyperlipidemia, and its importance in regulation and metabolism, since products of lipolysis such as free fatty acids and diacylglycerols play many critical roles, especially as mediators in cell activation and signal transduction.

2.1. Sources of Lipases

Lipase enzymes are distributed among higher animals, microorganisms and plants in which they fulfil a key role in the biological turnover of lipids. They are required as digestive enzymes to facilitate not only the transfer of lipid from one organism to another, but also the deposition and the mobilization of fat that is used as an energy reservoir within the organism. They are also involved in the metabolism of intracellular lipids, and, therefore, in the functioning of biological membranes.

Lipases can be obtained from animal, plant and microbial sources. Animal lipases include pancreatic and pregastric ones. Lipase can be extracted from porcine, pig and also human pancreas. This enzyme cleaves triglycerides, oils, fats, simple fatty acid esters and aryl esters. Animal lipases preferentially catalyze the hydrolysis of fatty acids with more than 12 carbon atoms and predominantly at the C-1 position of glycerol. The reaction rate decreases considerably in the substrate order tri-, di-, and monoglycerides. Porcine lipase was composed of a single chain of 449 amino acids and the calculated molecular weight of the protein moiety was about 50,000 and glycosidic residues gave a total molecular weight of 52,000 as observed by sedimentation equilibrium analysis. Serine residue that is involved at substrate binding is located at position 152 and it occurs in the amino acid sequence Gly-His-Ser-Leu-Gly. This sequence is homologous with other lipases (Antonian, 1988).

Other type of animal lipases includes pregastric lipases from goats, sheep, and calves. These enzymes preferentially catalyze the hydrolysis of short-chain fatty acids in milk fat and are used in the production of specially flavored cheeses.

Second type of lipases source is plant. Germinating oilseeds contain a considerable amount of lipases. Crude lipase preparations were isolated from seedling of rape (*Brassica napus*), mustard (*Sinapis alba)*, castor bean (*Ricinus communis*) and

cotyledons of lupine (*Lupinus albus)*; by acetone or buffer solution extractions. Each showed specificity for the 1,3 positions of triacylglycerols with maximum activity between pH 8 and 9 (Antonian, 1988). Also, in recent years, lipase is extracted from rice, which is one of the most important food cereals for the world's population.(Prabhu et al., 1999).

The last and most common lipase source is microorganisms that are produced by fermentation of different fungi and bacteria. The industrial products are mixtures of lipases and esterases. Bacteria like *Staphylococcus spp., Pseudomonas spp., Chromobacterium spp., Achromobacter spp., Alcaligenes spp.* are commonly used for lipase production.

Lipases from *Aspergillus species;* namely from *Aspergillus niger* and *Aspergillus oryzae.* The molecular masses are between 200,000 and 250,000 and optimum pH is between 4.5 and 6.5. They act on coconut oil, linseed oil and olive oil with yields of 48-93 %. Special types of such lipases are used in cheese ripening.

Lipases from *Candida rugosa:* Molecular masses 120,000, isoelectric point is pH 4.5 and optimum activity is between pH 6.5 and 7.5 (Petersen et al., 2001). They hydrolyze olive oil with a yield of 95-97 %.

Lipases from *Rhizopus*; namely from *R. arrhizus, R. javanicus, R. niveus, R. delemar. R. arrhizus* lipase has a molecular mass of 43,000 and isoelectric point is pH 6.3. The enzyme is a glycopeptide with 13-14 % mannose per molecule. There is 1.3-regiospecificity, the optimum of activity is at pH 5.0-7.0 and optimum temperature is at 30-45 ^{0}C.

Lipases from *Mucor species:* They catalyze the glycerol transesterification in 1-3 position. Different types are specific to long and short chain fatty acids.

Lipases from *Pseudomonas:* Molecular mass is 29,000 and isoelectric point is at pH 5.8 and optimum pH range is about 6.5-8.5 (Petersen et al., 2001). Since it is active and stable at alkaline pH, it can be used in detergents. For the same purpose, lipases from *Humicola languinosa* with pI value of 5.0 can be used (Petersen et al., 2001).

Microbial sources of lipases can be seen in Table 2.1.

Table 2.1. Sources of microbial lipases (Fadıloğlu, 1996)

Bacterial	
	Chromobacterium viscosum
	Lactobacillus spp.
	Micrococcus caseolyticus
	Pseudomonas fluorescens
	Pseudomonas fragi
	Pseudomonas spp.
	Streptococcus faecalis
	Streptococcus thermophilus
Fungal	
	Aspergillus niger
	Candida curvata
	Candida cylindracea
	Candida deformans
	Candida quillermondi
	Fusarium oxysporum
	Geotrichum candidum
	Geotrichum cyclopium
	Humicola lanuginosa
	Mucor miehei
	Penicillium camemberti
	Penicillium crysogenum
	Penicillium cyclopium
	Phycomyces nitens
	Rhizopus arrhizus
	Rhizopus delemar
	Rhizopus oligosporus
	Saccharomycopsis lipolytica
	Yarrowia lipolytica

2.2. Classification of Lipases

The main advantage of lipases, which differentiates enzymatic reaction from chemical reaction, is lipase specificity. Lipase specificity is controlled by the molecular properties of the enzyme, structure of the substrate, and factors affecting binding of the enzyme to the substrate. The fatty acid specificity of lipases has been exploited to produce structured lipids for medical foods and to enrich lipids with specific fatty acids to improve the nutritional properties of fats and oils. There are three main types of lipase specificity: positional, substrate, and stereospecificity.

2.2.1. Nonspecific Lipases

Certain lipases are responsible from the hydrolyses of all glyceride bonds formed between fatty acids and glyceride randomly; position of glyceride molecule is not important (Figure 2.1-a). Examples of nonspecific lipases include lipases derived from *Candida rugosa, Corymebacterium acnes, Chromobacterium spp.* and *Staphylococcus aureus.*

2.2.2. Positional Specificity

These lipases have specificity towards ester bonds in position *sn*-1, 3 of the triacylglycerol that is the glyceride bonds at terminal position. (Figure 2.1-b). Steric hindrance prevents the fatty acid in position *sn*-2 from entering the active site. In this reaction, through the acyl migration, 2-fatty acid is pushed to 1 or 3 position of the glycerol molecule. However, as this process is slow, the hydrolysis slows down and awaits the acyl migration to complete for enabling the lipase to attack the glyceride at the 1 and/or the 3 position. Lipases that are 1.3-specific include those from *Aspergillus niger, Mucor miehei, Rhizopus arrhizus* and *Rhizopus delemar.*

2.2.3. Lipases Specific to Chain Length of Fatty Acid

Some of the lipases have affinity for short-chain fatty acids (acetic, butyric, capric, caproic, caprylic, etc.) while some of them being specific toward medium and long chain fatty acids. For example, porcine pancreatic lipase is specific toward shorter

chain fatty acids, while lipase from *Penicillium cyclopium* is specific toward long chain fatty acids. As well, lipases from *Aspergillus niger* and *Aspergillus delemar* are specific toward both medium chain and short chain fatty acids. Other lipases have been found to be specific toward fatty acids of varying lengths. Marangoni et al. found that in the hydrolysis of butter oil, lipase from *Candida rugosa* showed specificity toward butyric acid, whereas *Pseudomonas fluorescens* lipase did not.

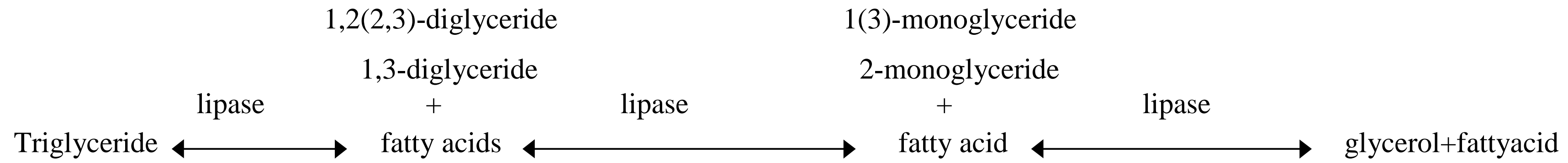
2.2.4. Lipases Specific to Fatty Acid

Many lipases are specific toward particular fatty acid substrates. Most of the lipases from microbial sources show little fatty acid specificity but lipase from *Geotrichum candidum* is specific toward long chain fatty acids containing *cis-9* double bonds. Lipases that are specific to fatty acid hydrolyse the glyceride bonds formed by *cis-9* unsaturated fatty acids. (Oleic, linoleic and linolenic acids) The chain specificity of *Candida rugosa* lipase is in the order of oleic > lauric > palmitic > myristic > stearic.

Table 2.2. Substrate specificity towards triglycerides (Fadıloğlu, 1996).

Source	Triglycerides
C. viscosum	C4-FA
Bacillus spp.	C8-FA
G. candidum	Δ^9-C18-FA
	C8-FA
	C16-FA
H. lanuginosa	C12-FA
P. nitens	C8-FA
S. lipolytica	Δ^9-C18-FA
C. cylindracea	Δ^9-C18-FA
P. cyclopium	Δ^9-C18-FA
	C16-FA

A Non-Specific Lipase



B. 1,3-Specific Lipase

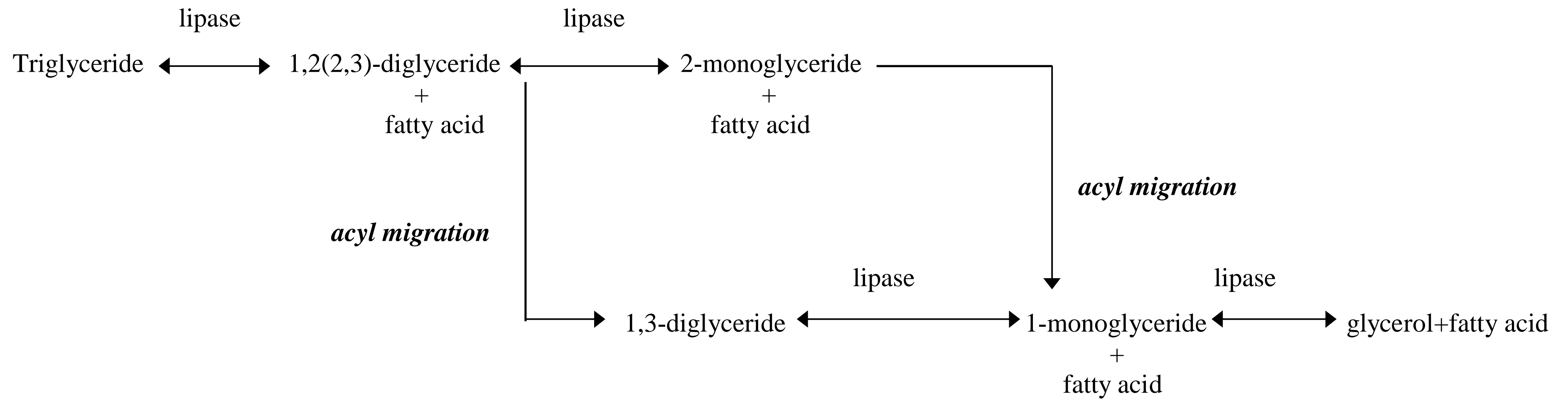


Figure 2.1. Reaction scheme lipase specificity.

2.3. Reactions Catalyzed by Lipases:

Lipases have been used as biocatalysts for a variety of reactions. Unlike other hydrophilic enzymes, lipases from different origins are uniquely stable in non-polar organic solvents and can accept a broad range of substrates of varying size and stereochemical complexities. Their flexible protein backbones, which assume a variety of conformations, give them the ability to carry out many profitable reactions such as hydrolysis, and also reverse of hydrolysis reaction; esterification, transesterification (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification. The equilibrium between the forward (hydrolysis) and the reverse (synthesis) reactions is controlled by the water activity of the reaction mixture.

2.3.1. Hydrolysis

Lipases catalyze the cleavage of ester bonds of triacylglycerols with the concomitant consumption of water molecules, which is called as hydrolysis. The American fatty acid industry splits fats using the Colgate-Emery process. In this process superheated steam is sparged into the fat. The usual conditions for splitting are the pressure of 700 psig and temperature of 240-260 ^{0}C or higher. The entire fat splitting operation is blanketed with nitrogen. Nevertheless, polyunsaturated fats undergo significant degradation and must be extensively purified by distillation for most uses. Also, the Colgate-Emery process is very energy intensive, using about 340 Btu of energy per pound of oil split. Therefore, an efficient and inexpensive method of rapidly hydrolyzing oleaginous materials of all types into their constituent fatty acids and glycerol has been developed which uses lipase enzyme as catalyst. Hydrolysis may be conducted in organic solvent at room temperature to yield a colorless, non-oxidised material. The fatty acid is produced as the free acid, rather than the acid salt, and may be removed from the lipase using organic solvent washes. Yields greater than 97 % can be obtained (Akoh and Min, 1998).

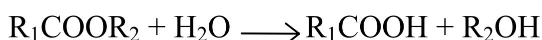
Therefore, the use of lipases for enzymatic splitting of fats in the presence of excess water is more appealing since the reaction proceeds under mild conditions of pressure and temperature with specificity, high yield and reduced waste. This technology is currently employed in the production of fatty acids, diglycerides,

monoglycerides, flavoring agents for dairy products and detergents for laundry and household uses.

2.3.2. Esterification

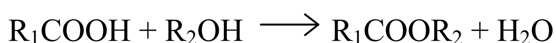
Esterification reactions between alcohol and free fatty acids, that is the reverse of the hydrolysis, are catalyzed by lipases in water-poor organic solvents under conditions of low water activity or even solvent free systems. Although ester synthesis can be done chemically with acid or base catalysis, the use of enzyme technology offers the advantages of mild conditions, reduced side reactions, and specificity. Examples of high-value chemicals obtained by lipase-catalyzed ester synthesis include the production of oleic acid esters of primary and secondary aliphatic and terpenic alcohols, and the production of geranyl and menthyl esters from butyric acid and geranol, or lauric acid and menthol, respectively (Villeneuve et al., 2000).

- Hydrolysis



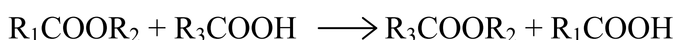
$R_1COOR_2 + H_2O \longrightarrow R_1COOH + R_2OH$

- Ester Synthesis:



$R_1COOH + R_2OH \longrightarrow R_1COOR_2 + H_2O$

- Acidolysis:

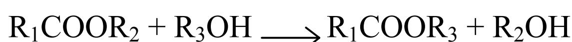


$R_1COOR_2 + R_3COOH \longrightarrow R_3COOR_2 + R_1COOH$

- Interesterification:

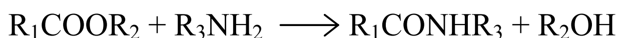
$R_1COOR_2 + R_3COOR_4 \longrightarrow R_3COOR_2 + R_1COOR_4$

- Alcoholysis



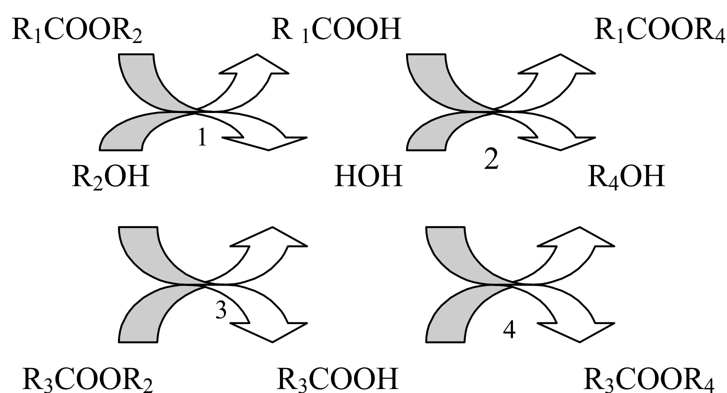
$R_1COOR_2 + R_3OH \longrightarrow R_1COOR_3 + R_2OH$

- Aminolysis



$R_1COOR_2 + R_3NH_2 \longrightarrow R_1CONHR_3 + R_2OH$

Figure 2.2. Reactions catalyzed by lipases.



1: Hydrolysis

2: Ester synthesis

1+2: Alcoholysis

1+3: Acidolysis

1+2+3+4: Transesterification

Figure 2.3. Schematic illustration of lipase reactions (Balcao et al., 1996).

2.3.3. Transesterification

The term transesterification refers to the process of exchanging acyl radicals between an ester and an acid (acidolysis), an ester and another ester (interesterification), or an ester and an alcohol (alcoholysis). Transesterification is accomplished industrially by heating a mixture of the anhydrous ester and another reactant species at relatively high temperatures. Alternatively, alkali metals or alkali alkylates may be used at lower temperatures. However, the application of lipases for the modification of fats and oils by transesterification offers again the advantages of mild conditions, reduced side reactions, and specificity. One example is the production of cocoa butter analogues from cheaper feedstocks. This reaction is accomplished via lipase-catalyzed transesterification reactions involving palm oil mid fraction and stearic acid, or palm oil mid fraction and tristearoylglycerol (Villeneuve et al., 2000).

2.3.4. Catalysis on "Unnatural" Substrates

Lipases are not limited to catalysis of the synthesis and hydrolysis of carboxylic acid esters. They can utilize compounds other than water and alcohols as nucleophiles.

Lipases are thus capable of catalyzing different reactions such as aminolysis, thiotransesterification, and oximolysis in organic solvents with selectivity. The selectivity of lipases in the aminolysis of esters in anhydrous media has been successfully used for peptide and fatty amide syntheses. These results hold promise for using lipase technology in the synthesis of optically active peptides, polymers, surfactants and new detergents at low cost (Villeneuve et al., 2000).

2.4. Three - Dimensional Structure of Lipases

Lipases can be derived from animal, bacterial, and fungal sources so they all tend to have similar three-dimensional structures. Between 1990 and 1995, crystallographers solved the high-resolution structures of 11 different lipases and esterases including 4 fungal lipases, a bacterial lipase and human pancreatic lipase. It has been seen that despite differences in size, sequence homology, substrates, activators, inhibitors, and other properties, most of them adopt a similar core topology. The characteristic patterns found in all lipases studied so far have included α/β structures with a mixed central β-sheet containing the catalytic residues. The interior topology of α/β hydrolase fold proteins is composed largely of parallel β -pleated strands (at least five in lipases), separated by stretches of α -helix, and forming, overall, a superhelically twisted-pleated sheet. Helical peptide sections packed on both faces of this sheet form much of the outer surface of the protein. Three-dimensional structure of *Candida rugosa* lipase can be seen in Figure 2.4.

In general, a lipase is a polypeptide chain folded into two domains, the C-terminal domain and the N-terminal domain. The N-terminal domain contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that can accommodate a long fatty acid chain (Akoh and Min, 1998).

Despite widely varying degrees of sequence homology between the members of this family, one sequence is exceptionally highly conserved: the pentapeptide Gly-X-Ser-X-Gly. The conservation of this serine, and the loss of catalytic activity upon its modification or replacement, argue that this amino acid is crucial to catalysis. Its topographic location is also conserved and significant: it sits at the apex of a tight bend ("nucleophilic elbow") in the protein chain, a bend that can be formed only when the amino acids at the -2 and +2 positions relative to the serine have small side chain groups and hence, the predominance of glycine at these locations.

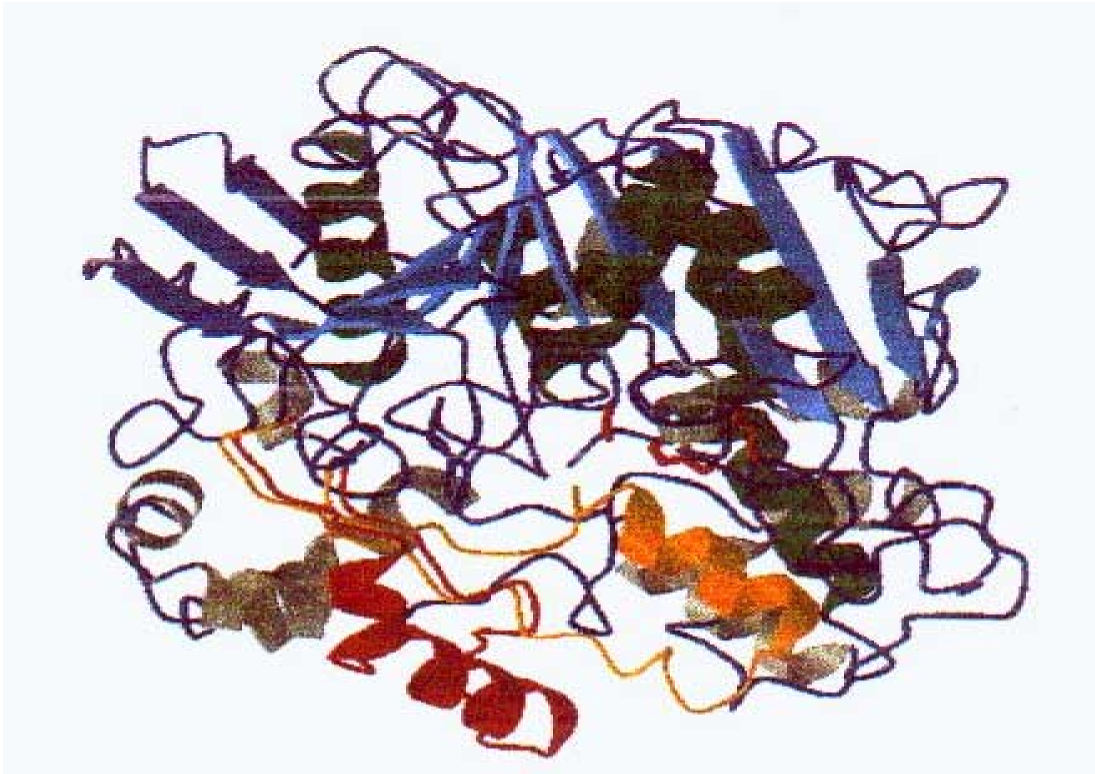


Figure 2.4. Three dimensional structure of *Candida rugosa* lipase (With open and closed states of the lid superimposed. The central mixed L-sheet is light blue and the smaller N-terminal L-sheet is dark blue. Helices, which pack against the central L-sheet, are dark green. The closed conformation of the lid is yellow and the open conformation is red. The residues forming the catalytic triad are shown in red).

In addition to the catalytic serine, the active centers of most lipases consist of a histidine and an acidic amino acid (Asp or Glu). The nucleophilic serine rests between a β-strand and an α-helix, while histidine and aspartic acid or glutamic acid rest on one side of serine. The three residues forming a catalytic triad whose topology is highly conserved among the lipases and is in many ways analogous to that of the serine proteases.

The catalytic mechanism assumed for triacylglyceride lipases is centered on the active site Ser. The nucleophile oxygen of the active site Ser forms a tetrahedral hemiacetal intermediate with the triacylglyceride. The ester bond of the hemiacetal is hydrolysed and the diacylglyceride is released. The active site serine acyl ester subsequently is believed to react with a water molecule, and the acyl enzyme is then cleaved and the fatty acid is dissociated. At this stage of the catalytic process, product release from the active site cleft is of special importance (Petersen et al., 2001).

2.4.1. Interfacial Activation

Lipases are unique enzymes in that they require interfacial activation for full catalytic performance, a fact that was first established in 1958 by Sarda and Desnuelle (Balcao et al., 1996). The phenomenon of interfacial activation was, infact, first observed in 1936 by Holwerda et al. and then in 1945 by Schonheyder and Volqvartz. (Verger, 1997).

In 1990, after the elucidation of first 3D structures of lipases, it was suggested that the phenomenon of interfacial activation might be due to the presence of an amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid. In the absence of an interface, lipase has elements of secondary structure, which makes it inaccessible to substrates. However, when a hydrophobic interface is present, lipase would yield "open structure" and enzyme would be active. In this way, lipases would seem to be strongly adsorbed to hydrophobic interfaces through several pockets of a large hydrophobic surface, which surrounds the catalytic side. This mechanism can be explained by lid conformation. Active site of lipases includes Asp-His-Ser residues. In solution, a helical segment covers the active site of lipase, but in the presence of lipids or organic solvent, there is a conformation change in which the lid opens, exposing the hydrophobic core containing the active site. Therefore, the requirement for an interface is essential for lipolytic activity. The outside of the lid is relatively hydrophilic, while the side facing the catalytic site is hydrophobic. Upon association with the interface, the lid folds back, revealing its hydrophobic site, which leads to increased interactions with the lipid at the interface. The substrate can then enter the hydrophobic tunnel containing the active site. The lid is held in place by mostly hydrophobic and some hydrogen bonds. The structure of the lid differs for lipases in the number and position of the surface lops. The ability of the lipase to bind to lipid at the interface is effected by the amphipathicity of the α helix in the lid. As the amphiphilic properties of the loop are reduced, the activity of the enzyme is decreased.

In this way, it can be assumed that interfacial activation leads very slight differences in lipase structures (e.g. differences between isoenzymes or isoforms) and possible post-translational changes (e.g. partial de-glycosylation) promote strong changes in the exact structure of open lipases and, hence, in their catalytic properties, mainly in selectivity (Sanchez et al., 1999).

In considering the action of the lipases at the interfaces, several factors should be considered, including the reversibility of adsorption, the possibility of inactivation, and the quality of the interface. Generally, lipases are considered to be reversibly adsorbed at the interfaces, since increasing surface pressure has been found to result in the desorption of lipases from the interface. The quality of the interface can effect the activity of lipases. Any factor that affects the affinity of the enzyme for the interface and also the packing and orientation of the molecules at the interface can affect activity.

2.5. Properties of Lipases

2.5.1.Inhibition and Activation of Lipase

Screening of the effect of the ions and reagents on lipase activity showed that heavy metal ions inhibit the lipase activity while most alkali metal ions enhance the activity. The most effective ion on lipase activity is the calcium. This ion stimulates the lipase-catalyzed hydrolysis of olive oil by:

1.Binding to the enzyme resulting in a change in enzyme conformation

2.Enhancing the adsorption of the lipase to the oil-water interface.

3.Removing the fatty acids produced by hydrolysis from oil-water interface to make lipase bind to other oil molecules thereby eliminating the possibility of product inhibition.

However, calcium ion does not effect the tributyrin hydrolysis since butyric acid is soluble in water even in the absence of calcium.

Many substances have been shown to inhibit the lipase activity; examples include anionic surfactants, certain proteins, Co^{2+}, Ni^{2+}, Hg^{2+}, Sn^{2+}, boronic acids, phosphorus-containing compounds such as diethyl-*p*-dinitrophenyl phosphate, phenylmethyl sulfonylfluoride, certain carbamates, β-lactones and diisoproplyfluoro phosphate (Akoh and Min, 1998). Also, Zn^{2+}, Mg^{2+}, EDTA and sodium dodecyl sulfate slightly inhibits the lipase activity (http://www.iisc.ernet.in/ ~currsci / july10/ articles 18.htm).

Activation or inhibition of lipase by ions or reagents depends on the substrate, enzyme source and assay conditions. Bile salt sodium taurodeoxycholate inhibits the pancreatic lipase activity when tributyrin is the substrate, and 10 kDa protein colipase could reverse the inhibition, the bile salt actually stimulated hydrolytic activity when

triolein was substrate. This was attributed to the fact that triolein is a more natural substrate for the lipase than tributyrin. Furthermore, stimulation of *Candida rugosa* lipase activity was attributed to the formation of calcium salts of fatty acid products in a normal phase emulsion, with olive oil as the substrate but not with tributyrin; calcium, however, had no effect in an invert emulsion. In a nonemulsion system, calcium had no effect on *Candida rugosa* lipase activity with olive oil as the substrate but tended to offset the inhibitory effects of bile acid. On the other hand, when sodium taurocholate was added to an emulsion assay of the lipase from *Candida rugosa* with olive oil as the substrate, activity was progressively inhibited from 0.1 mM to 0.8 mM concentration of the bile salt. Relatively high activity at the lowest concentration was attributed to the role of the bile salt in the stabilization of oil particles in the emulsion and providing high interfacial area for adsorption of the lipase, and inhibition was due to interaction with the enzyme such adsorption was reduced.

Table 2.3. Inhibitors and activators of microbial lipases (Fadıloğlu, 1996).

Source	Inhibitors	Activators
H. lanuginosa	Co^{2+}, Ni^{2+}, Cu^{2+}, Sn^{2+}, Hg^{2+}	Lipase $^{+}$, K^{+}, Ca^{2+}
P. nitens	Ag^{+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, $KmnO_4$, NBS	Mammalian Bile
C. deformans	Cu^{2+}, Zn^{2+}, PCMB, EDTA	Ca^{2+}, Mg^{2+}, Co^{2+}
A. niger	Ag^{+}, iodoacetamide	-----------------------
C. rugosa	Hg^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}	-----------------------
Bacillus spp.	Cu^{2+}, Zn^{2+}, Hg^{2+}	-----------------------
P. fluorescens	I_2, Fe^{3+}, NBS	Ba^{2+}, Ca^{2+}
C. viscosum	Cu^{2+}, Hg^{2+}, Sn^{2+}	Ca^{2+}, Mg^{2+}, Mn^{2+}, Bile Salts
B. bassiana	Fe^{2+}, Cu^{2+}, Tween-80	Mg^{2+}
Porcine Pancreas	Ca^{2+}, Sr^{2+}, Mg^{2+}	Versene, Zn^{2+}, Cu^{2+}, Hg^{2+}, iodine PCMB.

2.5.2. Optimum pH of Lipases

Lipases are catalytically active only at certain pH values, depending on their origin and the ionization state of residues in their active sites. Basic, acidic and neutral residues are included at the active sites of lipases and therefore, the residues in the catalytic site are active only in one particular ionization state. The pH optima for most lipases lie between 7.0 and 9.0, although lipases can be over a wide range of acid and alkaline pH values, from about 4 to 10.

Microbial lipases show profound stability around pH 6.0-7.5 with considerable stability at acidic pH up to 4.0 and alkaline pH up to 8.0. For example, *Pseudomonas* lipase is optimally active at pH 7.0 and stable at pH 5.0-10.5. As a result of the adaptable nature of the bacteria, the pH dependencies of activity and stability may depend on culture conditions. If the organisms are grown at alkaline pH, the lipases produced may have an alkaline pH optimum. The pH optima of some bacterial lipases are given in Table 2.4.

Table 2.4. pH optimum of microbial lipases (Fadıloğlu, 1996).

Source	Substrate	Optimum pH
C. cylindracea	Olive oil	5.0-7.0
	Tributyrin	7.0
P. fluorescens	Sesame oil	7.0
Bacillus spp.	Olive oil	5.5-7.2
G. candidum		
Lipase I	Olive oil	8.0
Lipase II	Olive oil	6.0
H. lanuginosa	Olive oil	7.0
P. nitens	Olive oil	6.0
C. rugosa	Olive oil	7.1
S. thermophilus	Tributyrin	9.0
S. faecalis	Butter oil	7.5
Pseudomonas spp.	Corn oil	8.5

2.5.3.Temperature Optima and Thermal Stability

The heat stability of enzymes is affected by at least two factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule.

Generally, lipases are known to be active over a wide range of temperature. Animal and plant lipases are usually less thermostable than extracellular microbial lipases. The pancreatic lipases loose activity on storage at temperatures above 40 ^{0}C while lipase from *Aspergillus niger* is stable at 50 ^{0}C. The optimum temperature and thermal stability of some microbial lipases are summarized in Table 2.5.

Table 2.5. Optimum temperature and thermal stability of microbial lipases (Fadıloğlu, 1996).

Source	Optimum Temp., ^{0}C	Stability, ^{0}C
P. fluorescens	45	n.d.
P. nitens	40	40 ^{0}C (10 min.)
Bacillus spp.	60	65 ^{0}C (30 min.)
C. deformans	40-50	n.d.
G. candidum	40	55 ^{0}C (15 min.)
S. thermophilus	45	45 ^{0}C (1 week)
H. lanuginosa	45-55	65 ^{0}C (60 min.)
C. viscosum	65	60 ^{0}C (10 min)
S. faecalis	40	40 ^{0}C (10 min)
C. rugosa	30-35	n.d.

2.5.4. Amino Acid Composition

Adsorption of lipases to lipid-water interface is hydrophobic in nature and this interaction is essential for lipase-catalyzed reactions. Therefore, amino acid composition of enzyme is important for the determination of structure-function relationships. Amino acid composition of microbial lipases can be seen in Table 2.6.

Table 2.6. Amino acid composition of microbial lipases (Fadıloğlu, 1996).

Amino acid residue*	*Bacillus spp.*	*P. nitens*	*C .viscosum*		*G. candidum*	*C. rugosa*
			A	B		
Phenylalanine	4	10	21	6	28	26
Tyrosine	10	13	29	8	18	17
Leucine	22	16	96	26	40	41
Isoleucine	7	11	33	8	18	23
Methionine	1	2	17	1	0	12
Valine	18	21	87	23	24	24
Half-cystine	1	4	2	0	0	5
Alanine	26	17	120	31	34	43
Glycine	24	17	112	18	40	46-47
Proline	9	10	34	8	26	28
Glutamic acid	16	20	89	19	30	33
Serine	14	18	80	22	28	39
Threonine	22	17	105	28	22	31-32
Aspartic acid	22	36	110	26	54	58
Arginine	7	8	34	9	20	12
Histidine	6	5	27	7	10	4
Lysine	4	11	51	4	16	17
Tryptophan	2	2	3	3	8	5
TOTAL	215	238	1050	247	416	464-466

*Amino acid residues were expressed as residues/mole enzyme

In general, these proteins may have a relatively high content of hydrophobic amino acid residues and lipid compared to other proteins. However, some lipases

contain hydrophobic amino acids in normal percentages as in *Pseudomonas* lipase or any lipid or carbohydrates as in *Chromobacterium* lipase B. Thus, their affinity arises from the tertiary structure of the protein giving the molecule an amphipathic structure such as found with detergents (Fadıloğlu, 1996).

2.5.5. The Molecular Weight

Molecular weight of lipases from different sources shows great variation but generally, they range between 20,000 and 60,000 Da. Some of their weight is high, for example, *Candida cylindraceae* lipase has a molecular weight of 120,000 Da. The reason of this can be explained by the self-association of the low molecular weight protein molecules. This enzyme has a single sub unit. Most of the enzymes have molecular masses of 30–40 kDa, but the lipases from *Geotrichum candidum* (GCL) and *Candida rugosa* (CRL) are larger, with masses of about 60 kDa.

The molecular weights of some microbial lipases are summarized in Table 2.7

Table 2.7. Molecular weights of some microbial lipases (Fadıloğlu, 1996).

Microorganism	MW (kDa)	Method
P. fluorescens	32.0	Gel filtration
Bacillus spp.	22.0	Gel filtration
		SDS-PAGE
A. japonicus	155.0	Gel filtration
G. candidum	53.0	Gel filtration
	61.6	SDS-PAGE
H. lanuginosa	39.0	Gel filtration
		SDS-PAGE
P. nitens	26.5	Gel filtration
		SDS-PAGE
C. deformans	207.0	PAGE
S. faecalis	20.9	Gel filtration
C. cylindracea	67.0	SDS-PAGE
	50.0	Gel filtration

In this table, it should be noted that lipases of all specified sources are monomer except *C. deformans* in which homogeneity was not specified.

2.5.6. The Isoelectric Point

The point at which the net charge is zero is termed the “isoelectric point” (pI). Near the pI, proteins tend to be less soluble; far from the pI, they tend to be more soluble. In aqueous solution, charged groups interact with polar water molecules and stabilize the protein, which is intrinsically hydrophobic. A low number of charged groups and a high number of aliphatic or aromatic side chains characterize a protein that is less soluble in water. As one moves farther from pI, the number of ionized groups increases and the solubility tends to increase. Therefore, isoleectric point is important as it affects the solubility of proteins as well as interaction between them (Whellcuright, 1991).

Table 2.8. Isoelectric pH of lipases from various sources (Fadıloğlu, 1996; Peterson et al., 2001).

Source	PI
H. lanuginosa	6.6
P. nitens	5.9
G. candidum	4.4 - 4.8
S. faecalis	3.6
P. fluorescens	4.8-5.1
S. lipolytica	5.2 (Lipase I)
	4.6 (Lipase II)
A. niger	4.0 (Lipase I)
	3.5 (Lipase II)
C. rugosa	4.5
C. antarctica	6.5
P. glumae	7.0

Table 2.9. Comparison of properties of various lipase preparations.

Source (species)	Positional specificity	Temperature (^{0}C)	pI	pH stability range
Porcine pancreas	1,3-specific	40 – 45	n.d.*	5.5 - 7.5
Rhizopus spp.	1,3-specific	35 – 40	n.d.	4.0 - 8.0
Mucor javanicus	1,3-specific	40 – 45	n.d.	4.5 - 6.5
Aspergillus niger	1,3-specific	40 – 50	4.1	5.0 - 7.0
Pseudomonas	Non-specific	50 – 60	6.2 – 5.7	4.5 - 10.0
Candida cylindracea	Non-specific	40 – 45	4.0	4.5 - 8.5
Candida rugosa	Non-specific	30-35	4.5	5.0-7.0

*: not determined

2.6. Industrial Applications of Lipases

The industrial enzyme market in 1989 was about $ 600 million, with lipases representing about 4 % of the market but it has been steadily increasing ever since (Balcao and Malcata, 1998) In the industry, application of lipases as catalysts in organic synthesis has been paid much more attention due to its several advantages over inorganic, metal-derived or chemical catalysts. Lipase catalyzed reactions resemble closely the pathways designed by nature for the metabolism of live beings. Thus, the reaction mechanisms and processes of lipases are viewed as more environmental friendly. Also, products with high purity and high added value are observed by the aid substrate specificity and stereospecificity properties of lipases. Moreover, the use of enzyme decreases the side reactions and simplifies post-reaction separation problems (Pandey et al., 1999). Activation energy of this enzyme is low, thus, require mild reaction conditions of pH and temperature, which lead to decrease in energy consumption and less thermal damage to reactants and products (Balcao et al., 1996). However, the use of lipases is not yet as significant as that associated with such other enzymes as proteases and carbohydrates. This may be because of, first, most detailed studies involving lipases are recent and the usual lag time prior to full commercial exploitation has not yet elapsed. Second, their cost is still relatively high, a constraint that will eventually be overcome in the coming years as a result of evolution encompassing their extraction and purification, as well as their production via genetic engineering (Balcao et al., 1996).

2.6.1. Lipases in the Food Industry

The use of enzymes in the food industry instead of traditional chemical processes has been developed in the past few years due to the increased interest in natural products. Nowadays, lipases are widely used in the production of a variety of products like cheese, butter, dressings, soups and sauces. In Italian cheeses, development of flavour, in cheddar cheese, acceleration of ripening and in processed blue cheese, improvement of flavour is achieved by the aid of the lipase enzyme. Addition of lipases primarily releases short-chain (C_4 and C_6) fatty acids that leads to the development of sharp, tangy flavour, the release of medium-chain (C_{12} and C_{14}) fatty acids tends to impart a soapy taste to the product. Butter oil partly hydrolyzed with

lipases has an enhanced creamy flavour and is added to a variety of foodstuffs including popcorn, cooking oils, fats, cereals, candies, snacks and baked goods.

Lipase-modified creams are added as dairy flavour enhancers to coffee whiteners, candies, doughs, soups and baked products. Also, lipolysis of milk fat is controlled with lipase. In dairy industry that involve the use of lipase, production conditions like lipase concentration, pH, temperature and emulsion content are set to allow controlled release of specific fatty acids, minimising the release of those that could cause off flavours which can not be obtained by conventional chemical interesterification. Moreover, lipase obtained from different sources shows different properties, different selectivity and thus, selection of lipase type is essential to obtain products with desired properties.

Milkfat is an important source of dietary fat but for a number of years it has been seen as bad for health because it contains reasonable amounts of cholesterol and primarily saturated fatty acid residues which have been implicated in promotion of coronary heart disease. The health hazards posed by saturated fats in human diets are largely due to lauric, myristic and palmitic acids and conversely, oleic acid (C18:1) is effective in lowering plasma cholesterol effects (Balcao and Malcata, 1998). So, in addition to flavour effects, lipase is used milkfat engineering to improve its nutritional quality by changing the combination of fatty acid residues in the glycerol backbone of triacylglycerols.

Lipase-mediated ester synthesis is considered to be natural to satisfy the increasing commercial demand for flavour esters. One of them is ethyl butyrate that contributes to the natural aroma of fruits like pineapple, and banana and is used widely in the food industry as a flavour enhancer (Chen, 1996). More recently, it has been discovered that, lipase is used as an index for the rapid identification of spoilage microorganisms and some toxins in food. Also, in a review, the role of lipases in psychrotrophs in food spoilage and their control is described (Pandey et al., 1999).

The position, chain length and degree of unsaturation greatly influence not only the physical properties but also the nutritional and sensory value of a given triglyceride. Cocoa butter contains palmitic and stearic acids and has a melting point of approximately 37 ^{0}C, leading to its melting in the mouth, which results in a perceived cooling sensation. In 1976, Unilever filed a patent describing a mixed hydrolysis and synthesis reaction to produce a cocoa-butter substitute using an immobilized lipase.

Table 2.10. Important areas of industrial applications of microbial lipases (http://www.iisc.ernet.in/~currsci/july10/articles18.htm).

INDUSTRY	**EFFECT**	**PRODUCT**
Bakery	Flavour improvement and shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis Hydrolysis	Chemicals Removal of cleaning agents like surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Dairy	Hydrolysis of milk fat Cheese ripening Modification of butter fat	Flavour agents Cheese Butter
Fats and oils	Transesterification Hydrolysis	Cocoa butter, margarine Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressings and whippings
Health food	Transesterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceutical	Transesterification Hydrolysis	Specially lipids Digestive aids

This technology is now commercialized by Quest-Loders Croklan based on immobilized *R. miehei* lipase, which carries out a transesterification reaction replacing palmitic acid with stearic acid to give the desired stearic-oleic-stearic triglyceride (Jaeger and Reetz, 1998).

Polyunsaturated fatty acids play an increasingly important role as biomedical and so-called `nutraceutical' agents. Many of them belong to the essential fatty acids, the uptake of which is required for membrane-lipid and prostaglandin synthesis, a fact that is often ignored when recommending a fat-free diet. Microbial lipases are used to enrich PUFAs from animal and plant lipids, such as menhaden, tuna or borage oil. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, anti-inflammatories and thrombolytics (Jaeger and Reetz, 1998).

2.6.2. Lipases in the Detergents:

An enzyme was first used to improve the effectiveness of a laundry detergent in 1913 by a German named Otto Rohm, the founder of the giant chemical company Rohm and Hass. The proteolytic enzyme he used, derived from milled animal pancreases, was quite crude and contained many impurities which, in turn, sometimes stained the textile although it was supposed to clean. Neither was the process of enzyme extraction economical enough to include it routinely in household detergents. However, nowadays the most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Enzyme sales in 1995 have been estimated to be US $30 million, with detergent enzymes making up 30 %. An estimated 1000 tons of lipases are added to the approximately 13 billion tons of detergents produced each year. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase(TM), which originated from the fungus *T. lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced -- Lumafast(TM) from *Pseudomonas mendocina* and Lipomax(TM) from *Pseudomonas alcaligenes*, both produced by Genencor International (Jaeger and Reetz, 1998).

In the industrial processes, lipase, protease, amylase and cellulase enzymes are added to the detergents where they catalyse the breakdown of chemical bonds on the addition of water (Pandey et al., 1999). Removal of oil/fatty deposits by lipase is

attractive owing to its suitability under washing conditions. To be a suitable additive in detergents, lipases should be both thermophilic and alkalophilic and capable of functioning in the presence of the various components of washing powder formulations. Lipases have also been used in the formulations prepared to clean drains clogged with food and/or non-food plant-material-containing deposit. Here they are used in association with pectinase.

The challenges that producers of detergent lipases need to meet include: (1) the high variation in the triglyceride content of fat stains, requiring lipases with low substrate specificity, (2) the relatively harsh washing conditions (pH 10.0-11.0 and 30-60 ^{0}C), requiring stable enzymes and (3) the effects of chemical denaturation and/or proteolytic degradation caused by detergent additives such as the surfactant linear alkyl benzene sulfonate (LAS) and proteases. Solutions to these problems are being sought by a combination of continuous screening for improved lipases and attempts to enhance lipase properties on the basis of protein engineering (Jaeger and Reetz, 1998).

2.6.3. Lipases in the Leather Industry

Leather processing involves the removal of subcutaneous fat, dehairing and stuffing. An enzyme preparation that contained lipases in combination with other hydrolytic enzymes such as proteases would open a new avenue in leather processing. Since the process is carried out at alkaline pH, alkalophilic lipases are used in combination with alkaline or neutral proteases and a surfactant or sequestrant (Pandey et al., 1999).

2.6.4. Lipases in Environmental Management

Bioremediation for waste disposal is a new avenue in lipase biotechnology. For instance, lipases have been used extensively (ex situ or in situ) in wastewater treatment for the degradation of contaminants such as olive oil from oil mills. Also, wastes from fast-food restaurants are treated with lipase for the removal of fats, oils and greases.

2.6.5. Lipases in the Cosmetics and Perfume Industry

The usage of lipases in cosmetics and perfume industry was due to its activity in surfactants and in aroma production, which are the main ingredients in cosmetics and perfumes. For example, monoacylglycerols and diacylglycerols, prepared by the lipase-catalysed esterification of glycerol, are useful as surfactants in cosmetics. Mixed-acid-type polyester and acylglycerol ester fatty acids were the main components in lipase-mediated cosmetics. Also, by the transesterification of 3,7-dimethyl-4,7-octadien-1-ol with microbial lipases rose oxide was prepared which is an important fragrance ingredient in the perfume industry (Pandey et al., 1999).

2.6.6. Lipases in Biomedical Applications

Lipases are very commonly used in pharmaceutical industry because of its regioselective property. Production of enantiomeric compounds, racemic esters, catalysis of synthetic reactions, kinetic resolution process for the preparation of optically active chiral compounds can be examples to the usage of lipases in pharmaceutical industry. Especially, preparation of homochiral compounds that are used against HIV and synthesis of anti-tumour agents, alkaloids, antibiotics and vitamins can be said to be the vital applications of lipases.

2.6.7. Lipases as Biosensors

Lipases are also used as biosensors in the fat and oil industry, in food technology and in clinical diagnosis since quantitative determination of triacylglycerols is of great importance. Biosensors can be of a chemical, biological and electronic nature but usage of lipases is cheaper and less time-consuming. Lipases are used as biosensors in food and beverage industry for the determination of fatty acids, in environmental control, pollution analysis, especially pesticide contamination and in clinical aspects, control of lipid level in the blood of patients with cardiovascular complaints.

2.6.8. Lipases in Pesticides

Lipases are used in pesticides because of their potential for decreasing costs and environmental contamination. In this area, lipases are used in immobilized form for cost-effectiveness.

2.6.9. Lipases in Pulp and Paper Industry

In the paper industry, presence of pitches causes severe problems. `Pitch' is a term used to describe collectively the hydrophobic components of wood, namely triglycerides and waxes, which cause severe problems in pulp and paper manufacture. Nippon Paper Industries in Japan developed a pitch-control method that uses a fungal lipase from *C. rugosa* to hydrolyze up to 90 % of the triglycerides (Jaeger and Reetz, 1998).

Chapter 3

IMMOBILIZATION

Enzymes have been used for several years to modify the structure and composition of foods but they have only recently become available for large-scale use in industry, mainly because of the high cost of enzymes. However, progress in genetics and in process technology may now enable the enzyme industry to offer products with improved properties and at reduced costs. Economical usage of lipases in industry requires enzyme immobilization, which enables enzyme reuse and facilitation of the continuous process. Immobilized enzymes are defined by Katchalski-Katzir at the first Enzyme Engineering Conference, held at Henniker, NH, USA, in 1971, as the confinement or localization of enzyme physically in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously (Katchalski-Katzir and Kraemer, 2000).

The concept of immobilizing proteins and enzymes to insoluble supports has been the subject of considerable research for over 30 years and consequently, many different methodologies and a vast range of applications have been suggested. Aims often include such factors as the reuse or better use of enzymes, especially if they are scarce or expensive, better quality products as there should be little enzyme in the product requiring inactivation or downstream purification, the production of biosensors, flow-through analytical devices or the development of continuous manufacturing processes. Although large tonnages of immobilized enzymes are used industrially, for example in the production of various syrups from starch, and there are several smaller-scale industrial applications, the introduction of such biocatalysts has been disappointingly slow. With many current manufacturing applications the cost of the enzyme itself is not a large proportion of overall production costs but the trend toward more complex processing operations and more sophisticated products in the pharmaceutical, food, chemical and other bioprocessing industries will require the use of a wider range of enzymes of greater purity and specificity and hence, higher value. Optimizing use and reuse of enzymes will be increasingly required.

Nelson and Griffin carried out the first investigation on immobilization in 1916. In their work, invertase was adsorbed both on charcoal and aluminium hydroxide

(Pitcher, 1980). Meanwhile, the first lipase immobilization study was carried out in 1956 by Brandenberger H. who covalently linked the lipase on ion-exchange resin. However, the first attempts for the usage of immobilized lipase for hydrolysis or ester synthesis reactions were done by Iwai et al. in 1964 (Akşamoğlu, 1997).

The first industrial application of immobilized enzymes was reported by Tosa et al. 1967 at the Tanabe Seiyaku in Japan who developed columns of immobilized *Aspergillus oryzae* aminoacylase for the resolution of synthetic, racemic -amino acids into the corresponding, optically active enantiomers. Around 1970, two other immobilized systems were launched on a pilot-plant scale. In England, immobilized penicillin acylase, also referred to as penicillin amidase, was used to prepare 6-amino penicillanic acid (6-APA) from penicillin G or V, and in the USA, immobilized glucose isomerase was employed to convert glucose into fructose. These successful industrial applications prompted extensive research in enzyme technology, leading to a steady increase in the number of industrial processes based on sophisticated, immobilized-enzyme reactors (Katchalski-Katzir and Kraemer, 2000).

3.1. Advantages of Immobilization

Immobilization process seems to offer mainly the economical advantages. However, there are a number of advantages to attaching enzymes to a solid support and a few of the major reasons are listed below:

- Enzymes can be reused.
- Processes can be operated continuously and can be readily controlled.
- Products are easily separated.
- Effluent problems and materials handling are minimized.
- In some cases, enzyme properties (activity and stability) can be altered favorably by immobilization.
- Provides higher purity and product yields, product inhibition is less apparent.
- Greater pH and thermal stability.
- No contamination due to added enzyme.
- Continuous operation.
- Greater flexibility in reactor design.

Despite these advantages, industrial application is still limited by:

- the comparatively low cost of soluble enzymes
- traditional attitudes
- the investment needed for introducing new equipment to already implanted processes
- the nature and cost of the immobilizing support and the immobilizing process (including losses of activity)
- the performance of the system

3.2. Factors Affecting Immobilization Performance

There are many factors that influence the performance of an immobilized enzyme preparation. Some of the most important factors are the choice of a carrier and the selection of an immobilization strategy.

3.2.1. Support Materials

An enormous number of different types of matrices have been used in laboratory immobilization studies. However, selection of the optimum support is the major parameter that effects the immobilization performance. The properties that an enzyme carrier should has can be listed as follows:

- Large surface area
- Permeability
- Insolubility
- Chemical, mechanical and thermal stability
- High rigidity
- Suitable shape and particle size
- Resistance to microbial attach
- Regenerability

However, the exact nature of the process design, the physical properties of the feedstock and the product, the reaction conditions and many other factors will place constraints on the type of matrix, which will be most suitable. In an industrial operation,

maximized enzyme-matrix life span is vital component. In Table 3.1., factors that effect the matrix selection can be seen.

Table 3.1. Factors to be considered in the selection of an immobilization matrix.

Factor Under Consideration	Examples
Cost of matrix	Beaded dextrans and controlled-pore glass are expensive compared with ceramics and celluloses.
Chemical resistance of the matrix	Silica-based matrices are significantly soluble at pH values above 8.0. Cellulose and dextran-based matrices can be degraded enzymically. Some organic polymers change shape in organic solvents.
Physical properties of the matrix	Ceramic matrices have a high resistance to pressure compared with beaded dextrans.
Complexity of the immobilization chemistry	Ion exchange adsorption interactions can be generated rapidly and minimize the inactivation of unstable enzymes. Covalent attachment can be laborious, expensive and destructive.
Stability of the enzyme-matrix association	Enzymes immobilized by physical means undergo slow leakage compared with covalently attached enzymes.
Special requirements of the process	Pressure sensitive matrices (e.g. agarose) are unsuitable for plug-flow reactors. Magnetic matrices are useful for the recovery of enzyme in CSTR* systems.

*Continuous stirred tank reactor.

3.3. Immobilization Techniques

The selection of an immobilization technique is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of the lipase utilization, deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase. Chemical methods feature the formation of covalent bonds between the lipase and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the lipase within the support. Methods for enzyme immobilization can be classified into three main categories**:**

- Entrapment
- Carrier binding
- Cross linking

3.3.1. Carrier Binding

Carrier-Binding method is the oldest immobilization method for enzymes and is defined as the binding of enzymes to water-insoluble carriers. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depends on the nature of the carrier. The following picture shows how the enzyme is bonded to the carrier in this method**:**

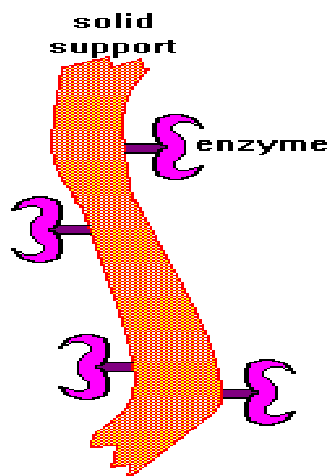
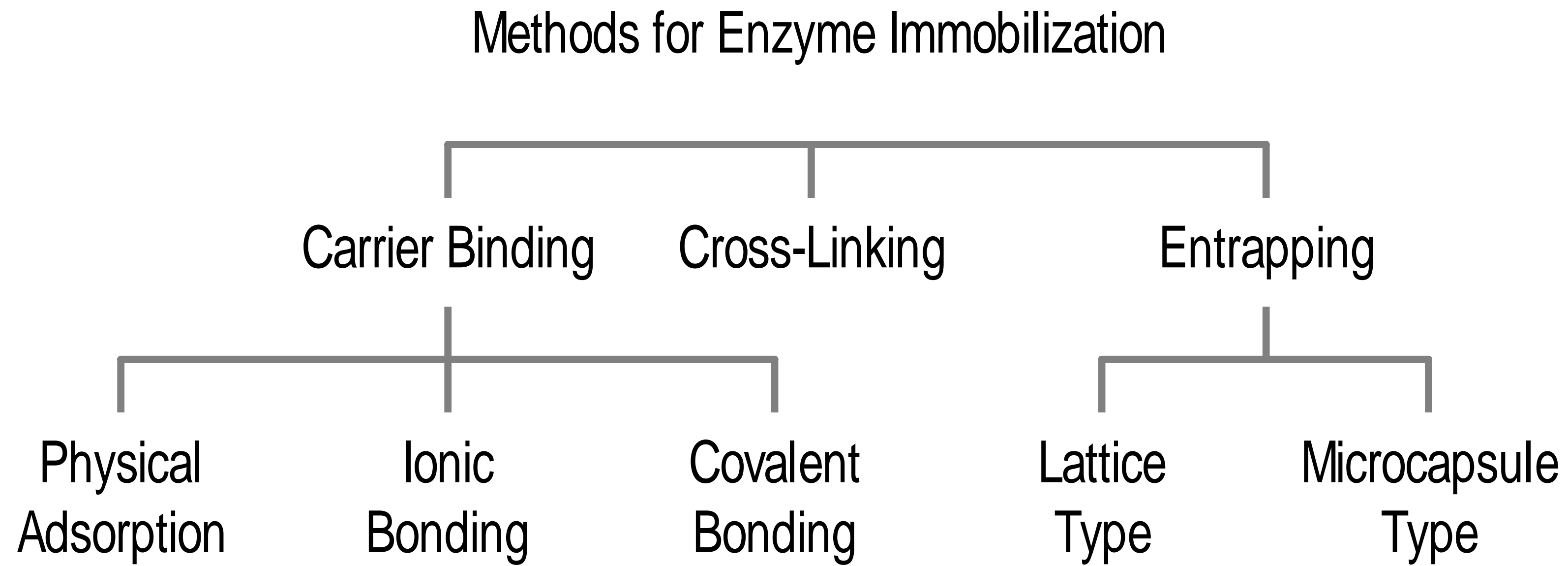


Figure 3.1. Schematic illustration of carrier-binding method (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

Table 3.2. Enzyme immobilization methods.



The selection of the carrier depends on the nature of the enzyme itself, as well as the:

- Particle size,
- Surface area,
- Molar ratio of hydrophilic to hydrophobic groups,
- Chemical composition.

In general, an increase in the ratio of hydrophilic groups and in the concentration of bounded enzyme results in a higher activity of the immobilized enzymes. The most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.

According to the binding mode of the enzyme, carrier-binding method can further be sub-classified into:

1. Physical Adsorption
2. Ionic Binding
3. Covalent Binding

3.3.1.1. Physical Adsorption

The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminium hydroxide. This method for the immobilization of enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme, or destruction of its active center. If a suitable carrier is found, this method can be both simple and cheap. Also, they lend themselves a minimal resistance in the reaction mixtures, and possible supports for physical adsorption are mechanically durable and re-useable (Oliveira et al., 2000). Thus, this method is found to be the most suitable for large-scale immobilization.

However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during utilization, because the binding force between the enzyme and the carrier is weak. The enzyme is immobilized onto a solid support by low energy binding forces, e.g., Van der Walls interactions, hydrophobic interactions, hydrogen bonds, ionic bonds. Many carrier material exist, the choice of one often depending on properties that are important for potential industrial applications: mechanical strength, chemical and

physical stability, hydrophobic/hydrophilic character, enzyme load capacity and cost. Initially, mineral supports such as porous glass beads, diatomaceous earth, silica and alumina were used. More recently, the most used supports are ion exchange resins, celite and biopolymers (Villeneuve et al., 2000).

The success and efficiency of the physical adsorption of the enzyme on a solid support is dependent of several parameters. The size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface (porosity, pore size) are crucial. Typically, the use of a porous support is advantageous since the enzyme will be adsorbed at the outer surface of the material and within the pores as well. An efficient immobilization is also dependent on the enzyme concentration. The amount of adsorbed enzyme per amount of support increases with the enzyme concentration reaching a plateau at the saturation of the carrier. This operation is usually carried out at constant temperature, and, consequently, adsorption isotherms are obtained which follow the Langmuir or Freundlich equations. The pH at which the adsorption is conducted is equally important since ionic interactions are crucial in such an immobilization. Usually, the maximum adsorption is observed for pH values close to the isoelectric point of the enzyme. Finally, addition of water miscible solvents during the immobilization process favors the adsorption by reducing the solubility of the enzyme in the aqueous phase.

Lipase immobilization by physical adsorption finds applications in oils and fats bioconversion. Their unique specificities allow the design of synthetic routes that predetermine product structure and distribution whereas chemical catalysts generally lead to random reaction product mixtures. Thus, the use of lipases makes it feasible to obtain new products with predetermined physical and chemical properties.

During the immobilization of lipase by physical adsorption, driving force is mainly hydrophobic interaction because of the structural properties of the enzyme. Hydrophobic interaction arises from the repulsion of polar and non-polar molecules. Hydrophobic interactions are not a binding of hydrophobic groups to each other, these interactions are forced on the non-polar compounds by the polar environment such as water. It is the structure of the water that creates hydrophobic interactions. If the structure of the water changes by dissolving salts or organic solvents in water, then hydrophobic interactions would be affected. Generally, increasing ionic strength increases hydrophobic interactions. In addition to ionic strength, temperature and pH also affects the strength of interaction.

3.3.1.2. Ionic Binding

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic polymers having ion-exchange centers are usually used for carriers. The binding of enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme, and so yields immobilized enzymes with high activity in most cases. As the binding forces between enzyme protein and carriers are less strong than in covalent binding; leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH.

The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages is much stronger for ionic binding although less strong than in covalent binding.

3.3.1.3. Covalent Binding

The covalent binding method is based on the binding of enzymes and water-insoluble carriers by covalent bonds. The functional groups that take part in this binding of enzyme to carrier can be amino, carboxyl, sulfhydryl, hydroxyl, imidazole or phenolic groups which are not essential for the catalytic activity. In order to protect the active site, immobilization can be carried out in the presence of its substrate or a competitive inhibitor. Activity of the covalent bonded enzyme depends on the size and shape of carrier material, nature of the coupling method, composition of the carrier material and specific conditions during coupling.

The main advantage of the covalent attachment is that such an immobilization is very solid. Unlike physical adsorption, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength. Moreover, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). Finally, a large number of different supports and methods to activate them are available. However, experimental procedures are obviously more difficult to carry out than for physical adsorption. The 3-D structure of the protein is considerably modified after the

attachment to the support. This modification generally leads to a significant loss of the initial activity of the biocatalyst (Villeneuve et al., 2000).

Examples of derivatized lipases obtained through this procedure include PEG-modified lipase, fatty acid-modified lipase, amidinated lipase, and detergent-modified lipase (Villeneuve et al., 2000).

3.3.2. Cross-Linking

It can be defined as the intermolecular cross-linking of enzymes by bifunctional or multifunctional reagents and it is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used. The immobilization is performed by the formation of intermolecular cross-linkages between the enzyme molecules by means of bi or multifunctional reagents. The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

This method can be applied in three different manners:

- Mixing the prepolymers with a photosensitizer (e.g., benzoin ethyl ether), melting, mixing with an enzyme solution and gelling by exposure to near ultraviolet radiation.
- Freezing a monomer solution containing the enzyme in the form of small beads. Polymerization is then started using gamma radiation.
- Mixing the enzyme in a buffered aqueous solution of acrylamide monomer and a cross-linking agent. Polymerization can be initiated by the addition of some chemicals.

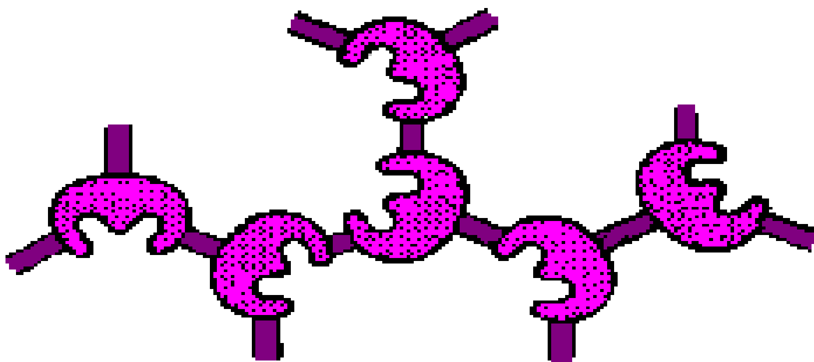


Figure 3.2. Schematic illustration of cross-linking method (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

3.3.3. Entrapment

Incorporating enzymes into the lattices of a semipermeable gel or enclosing the enzymes in a semipermeable polymer membrane. The entrapment for immobilization is based the localization of an enzyme within the lattice of a polymer matrix or membrane in such a way as to retain protein while allowing penetration of substrate. This method differs from the covalent binding and cross-linking in that enzyme itself does not bind to the gel matrix or membrane; and thus, has a wide applicability. The conditions, used in the chemical polymerization reaction, are relatively severe, resulting in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required.

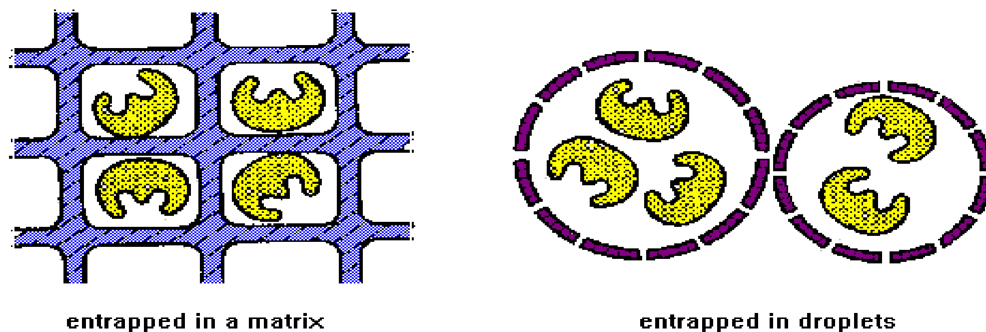


Figure 3.3. Schematic illustration of entrapment methods (http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

Entrapment can be classified into lattice and microcapsule types.

- Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyacrylamide, polyvinylalcohol, etc. and natural polymer (starch) have been used to immobilized enzymes using this technique.
- Microcapsule-Type entrapping involves enclosing the enzymes within semipermeable polymer membranes. This is probably the less developed immobilization technique, is very similar to entrapment, although in this case, it is the enzyme and its whole environment that are immobilized. Microencapsulation creates artificial cells delimited by a membrane. Large molecules such as enzymes are not able to diffuse across the synthetic membrane whereas small molecules, e.g., substrates and products, can pass through it (Villeneuve et al., 2000). The preparation of enzyme

microcapsules requires extremely well controlled conditions; and the procedures for microcapsulation of enzymes are liquid drying, phase separation and interfacial polymerization method.

The advantage of such an immobilization technique is that the enzyme does not chemically interact with the polymer; therefore, denaturation is usually avoided. However, mass transfer phenomena around the membrane are problematic. The diffusion rate across the membrane of the substrate and the product is often the limiting parameter. Generally, high substrate concentrations are necessary in order to limit its influence. Finally, entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst (Villeneuve et al., 2000).

3.4. Lipase Immobilization

Chemical modifications of lipases by either reduction of amino acid residues, covalent coupling to an insoluble carrier, attachment to a matrix by covalent bonds, or by formation of cross-linked lipase matrix are certainly excellent tools for increasing the stability and improving the performance of these enzymes in hydrophobic environment. These procedures should produce enzymes capable of catalyzing not only the reverse reaction of hydrolysis, ester synthesis, but also reactions of ester exchange and aminolysis in organic solvents. The immobilized lipases can easily be recovered and reused, or alternatively reused in a continuous process involving packed bed reactors. To render this enzyme very active in organic solvents, the procedures of immobilization should preserve the hydration shell around the lipases and keep the enzymes physically separated from the external organic environment. This strategy enables a micro-heterogeneous environment with a high interfacial area. Thus, chemical modifications should be performed under mild conditions with less damage to the tertiary structure of the lipases. It should also be emphasized that medium engineering, i.e., modification of immediate surroundings of the biocatalyst in non-aqueous solvents to provide a better microenvironment for the enzyme, may further increase the performance of a given modified biocatalyst.

The data compiled in and show that the activity of the different modified lipases vary tremendously depending upon the binding procedure applied and the support selected. Values of the percent activity retained range from 1 to 150. In some cases,

transesterification and hydrolytic activities increase concomitantly, but in other cases, hydrolysis is favored, while the transesterification activity decreases, and vice versa. The same observation applies also to solvent solubility and specificity. These observations may reflect either enzyme denaturation, the contribution of the testing parameters, such as water content, nature of the solvent, temperature, ion concentration, or other unknown phenomenon. Thus, one can conclude that the immobilization of lipase by chemical means is still a trail and error procedure.

Table 3.3. Comparison of immobilization methods (Akşamoğlu, 1997).

CHARACTERISTIC	CROSS-LINKING	ADSORPTION	IONIC-BINDING	COVALENT BINDING	ENTRAPPING
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 3.4. Some examples to the lipase immobilization methods and supports.

Immobilization Method	**Lipase Source**	**Matrix**	**Reference**
Gel Entrapment	*Candida cylindracea*	Polymer	Chen, 1996
Adsorption	*Candida cylindracea*	Zeolite	Lie et al., 1991
Adsorption	*Candida rugosa*	Polymer	Basri et al., 1994
Adsorption	*Candida rugosa*	Polypropylene	Montearo et al., 1993
Covalent Attachment	*Candida cylindracea*	Nylon pellets	Zaidi et al., 1995
Adsorption	*Candida cylindracea*	Rice Hull Ash	Tantrakulsiri et al., 1997
Adsorption	*Mucor miehei*	Celite, Silica	Perraud et al., 1995
Adsorption	*Expansum penicillium*	Ca-alginate beads	Yang et al., 1994
Covalent Attachment	*Pseudomonas fluorescens*	Copoly(ethylene/acrylic acid) fiber	Emi et al., 1994
Adsorption	*Resinase A*	Pulp fibers	Fischer et al., 1992
Adsorption	*Humicola lanuginosa*	Glass beads	Gunnlaugsdottir et al., 1998
Entrapment, Adsorption	*Pseudomonas* spp.	Egg shell and alginate	Vemuri et al., 1998
Entrapment	*Aspergillus niger, Candida antarctica, Rhizopus arrhizus, Rhizomucor miehei*	Sol-gel materials	Reetz et al., 1996
Gel Entrapment	*Rhizopus arrhizus*	Polymer	Yang et al., 1994

Chapter 4

MATERIALS AND METHODS

4.1. Materials

Lipase isolated from *Candida rugosa* (875 Units/mg enzyme) was purchased from Sigma Chemical Co. and used without purification. Sigma Chemical Co. defined one unit of lipase activity as that would hydrolyze 1.0 microequivalent of fatty acid from olive oil in 1 hour at pH 7.2 at 37 ^oC. Some important properties of *Candida rugosa* lipase can be seen in Table 4.1.

Table 4.1. Properties of *Candida rugosa* lipase (Fadıloğlu, 1996)

Properties	
Molecular Weight (kDa)	65
Isoelectric Point (pI)	4.5
pH Optimum	6.5-7.5
Optimum Temperature (^0C)	30-35

Octyl-Sepharose CL-4B was supplied from Sigma Chemical Co. and used as the hydrophobic support material. Particle size of this material in hydrated form was in the range between 45-165 μm. Chitosan was kindly supplied by Japan Organo Co. and used as hydrophilic support. Particle size of chitosan in hydrated form was between 400-600 μm and the pore size in dry state was 0.1-0.2 μm.

Commercial olive oil (Tariş Oil Co., Riviera type olive oil, free fatty acid maximum 1.5 %), sunflower oil (Cidersan Oil Co., Damla sunflower oil, free fatty acid maximum 0.3 %, Refined, Winterized and Deodorized) and corn oil (Cidersan Oil Co., Damla corn oil, free fatty acid maximum 0.3 %, Refined, Winterized and Deodorized) were purchased from local market and used without purification. The reagents used were all of reagent quality and high purity.

Distilled and deionized water was used in all preparations, throughout this study.

All the analyses were carried out at least duplicate and mean values are reported.

4.2. Methods

4.2.1. Protein Assay

The protein content of lipase was determined spectrophotometrically using the kit produced by Sigma Chemicals (Cat. No: P 5656).

The Lowry procedure has been found to be one of the most reliable and satisfactory method for quantifying soluble proteins. The procedure described here is based on Peterson's modification of the Micro-Lowry method (Peterson, 1977) and the method of Lowry et al (1951).

This method is based on the principle that an alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple-blue color when the phenol reagent is added. Absorbance is read at a suitable wavelength between 500 nm and 800 nm. The protein concentration is determined from a calibration curve.

Reagents used are Lowry and Folin & Ciocalteu's Phenol reagent while protein standarts are prepared from Bovine Serum Albumine, Fraction V [BSA].

Protein standard solution, in the range of 0 - 400 μg/ml was prepared in triplicate to obtain a standard curve. Samples were diluted to 1.0 ml with water so that the protein fell within the range of the standards. 1.0 ml Lowry reagent solution was added to each tube and mixed well. The solutions were allowed to stand at room temperature for 20 minutes. With rapid and immediate mixing, 0.5 ml Folin & Ciocalteu's Phenol reagent working solution was added to each tube and color allowed to develop in a dark room for 30 minutes.

The solutions in each tube were transferred to cuvettes and the absorbance of the standards and samples versus blank were measured at a wavelength of 750 nm. Readings were completed within 30 minutes.

The absorbance values of the standards were plotted versus their protein concentrations to prepare a calibration curve (see Appendix 1). The protein concentrations of the other samples were determined from the calibration curve, and the result was multiplied by the appropriate dilution factor to obtain the protein concentration in the original sample.

4.2.2. Determination of Lipase Activity

The activity of the free and immobilized lipase was determined by hydrolyzing olive oil at room temperature and at pH 7.0 using a pH -Stat equipment (Metrohm). In a 25 ml beaker, 1 ml 1.0 N NaCl and 50 mM $CaCl_2$ solution and 4 ml of 25 mM phosphate buffer at pH 7.0 placed unless otherwise stated. Then, 1 ml of free or immobilized enzyme solution in varying concentrations was added. After the equilibration of the pH value, 5 ml of olive oil was added. The pH was maintained by the addition of 50 mM NaOH solution (Yang and Chen, 1994). Activity (U/mg solid) was calculated by the following formula:

$$A = \frac{\text{Normality of NaOH} * 1000 * \text{Amount of NaOH expended (ml)}}{\text{Amount of Enzyme (mg)}}$$

Here, one unit of lipase activity is defined as that would hydrolyze 1 miliequivalent of fatty acid from olive oil in 15 minutes at pH 7.0 at 20 ^{0}C.

4.2.3. Stability of Soluble Lipase

In order to find the optimum working conditions that would maximize the soluble lipase activity, effect of some parameters were investigated. These parameters include ionic strength, pH and temperature.

4.2.3.1. Effect of pH

The pH values investigated were 4.0, 5.0, 6.0, 6.5, 7.0, 8.0 and 9.0. In these analyses, different buffers were used, namely, for pH 4.0 and 5.0, acetate buffer; for pH 6.0, 6.5, 7.0 and 8.0, phosphate buffer and for pH 9.0, Tris-HCl buffers. However, it should be noted that concentration of each buffer was same, which is 0.025 M.

The enzyme solutions (5 mg/ml *Candida rugosa* lipase, 38.5 U/ml solution) were incubated for 24 hours at 20 ^{0}C in 9 ml of buffer solutions mentioned above in the presence of 0.5 M NaCl. During incubation, solution was continuously stirred within an orbital shaker (Ika Labortechnik – KS 125-Basic) at a speed of 300 rpm. After the

collection of samples, pH of the hydrolysis medium was adjusted with 0.05 M NaOH, the residual lipase activity was measured under the standard assay conditions.

4.2.3.2. Effect of Temperature

Effect of temperature on soluble enzyme activity was investigated with the incubation of enzyme solutions at 4, 20, 30, 37, 40 and 50 ^{0}C for 24 hours. The procedure indicated in Section 4.2.3.1. is followed for sample preparation and determination of residual activity. However, in these analyses, 0.025 M pH 7.0 phosphate buffer containing 0.5 M NaCl was used as incubation medium.

4.2.3.3. Effect of Ionic Strength

In order to find the optimum salt concentration that will maximize the activity of soluble enzyme, effect of 0, 0.25, 0.5, 1.0, 1.5 and 2.0 molars of NaCl solutions were examined. Procedure for sample preparation and residual activity determination was followed also in these analyses. These tests were carried out at room temperature (20 ^{0}C) with the 0.025 M pH 7.0 phosphate buffer under constant stirring at 300 rpm by adding 1 ml NaCl solutions of given concentrations.

4.2.4. Lipase Immobilization

In a standard experiment, *Candida rugosa* lipase was dissolved in buffer of different pH values in varying concentrations. Lipase solution (9 ml) and salt solution (1 ml) were mixed and appropriate volume of support solution was added. Samples were collected at varying time intervals and immobilized and non-immobilized fractions were separated by filtration. After the removal of filtrate, immobilized fraction was washed with 20 ml of deionized water twice. Non- immobilized enzyme fraction and immobilized enzyme fraction is assayed for olive oil hydrolysis separately. Results of these analysis were checked for mass balance and it was seen that 3-4 % of enzyme activity was lost. The reason for such a loss may be related to the washing step where the weakly bound enzyme was separated. Also, some of the enzyme may be adsorbed by the filter paper.

4.2.4.1. Effect of Enzyme Loading

In order to find the optimum enzyme concentration that should be loaded for immobilization, lipase solutions with concentrations of 3.055, 30.55, 61.1, 183.3, 305.5 and 916.5 U/ml were applied on octyl-sepharose. Enzyme solutions were prepared with 0.025 M phosphate buffer, pH 7.0 containing 1.0 M NaCl. The prepared solution was incubated at 4 ^{0}C and 20 ^{0}C under constant stirring rate of 300 rpm.

In chitosan immobilization studies, the enzyme concentrations studied were 1.1, 11, 21.8, 109, 218 and 327 U/ml. The pH of each medium was equilibrated with 0.025 M phosphate buffer, pH 6.0. In this case, there was no salt addition. The immobilization was carried out under constant temperature 20 ^{0}C and constant stirring rate of 300 rpm.

In both cases, for different initial concentrations of enzyme, samples (1 ml) were taken when equilibrium was reached. The residual and initial lipase activities in solution were determined by pH-stat and the amount of enzyme loaded per unit volume of support was calculated from the mass balances.

4.2.4.2. Time Course of Immobilization

Time course of immobilization was assayed for each enzyme concentration given above. During the kinetic studies of octyl-sepharose immobilization, samples were collected in every 20 second during the first five minute. Then, samples were collected in every 5 minute up to the end of one hour period. These analysis were carried out at two different temperatures, 4 and 20 ^{0}C under constant stirring at 300 rpm. Immobilization medium contained 0.025 M phosphate buffer, pH 7.0 and 1.0 M NaCl.

In the case of chitosan, time course of immobilization was investigated for only one enzyme concentration, 61.1 U/ml. Since the time required to reach the plateau value was longer, samples were collected at 2, 3, 4, 15, 20. and 24th hours of incubation at 20 ^{o}C with constant stirring at 300 rpm. In this case, pH of the medium was 6.0 which was obtained with 0.025 M phosphate buffer and there was no salt addition.

In both cases, supernatants obtained from each experiment were analyzed for lipase activity. The calculations were carried out from the initial and final amount of uptakes.

4.2.4.3. Effect of pH

These analyses were performed under constant temperature with constant stirring at 300 rpm. Enzyme concentration was 38.5 U/ml and the support added was 1 ml octyl-sepharose or chitosan. The pH values investigated were 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. In these analyses, buffers used were acetate, phosphate and Tris-HCl buffers as mentioned in Section 4.2.3.2. During immobilization on hydrophobic and hydrophilic supports, 0.025 M phosphate buffer, pH 7.0 containing 0.5 M NaCl and 0.025 M phosphate buffer, pH 6.0 were used, respectively.

4.2.4.4. Effect of Temperature

In order to find the optimum temperature for immobilization, lipase in the concentration of 38.5 U/ml and 1 ml octyl-sepharose solution or chitosan were added to immobilization media. Temperatures investigated were 4, 20, 30, 37, 40, and 50 ^{o}C for immobilization on octyl-sepharose and 4, 20 and 30 ^{o}C for immobilization on chitosan. Immobilization medium included 0.025 M pH 7.0 phosphate buffer and 0.5 M NaCl in the case of octyl-sepharose and 0.025 M pH 6.0 phosphate buffer in the case of chitosan. Stirring rate was 300 rpm.

4.2.4.5. Effect of Salt Concentration

Salt concentration is an important factor for immobilization especially for hydrophobic interactions and also for enzymatic activity. In order to find the salt concentration that will maximize the immobilized enzyme ratio, 0, 0.25, 0.5, 1.0, 1.5 and 2.0 molars of NaCl solutions were added to the incubation medium. These tests were carried out at room temperature. Immobilization medium included 0.025 M pH 7.0 phosphate buffer in the case of octyl-sepharose and 0.025 M pH 6.0 phosphate buffer in the case of chitosan. Stirring rate was 300 rpm, again.

4.2.4.6. Effect of Solid-Liquid Ratio

These analyses were performed by keeping support solution volumes or weights constant, but changing the lipase solution volumes. Support volume was 1 ml and the

concentration of lipase solution was 5 mg/ml in all cases. The volumes of lipase solutions added to immobilization media were 4, 9, 14, 19, 24 and 29 ml for octyl-sepharose immobilization and 4, 9, 14, 19 and 24 for chitosan immobilization. Immobilization medium included 0.025 M pH 7.0 phosphate buffer and 0.5 M NaCl in the case of octyl-sepharose and 0.025 M pH 6.0 phosphate buffer in the case of chitosan. Stirring rate was 300 rpm.

4.2.4.7. Storage Stability

In order to investigate the storage stability, lipase was immobilized under the conditions that were found to be optimum for maximum yield. Prepared materials were placed in aluminum foils and enclosed to prevent their contact with air. Also, some of the materials were put into beakers and 4 ml of 0.025 M pH 6.0 phosphate buffer was added to each of them. The beakers were then sealed with parafilms. All the samples were kept in a refrigerator. Hydrolysis tests were applied at certain time intervals to find the retained activity. Stability of immobilized lipases kept under dry conditions and in buffer was also compared.

4.2.5. Hydrolysis Reaction

The prepared immobilized enzyme would be used in hydrolysis reaction. So, it was also important to find the optimum substrate type and concentration used in hydrolysis.

4.2.5.1. Effect of Substrate Type

In all tests, olive oil was used as the substrate. However, there are several types of commercial oils such as sunflower and corn oil. In order to compare the hydrolysis efficiency of these two oils with olive oil, chitosan immobilized and free lipase was exposed to hydrolysis reaction with them. The reaction conditions were same with those of olive oil.

4.2.5.2. Effect of Substrate Concentration

During the hydrolysis reaction, amount of olive oil added was 5 ml as given in the literature (Yang and Chen, 1994). However, it was also estimated that the concentration of substrate would effect the hydrolysis ratio. Therefore, 0.5, 1, 2, 3, 4, 5 and 7 ml of olive oil were added to the medium and relative hydrolysis was investigated. Also, like olive oil, effect of different concentrations of sunflower and corn oils were also tested. The volumes used for these tests were 1, 3, 5 and 7 ml. All these tests were carried out with free and also chitosan immobilized lipases.

Chapter 5

RESULTS AND DISCUSSION

In this chapter, experimental results about the immobilization of lipase enzyme on a hydrophobic (octyl-sepharose) and on a hydrophilic support (chitosan) were presented together with the emphasis on hydrolysis and enzymatic activity. Experimental results include the effects of temperature, pH, salts, and gel to liquid volume on immobilization process. Enzyme loading and immobilization kinetic studies were conducted using the optimum operating conditions obtained from these results. Effect of substrate type and concentration on hydrolysis efficiency were also investigated. All experiments were done in duplicate at least and mean values were reported.

5.1. Protein Content

As mentioned before, commercial preparation of *Candida rugosa* lipase was examined for protein content using the Lowry Method with bovine serum albumin as the protein standard. The specific activity of an enzyme preparation is usually expressed in terms of Units/mg protein. In this study, the specific activity of *Candida rugosa* lipase enzyme was measured under standard assay conditions. The assay procedure is given in Chapter 4.2.1. It was found that commercial preparation of *Candida rugosa* lipase contained 8.67 % protein by mass. Specific activity of *Candida rugosa* lipase was calculated as 88.8 U/mg protein. This result complies well with that of Gitlesen et al. (1997) who also found 9.0 % protein in a commercial lipase from *Candida rugosa*.

5.2. Stability of Lipases

In this section, pH, temperature and salt stability of lipase enzymes are presented since they are the process variables, whereas the others are fixed by the nature of the immobilization system used in the study.

The stability of *Candida rugosa* lipase found in this study provides key information for choosing the operating conditions for immobilization process. pH,

temperature and ionic strength are very important for this study to reveal information about the optimum processing conditions for immobilization.

5.2.1. pH Stability

The tertiary structure of a protein depends on interactions such as hydrogen bonding between R groups. A change in pH can alter the ionization of these side chains and disrupt the native conformation and in some case denature the enzyme. Hence, each enzyme has an optimal pH range that help maintenance of its native conformation in an environment, which it operates.

The degree of ionization of the surface R groups of amino acid residues is a function of the medium's pH. The main factor involved here is the titration of the ionizable group which maintain surface charge, act in the active site, or stabilize the enzyme; resulting in an optimum pH for the enzyme. Immobilization often shifts the pH optimum; which may be desirable.

pH stability of *Candida rugosa* lipase was investigated in the range of 4.0-9.0 and results are given in Figure 5.1. The stability of the enzyme was expressed as the % activity remaining after 24 hours at the corresponding pH, at 20 ^{0}C, based on the original enzyme activity. The experimental procedure was explained in Section 4.2.3.2.

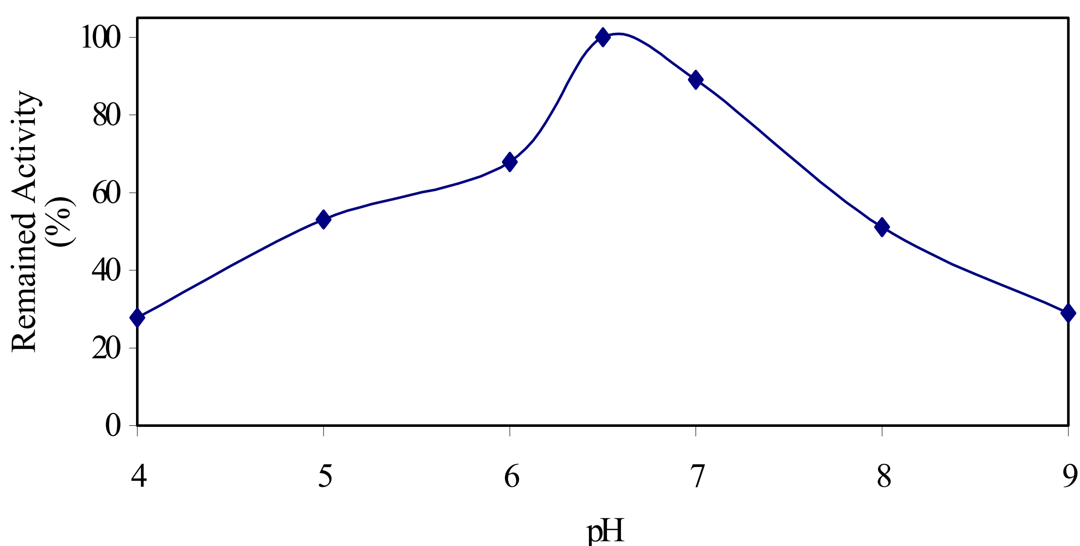


Figure 5.1. pH stability of *Candida rugosa* lipase (Working Conditions: 20 ^{0}C/24 hours incubation, 0.5 M NaCl).

As seen in Figure 5.1, lipase is very stable in the pH range of 6.0-7.0 and the full activity is remained after 24 hours. Below and above this pH range, the activity decreased and nearly 70 % of enzyme activity was lost at the pH values of 4.0 and 9.0.

Lipase enzyme preparation seemed to be very unstable both in alkaline (pH>7.0) and highly acidic (pH<5.0) solutions. This may be explained by the structural changes occurring in enzyme proteins caused by pH variation. It was indicated that for most of enzymes the variation of activity with pH, within a range of two or three pH units each side of the isoelectric points, is generally a reversible process (Akova and Üstün, 2000). However, extremes of pH would cause irreversible denaturation. In alkaline solutions (pH>8.0), there may be partial destruction of cystine residues caused by β-elimination, whereas in acidic solutions (pH<4.0) hydrolysis at the labile peptide bonds sometimes found next to aspartic acid residues may occur (Akova and Üstün, 2000). Also, at acidic pH, the enzyme may form high molecular weight aggregates or unspecific associations with other accompanying proteins due to its hydrophobic nature and favored by the proximity of its isoelectric point (Montero et al., 1993).

The results of this study was similar with that of Montero et al. (1993), who found soluble *Candida rugosa* lipase to be fully active between pH 6.2 to 7.7. At the same time, pH 8.0 was found to be the point where the soluble enzyme lost its activity. However, another study by Fadıloğlu and Söylemez (1997) reported the optimum pH for the soluble *Candida rugosa* lipase as 7.0. The authors observed the inactivation of the enzyme in acetate buffer after 30 minutes of incubation at pH 6.0 and 8.0. Especially, inactivation at pH 6.0 was found to be irreversible. In this study, after incubation of 24 hours in 0.025 M phosphate buffers with pH values of 6.0 and 7.0, 60 % and 50 % of the enzyme activity was remained, respectively. Therefore, the extended stability of lipase has been caused by the nature of the buffer solutions.

5.2.2. Temperature Stability

Experiments to determine the temperature stability of lipase were conducted and the extent of activity loss under various temperature regimes was determined. The studies showed that, lipase was stable when stored at the temperature range of 4-37 ^{0}C over a period of 24 hours in pH 7.0 phosphate buffer containing 0.5 M NaCl. However, 5 % and 70 % of the activity was lost at 40 and 50 ^{0}C, respectively (Figure 5.2). This is mainly due to the denaturation of the enzyme structure at these temperatures.

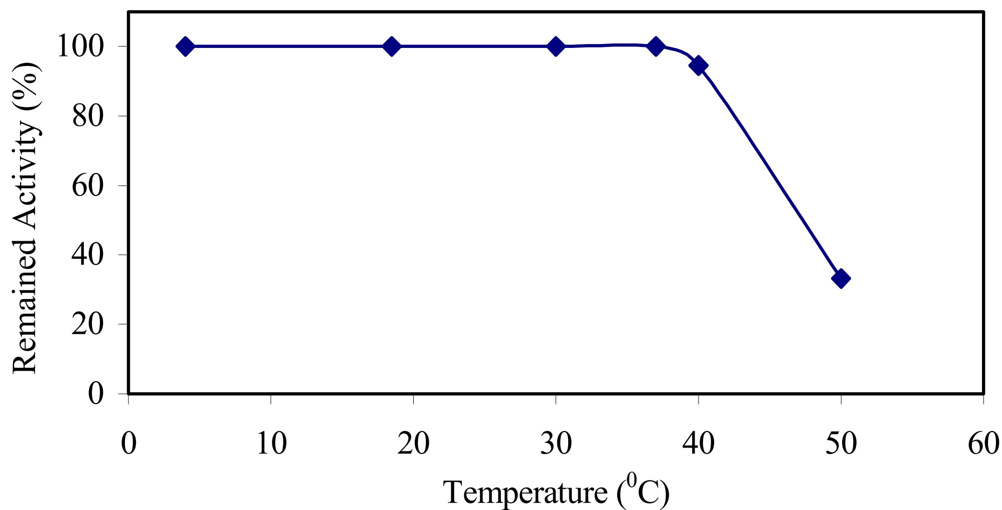


Figure 5.2. Temperature stability of soluble lipase activity (Working Conditions: 24 hours incubation, 0.5M NaCl, pH 7.0).

Several investigators have reported the optimum temperature of soluble *Candida rugosa* lipase as 37 ^{o}C (Montero et al., 1993; Xu et al., 1995). Also, Montero et al. (1993) found that treatment at higher temperatures led to the inactivation of the enzyme; at 55 ^{o}C, only 25 % of the enzyme activity remained. According to Fadıloğlu (1996), *Candida rugosa* lipase exhibits its maximal activity at 40 ^{o}C. In this study, enzyme showed considerable activity in a temperature range between 4-37 ^{0}C.

5.2.3. Effect of Ionic Strength on Soluble Enzyme Activity

Since the activity of lipases is modified by ions, especially Na^+ and Ca^{2+} (Sanchez et al., 1996), in order to examine the effect of ionic strength, different concentrations of NaCl (0.25 - 2 M) were added to soluble enzyme solutions and the remained activity of each sample was determined by the method given in Section 4.2.1

As seen in Figure 5.3, low and high ionic strengths caused activity loses; at zero salt concentration only 45 % of activity was recovered. Salt concentrations higher than 1.0 M also resulted with the activity loss and only 37 % of activity were remained with 2.0 M NaCl after 24 hours. In spite of long incubation period, 100 % of lipase activity was maintained in the solutions containing 1.0 M NaCl. This can be explained by the activating effect of Na^+ ions on the lipase enzyme and therefore as Na^+ ions increases in the solution up to 1.0 M, higher activity of lipase remains.

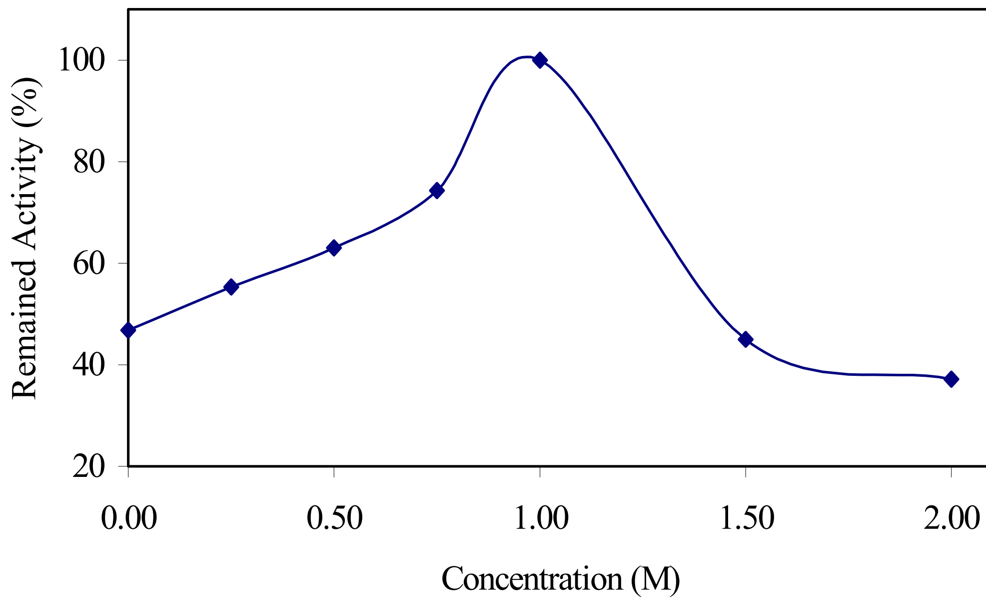


Figure 5.3. Effect of ionic strength on soluble enzyme activity (Working Conditions: 20 ^{0}C / 24 hours incubation, pH 7.0).

Sánchez et al (1996) examined the effect of Na^{+} ions on the activity of soluble *Candida cylindraceae* lipase. They found that the soluble lipase showed a slight activation when the NaCl concentration was increased. At 1.0 M NaCl, enzyme showed 100 % activity and it lost its activity by an extend of 40 % at zero salt concentration, which was similar to the results observed in this study.

5.3. Immobilization of Lipase on Hydrophobic Support (Octyl-Sepharose)

In this section, effect of environmental conditions on the immobilization of *Candida rugosa* lipase, time course of immobilization and effect of enzyme loading are presented. The optimum values obtained from the optimization studies for immobilization were used to investigate the effect of enzyme loading and immobilization kinetics.

5.3.1. Effect of Solid-Liquid Ratio

Besides other factors, solid-liquid ratio also affects the amount of enzyme adsorbed by the support during the immobilization process. The experiments were carried out with constant support volume (1 ml) and constant enzyme solution concentration (38.5 U) but the volume of the solution phase was changed in each experiment (4-29 ml).

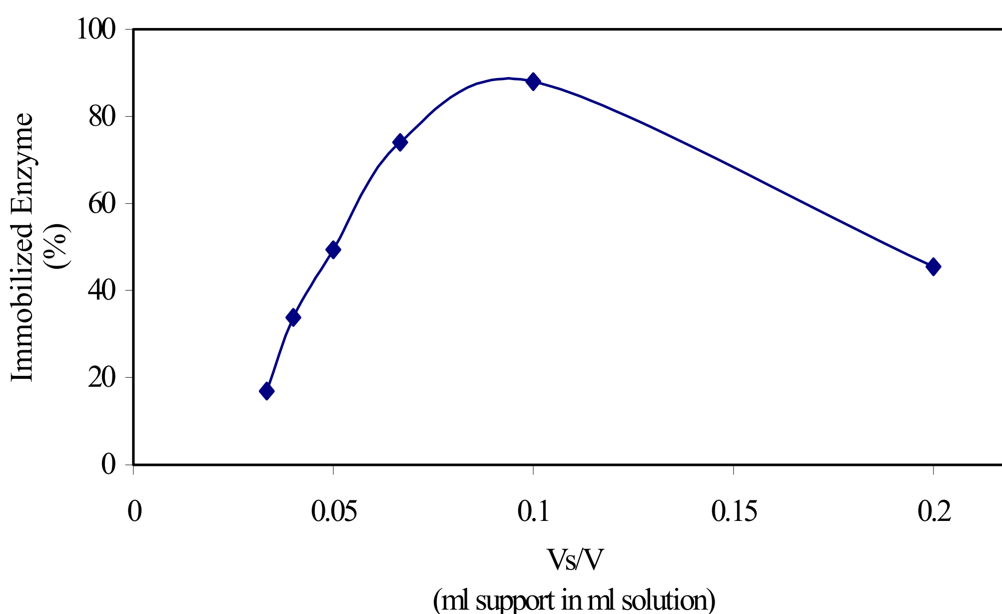


Figure 5.4. Effect of solid-liquid ratio on lipase immobilization on octyl-sepharose (Working Conditions: 4 ^{0}C/1 h. incubation, 0,5 M NaCl, pH 7.0).

Investigation of the effect of gel-liquid ratio on immobilization on octyl-sepharose is shown in Figure 5.4. When support was added to the solution phase with a ratio of 0.1, free enzyme activity was decreased and amount of the immobilized enzyme was maximized. Immobilization ratio was found around 45.5 % when Vs/V = 0.05 and this percentage decreased to 17 % when Vs/V = 0.033.

These experiments showed that between 0.1 and 0.2 Vs/V ratio the amount of enzyme immobilized increased, whereas at higher Vs/V ratios a decline occurred in the amount of immobilized enzyme. Since the amount of the enzyme immobilized was maximized when 9 ml. solution was added to 1 ml. of octyl-sepharose, Vs / V ratio was used as 0.1 in consecutive immobilization studies.

Sabuquillo et al. (1998) immobilized lipases from *Candida rugosa* and *Rhizopus niveus* on phenyl-, butyl- and octyl- sepharose matrices. In this study, it was observed that a two fold decrease in solid-liquid ratio leads almost 5 fold increase in time required to reach the same immobilization yield. This means that in the same time period, as the Vs/V decreased, immobilization yield decreased.

5.3.2. Effect of pH on Immobilization

The effect of buffer pH on octyl-sepharose/lipase system was investigated in the pH range of 4.0-9.0. As indicated in Section 5.2.1, *Candida rugosa* lipase was stable between pH 5.0 and 7.0. Thus, in these experiments, the studies were conducted in this pH range.

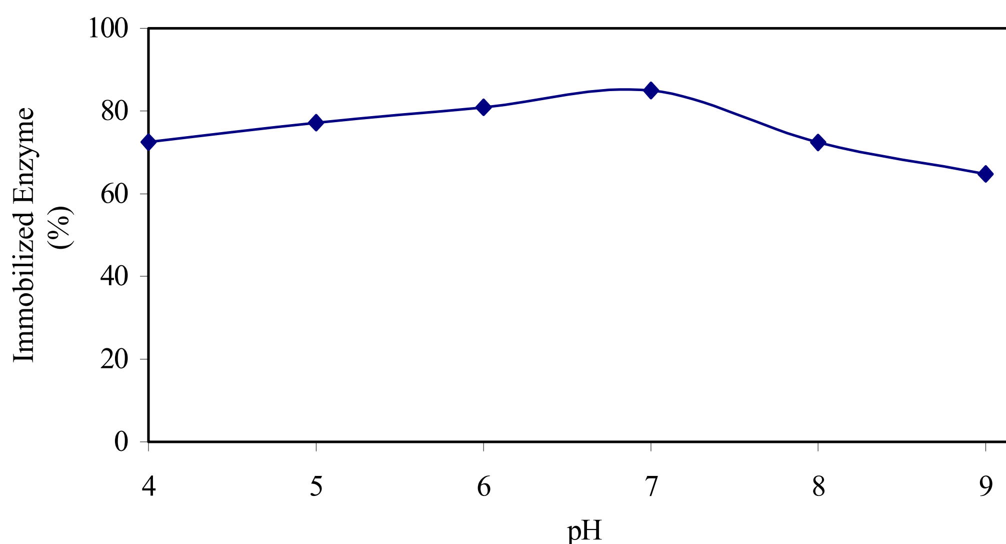


Figure 5.5. Effect of pH on immobilization of lipase on octyl-sepharose (Working conditions: 20 ^{0}C/1 h. incubation, 0.5 M NaCl).

Figure 5.5 shows the effect of pH on the immobilization of lipase enzyme on the hydrophobic support, octyl-sepharose. This figure indicates that lipase/octyl-sepharose system is strongly affected by pH. The maximum amount of immobilized enzyme was achieved at pH 7.0 with immobilized enzyme activity of 294 U/ml support. This value corresponds to the 85 % of adsorbed enzyme by this support. At pH values below 7.0, low values of immobilization were obtained. For example, at pH 4.0, nearly 72 % of

enzyme was adsorbed by 1 ml support and as pH increased, the amount of enzyme immobilized by octyl-sepharose increased.

Similar to this study, Arıca et al. (2001) investigated the optimum pH value for lipase immobilization on phenylalanine containing hydrogel membranes. These workers obtained the highest immobilization yield with 0.1 M, pH 7.0 phosphate buffer.

Usually, an increase in the pH value up to 9.0-10.0 decreases the hydrophobic interaction between proteins and hydrophobic supports. This is due to the increased hydrophilicity promoted by the change in the charge of the protein (Querioz et al., 2001). In this study, lipase immobilized at pH 9.0 showed only 25 % of the activity measured by the lipase immobilized at pH 7.0. Therefore, decreased interaction at elevated pH values is not very surprising.

Also, it is known that a protein with zero net charge (at pI) will have maximum hydrophobicity. However, at pH where protein and adsorbent have similar charges, repulsion may occur and this may cause the reduction of interaction. Moreover, close to isoelectric point, enzyme spontaneously tends to form less active aggregates. As seen in Figure 5.5, lipase immobilized at pH 4.0-5.0 showed only 30-50 % of the activity measured at pH 7.0. Such a result, therefore, can be explained by aggregate formation and enzyme-support repulsion near the isoelectric point (4.5) of lipase enzyme from *Candida rugosa*.

Lipases undergo structural changes in some pH values and this lead to inactivation of the enzyme or change in its activity due to perturbation in the vicinity of the active site. Similarly, these reactions might have occurred in the *Candida rugosa* lipase proteins, causing low activity at pH<6.0 and pH>8.0. These resulting structural changes in the enzyme molecule were naturally reflected in the adsorbed enzymes as well, affecting their activity in the same direction (Akova and Üstün, 2000). It is also an expected consequence that the enzyme adsorbed at the optimum pH level would show the highest activity. Therefore, pH 7.0 was established as the optimal pH of immobilization medium.

Montero et al. (1993), had immobilized *Candida rugosa* lipase on microporous polypropylene and they found that maximum immobilization yield was achieved between pH 5.7 and 6.5. When pH was above 7.0, immobilized enzyme yield rapidly decreased. Also, at pH 4.4, close to pI of enzyme, only 48 % of the enzyme were immobilized. These results may be explained by the formation of less active aggregates at acidic pH that is close to its pI. Also, at acidic pH, the enzyme may form high-

molecular weight or unspecific associations with other accompanying proteins due to its hydrophobic nature.

Considering these results, it may be assumed that at pH values close to pI, enzyme molecules were aggregated or combined with other proteins, which led to decrease in immobilization yield. Nevertheless, the significant change in immobilization yield was not observed in the range of pH studied. This can be attributed to the fact that pH is not the major factor influencing the immobilization by hydrophobic interactions.

5.3.3. Effect of Temperature on Immobilization

As observed in most of the chemical reactions, an increase in temperature increases the rate of reactions. However, the stability of a protein also decreases due to the thermal inactivation. If the enzyme is immobilized on a suitable support, thermal stability is usually improved. Therefore, determination of the optimum temperature at which the enzyme does not loose its activity is very important in finding the optimum operating conditions for immobilization by adsorption.

In this study, the influence of temperature on immobilization system of lipase/octyl-sepharose was investigated. This experimental procedure was conducted between 4 – 50 ^{0}C. Results are presented in Figure 5.6.

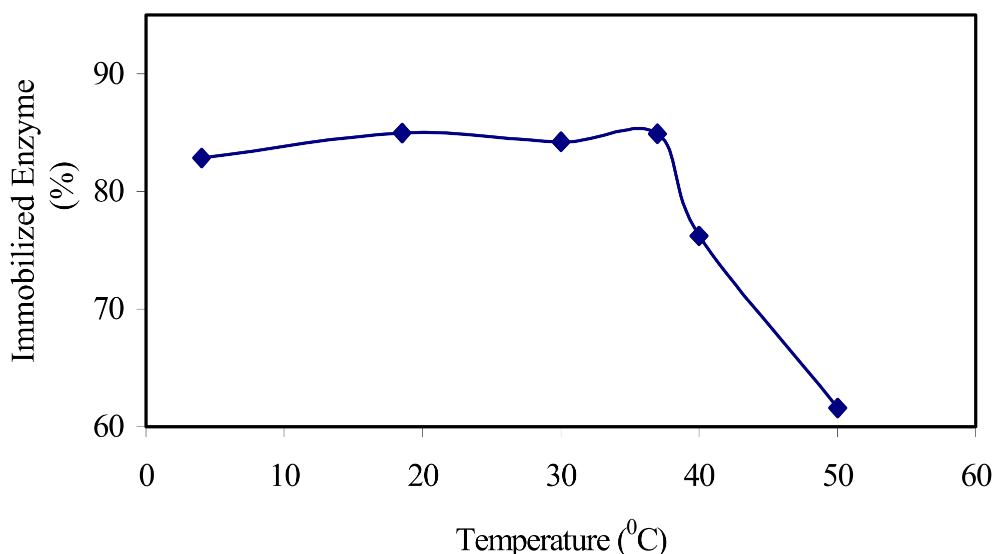


Figure 5.6. Effect of temperature on immobilization of lipase on octyl-sepharose (Working Conditions: 1 h. incubation, 0.5 M NaCl, pH 7.0).

As seen in this figure, 86.8 % of the enzyme was immobilized at 4 ^{0}C while this ratio was increased up to 89 % at 37 ^{0}C. This may be caused by the increase in hydrophobic interaction with increasing temperature. However, if the temperature increases to a value where the enzyme denaturation starts, immobilization ratio starts to decrease. Because of this phenomenon, the ratio of immobilized enzyme was only 65.6 % at 50 ^{0}C. As a result, optimum temperature for immobilization was 37 ^{0}C with an activity of 308 U/ml support.

Temperature can also influence adsorption but this is usually not readily apparent in systems of interest where adsorption is normally strong and temperature ranges are relatively small.

The results of this work were similar with those reported by Basri et al. (1994a, 1994b), who observed that the hydrophobic interaction is enhanced at high temperatures up to the point where the enzyme denaturation starts. Also, Knezevic et al. (1998) has investigated the kinetics of the immobilization of *Candida rugosa* lipase on zeolite type Y. At 0 ^{o}C, 8.2 mg protein was adsorbed per gram of zeolite with 33 % efficiency. However, at 30 ^{o}C, this amount decreased to 3 mg. These results shows that the amount of lipase bound on the solid surface decreases with the increase in temperature. Since chemisorption increases with temperature, these results supported the hypothesis of physical adsorption on the zeolite with a number of interactions between the surface and protein molecule such as hydrophobic interactions, hydrogen binding and Van der Walls interactions. In the same manner, in this study, decrease in immobilization yield at temperatures higher than 37 ^{0}C supports the hypothesis that the main driving force for immobilization of lipase on octyl-sepharose was hydrophobic interactions.

In another study conducted by Arıca et al. (2001), the effect of temperature on the lipase adsorption capacity was investigated with poly (HEMA-MAPA-3) membranes. An increase in the adsorption medium temperature from 4 to 45 °C led to an increase in the adsorption capacity of the membrane (up to 42 %) for lipase. An increase in the adsorption capacity of the membrane for lipase at a higher temperature indicated that the interaction between enzyme and phenylalanine groups of the membrane was mainly hydrophobic. At higher temperatures during the unfolding process, the proteins expose buried hydrophobic amino acid residues on the surface. Thus, the contact area between the protein and the hydrophobic group of the matrix

should increase, resulting in an increase in the hydrophobic interaction of proteins for the adsorbent at higher temperatures.

In fact, hydrophobic interaction is an entropy-driven process; since the enthalpy (ΔH) may be a small positive or negative value, free energy change (ΔG) is controlled by a positive entropy change and thus increases with temperature (Querioz et al., 2001). Therefore, it is possible that hydrophobic interaction was considerably enhanced at high temperatures causing rapid adsorption of the enzyme. The increased interaction, however, probably caused by the subsequent denaturation of the enzyme (Basri et al., 1994a).

5.3.4. Effect of Ionic Strength on Immobilization

Salt concentration is an important parameter for immobilization processes, especially when the hydrophobic interactions are concerned. It was a known property that the hydrophobic interaction increases with increasing salt concentration. However, salt concentration also affects the enzymatic activity and therefore this parameter also requires optimization. In this study; enzyme activity was found to be optimum when 1.0 M NaCl is used.

Figure 5.7 shows the effect of ionic strength on the immobilization system of lipase/octyl-sepharose using sodium chloride solutions of 0 – 2.0 M.

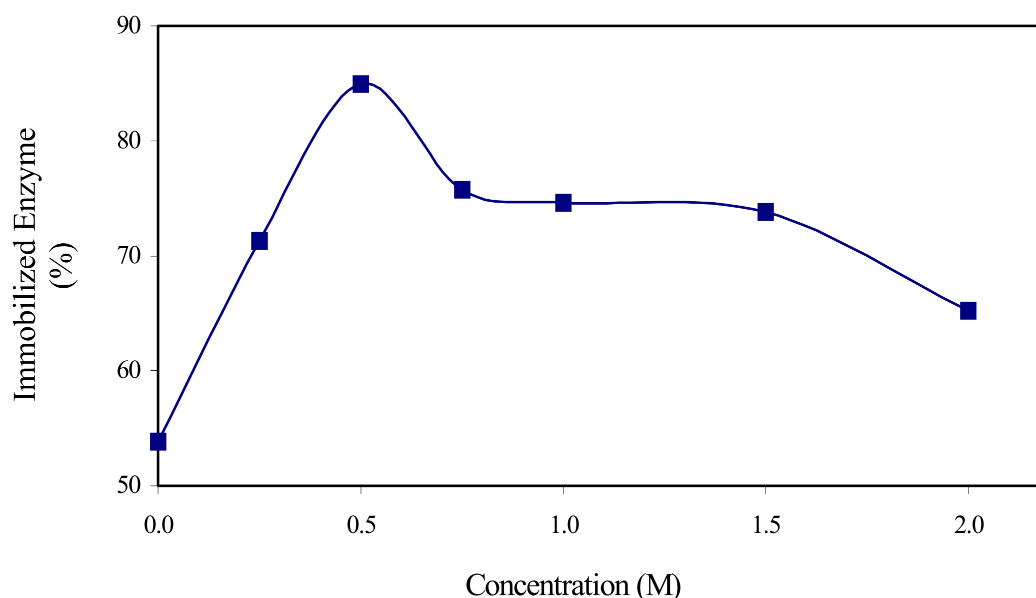


Figure 5.7. Effect of NaCl concentration on immobilization of lipase on octyl-sepharose (Working Conditions: 20 ^{0}C/1 h. incubation, pH 7.0).

During the immobilization process, maximum amount of enzyme binding was obtained with 0.5 M NaCl. At this concentration, 85 % of the enzyme was immobilized and the activity of immobilized lipase was 294 U/ml support. Also, at zero salt concentration, 54 % and at 2.0 M salt concentration, 65 % of enzyme was adsorbed on the support. Since the immobilized enzyme ratio is lower at high and low ionic concentrations than that of moderate, it can be concluded that adsorption of *Candida rugosa* lipase on octyl-sepharose reaches to the maximum value in the presence of 0.5 M NaCl. Also, since increasing concentrations of salt lead to increase in the immobilized enzyme ratio, it can be said that enzyme-support interaction is caused by hydrophobic interactions.

Detailed examination of Figure 5.7 shows that the immobilized enzyme ratio was maximized by the addition of 0.5 M NaCl, activity of immobilized lipase was maximized at 1.0 M NaCl which was caused by the relative higher activity of soluble lipase. Therefore, time course and effect of enzyme loading experiments were carried out in the presence of 1.0 M NaCl and the results are presented in Sections 5.3.5 and 5.3.6.

Generally, high ionic strength solutions of electrolytes do tend to cause the desorption of adsorbed proteins. The extend of this problem depends on the specific enzyme and support material involved, pH, temperature and perhaps other variables.

Salts such as sodium, potassium or ammonium sulfates and chlorides promote the matrix-protein interactions due to the molal surface tension increment effects. As well as salt type, its concentration also strongly influences the selectivity in enzyme immobilization and this influence is different and depend both on the support and buffer salt. In hydrophobic interaction, the use of high salt concentration in the buffer promotes the support-enzyme interaction and consequently the enzyme immobilization. The amount of the bound enzyme increases with the enhancement in ionic strength (Querioz et al., 2001; Diogo et al., 1999).

The presence of the salts increases the free energy of the proteins and this free energy augmentation is proportional to the hydrophobic surface area of the enzyme molecules. Intermolecular association of hydrophobic groups minimizes the increase in free energy by decreasing the hydrophobic contact area of the enzyme with the polar solvent media. Therefore, when a hydrophobic support is introduced into the system, enzymes bound to the support because it minimizes the surface contact area of enzyme and support with the salt solvent medium and produce a minimum increase in free

energy. So, in a medium of high salt concentration the bound form of enzyme is thermodynamically more stable than the unbound protein. This explains the enzyme binding to hydrophobic surfaces at high salt concentration (Querioz et al., 2001).

However, in this study, increase in salt concentration above 0.5 M led to a decrease in immobilization yield, which was quite different from standard hydrophobic interaction phenomena. Such a result was also obtained by Bastida et al. (1998), Sabuquillo et al. (1998) and Montero et al. (1993) who observed a decrease in immobilization yield by increasing the ionic strength above a point. Therefore, it can be concluded that adsorption of lipases on octyl-sepharose at low ionic strength seems to be very selective and also follow a very specific mechanism of adsorption (Bastida et al., 1998).

5.3.5. Time Course of Lipase Immobilization on Hydrophobic Support (Octyl-Sepharose)

The rate of lipase immobilization on octyl-sepharose was investigated at two different temperatures using different enzyme concentrations. In these analysis, working conditions were pH 7.0, 1.0 M NaCl and Vs / V = 0.1, since these are found to be the optimum values obtained from the previous studies. Lipase solutions prepared in concentrations of 3.055, 30.55, 61.1, 183.3. and 305.5 U/ml were tested at 4 ^{0}C and 3.055, 61.1, 305.5 and 916.5 U/ml were conducted at room temperature, 20 ^{0}C. Results are given in Figure 5.8 and Figure 5.9.

As mentioned above, the minimum concentration investigated was 0.25 mg/ml and at this concentration and at the twentieth second, only 20 % of the enzyme was adsorbed. The equilibrium was reached at the tenth minute where 68.5 % of the enzyme was bound to the support with the immobilized lipase activity of 18.8 U/ml support.

When the enzyme concentration increased to 2.5 mg/ml, it was seen that 47 % of the lipase was bound to the support in the first twenty second contact time. Later, immobilization was continued and at the end of the tenth minute, 93 % of the enzyme was immobilized with the activity of 255.7 U/ml support. At this concentration, it took nearly 10 minutes to reach the equilibrium.

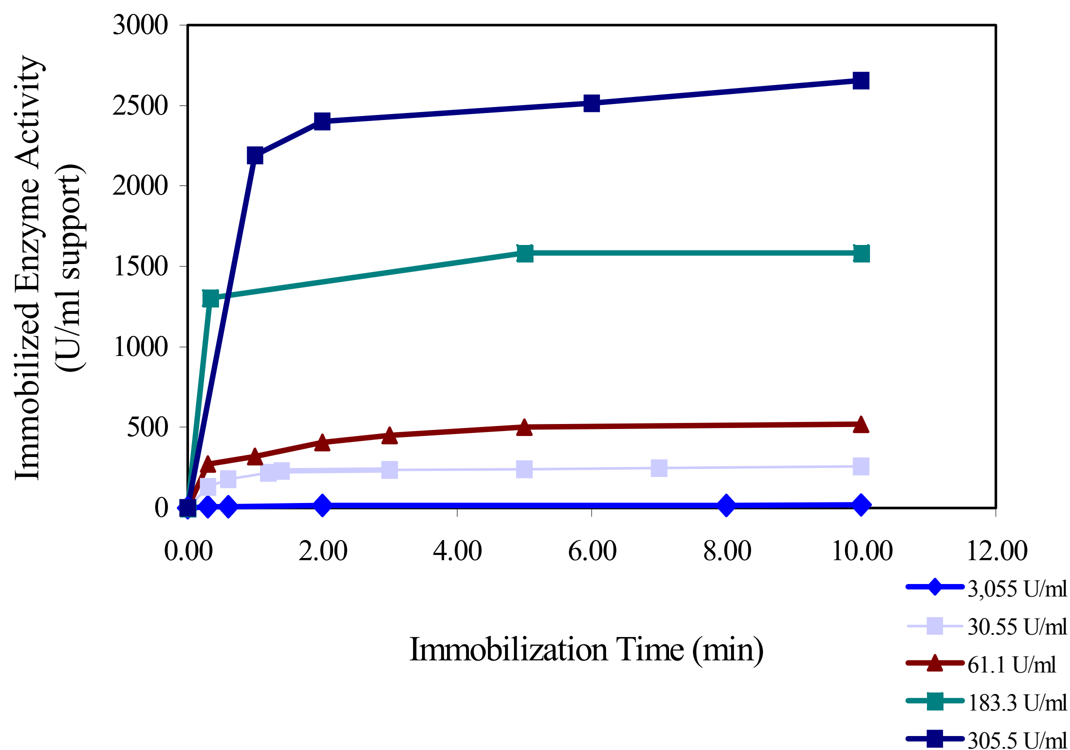


Figure 5.8. Time course of lipase immobilization at 4 ^{0}C (Working Conditions: pH 7.0, 1.0 M NaCl).

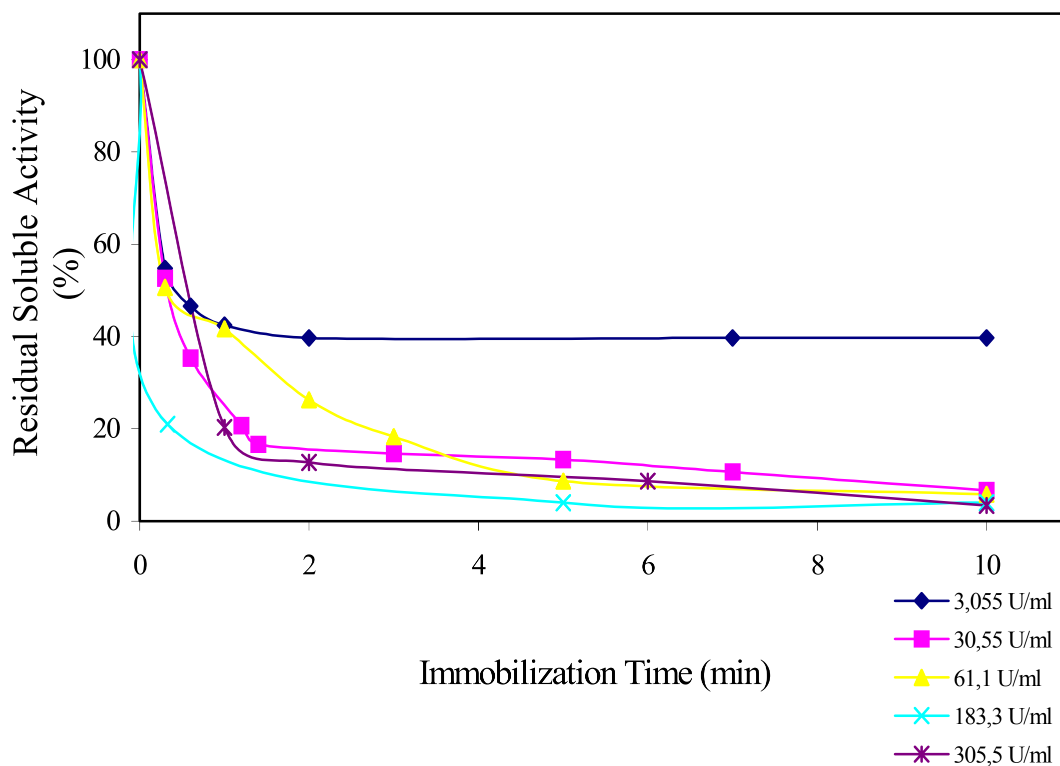


Figure 5.9. Time course of lipase immobilization at 4 ^{0}C. (Working Conditions: pH 7.0, 1.0 M NaCl)

Analysis at 5 mg/ml enzyme concentration showed that after 20 seconds of incubation, 49 % of the lipase was bound to the support. The reaction was reached to the equilibrium at the end of fifth minute and 94 % of the enzyme was immobilized. Activity of immobilized lipase was calculated as 517 U/ml support.

Time course of immobilization at 15 mg/ml. enzyme concentration was carried out at 4 ^{0}C and 79 % of immobilization was obtained at the end of the twentieth second. Then, it was slow for nearly one minute and after fifth minute, equilibrium was reached and 96 % of the lipase was immobilized. Activity of lipase immobilized was 1303.3 U/ml support.

If the enzyme concentration increases to 25 mg/ml, it can be seen that the amount of the enzyme immobilized at the twentieth second was 79 % and at the eighth minute equilibrium was reached with a ratio of 96 % immobilization. At this concentration, activity of immobilized lipase was 2639.5 U/ml support.

As seen from the figures, there is an initial period, which seems to be less than one minute of rapid immobilization responsible for about 80 % of total final adsorption. The amount of lipase adsorbed at 4 ^{0}C on all surfaces are close to the respective saturation values in a few minutes. This stage is followed by a slower approach to a limiting final value in 10 minutes. In general, the amount of immobilization increases with the increase in the initial enzyme concentration (p_o). The increase in the po from 0.25 to from 5 and 5 to 25 mg/ml have a progressively increasing effect on the final amount of enzyme immobilized as seen in Figure 5.9.

The other temperature value investigated was 20 ^{0}C. First concentration tested at this temperature was 0.25 mg/ml and again adsorption was quite rapid. At the twentieth second, 45.2 % of the enzyme was adsorbed. At the end of the second minute, reaction reached to equilibrium and maximum amount of the enzyme adsorbed was 60.3 %. At equilibrium, activity of immobilized lipase was found to be 16.6 U/ml support.

The analysis of time course of immobilization at 5 mg/ml enzyme concentration showed that after 20 seconds of incubation, at room temperature, nearly 80 % of the enzyme was immobilized. The equilibrium phase has been reached at the fourth minute and 91 % of the enzyme was immobilized. The final activity of immobilized lipase was 500.4 U/ml support.

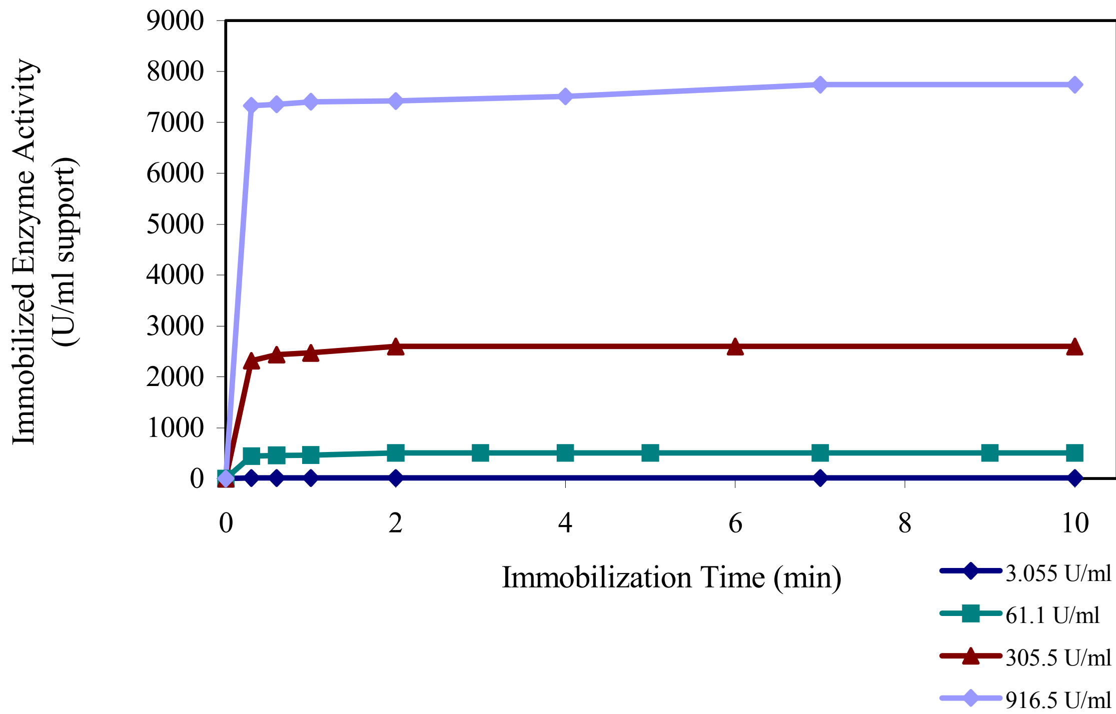


Figure 5.10. Time course of lipase immobilization at 20 ^{0}C (Working Conditions: pH 7.0, 1.0 M NaCl).

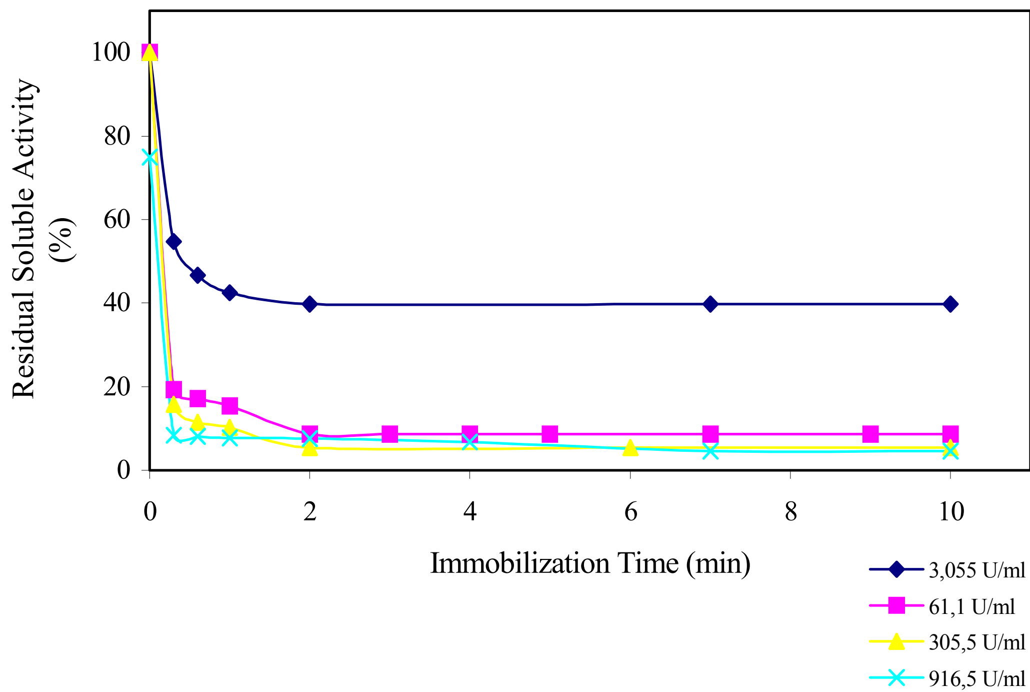


Figure 5.11. Time course of lipase immobilization at 20 ^{0}C (Working Conditions: pH 7.0, 1.0 M NaCl).

If the enzyme concentration was increased to 25 mg/ml, it was seen that 88 % of the enzyme was immobilized at the end of the first twenty minutes at room temperature. The maximum immobilized enzyme ratio was a little less that of at 4 ^{0}C, which was 95 %. This immobilization ratio resulted with the immobilized lipase with the activity of 2612 U/ml support.

Studies with 75 mg/ml enzyme concentration were completed with 88 % immobilization ratio in the first twenty seconds time. The equilibrium was reached at the seventh minute and totally 93 % of the enzyme was bound to the support. Immobilized lipase had an activity of 7740 U/ml support.

As seen in Figure 5.11, generally, when the initial concentration of enzyme was increased, time to reach the equilibrium was decreased. Also, in this case, the initial period of nearly 20 seconds of rapid immobilization was responsible for about 75-85 % of the total final adsorption. The amount of lipase adsorbed at 20 ^{0}C on all surfaces are reached to the saturation values in nearly 2 minutes time. Further incubation did not lead to additional increases in immobilization yield.

This study showed that temperature was not greatly effective on the immobilization yield but it effects the rate of immobilization. In this study, it was observed that temperature increase led to a decrease in time required to reach the equilibrium values.

Briefly, the amount of the activity adsorbed by octyl-sepharose increased very rapidly in a few minutes and reached to a plateau after a longer period of incubation. Such results suggest that the loading of protein was initially localized near the outer surface of the support instead of being uniformly distributed throughout the pores of the support. The rapid immobilization determined in this study was supported by the findings of Montero et al. (1993); who found that in 1 minute time, as much as 64 % of *Candida rugosa* lipase was immobilized on polypropylene support. Also, Lafunte et al. (1998) has achieved complete immobilization of lipases from different sources like *Candida rugosa, Candida antarctica A* and *B, Mucor miehi,*etc. on octyl-sepharose in 10 minutes time at low ionic strength with Vs/V= 1/10. Moreover, Fischer et al. (1992) has determined an immobilization yield of 75 % on pulp fibers in 1 minute time.

Octyl-sepharose is a highly hydrophobic support while phenyl-agarose has medium and butyl-sepharose has low hydrophobicity. In a study by Sabuquillo et al. (1998), lipase effect of supports with different hydrophobicity on the immobilization of lipase from *Candida rugosa* was investigated. It was seen that lipase was completely

immobilized on octyl-agarose, while 25 % and 60-65 % of immobilization yields were achieved by butyl-agarose and phenyl-agarose, respectively. Also, the time to reach the equilibrium was changed with respect to the hydrophobicity, as the hydrophobicity increased, equilibrium time decreased from 5 hours to 1 hour. Such a result was not valid only for *Candida rugosa* lipase, immobilization of *R. niveus* on the same supports resulted in the same differences. Therefore, it can be concluded that immobilization yield and time required to reach the equilibrium is related with the hydrophobicity of support.

5.3.6. Effect of Enzyme Loading

Effect of enzyme loading on immobilization depends on the temperature of the immobilization medium and, therefore, two different temperatures (4 ^{0}C and 20 ^{0}C) were examined in this section.

Figure 5.12 indicated the results of the analysis performed at 20 ^{0}C. The amount of immobilized lipase increased rapidly as the initial concentration of the enzyme increased and a plateau value could not be reached. Higher concentrations may have been worked but, at these high concentrations, viscosity increases and solubility of lipase decreases and working at these concentrations is very difficult. In the studied range, maximum amount of immobilized enzyme was 7740 U/ml support when the free enzyme concentration was 12.2 U/ml.

Adsorption isotherm models can be used to explain the enzyme loading for immobilization, since they show the distribution of enzyme concentration between the liquid phase at equilibrium (p) and support phase (q). There are several equations to describe the adsorption isotherms. These are Linear, Freundlich and Langmuir isotherms:

Linear isotherm equation: $q = p \times C$

Freundlich isotherm equation: $q = p^{1/n} \times C$

Langmuir isotherm equation: $q = \dfrac{q_m \times p}{K + p}$

Linearized forms of these equations are,

Freundlich: $$\log(q) = \frac{1}{n}\log(p) + \log(C)$$

Langmuir: $$\frac{1}{q} = \frac{K}{q_m} \times \frac{1}{p} + \frac{1}{q_m}$$

where q is the equilibrium concentration of immobilized enzyme (U adsorbed/ml support); q_m is the maximum adsorption capacity (U adsorbed/ml support); p is the equilibrium concentration of free enzyme (U free enzyme/ml solution); K is the dissociation constant for adsorbent-adsorbate complex (U/ml solution). 1/n is the appropriate constant for Freundlich isotherm, which indicates the effect of concentration on the adsorption capacity and represents adsorption intensity. C is the appropriate constant indicating the distribution of enzyme in the solid phase and, thereby, the adsorption capacity of the adsorbent. For Langmuir isotherm, regression analysis was performed to determine the slope and the y-intercept, thus allowing calculations of q_m as $slope^{-1}$ and K as slope $\times$ q_m. For Freundlich isotherm, regression analysis gave the n as $slope^{-1}$ and log (C) as the intercept. Finally, the C value for linear isotherm was calculated from the slope of q versus p diagram. Using these models, it is possible to calculate the parameters, which are quite important for the design and scale-up of immobilization processes.

As seen in Figure 5.12, in the studied range of enzyme concentration, the experimental data fitted well to the linear isotherm with the C value of 138.22. Generally, it was reported that immobilization of lipase follows the Langmuir isotherm which was different from the results of this study. However, in diluted solutions, it was seen that the data fitted well to the Langmuir isotherm with a correlation factor of 0.9815. Therefore, it can be said that the studied concentration range of the enzyme solution defines the type of the isotherm that the data would fit.

Before the establishment of the isotherms, data were investigated for the suitability to the different isotherms and the equation that yielded in the highest R^2 value is accepted as the most reliable isotherm for that case. In Table 5.1, the results of this investigation can be seen.

A solid surface has only a limited area to which protein molecules can adsorb. The adsorbed amount depends on the size of the protein molecule, the surface area of

the carrier and the number of sites available for protein adsorption. The area available for protein adsorption and the equilibrium behavior of the lipase/support in the immobilization systems depends on the physical and structural properties of the support (e.g. porosity, pore size) and the physical and chemical properties of the lipase. The adsorption isotherms of compact molecules as globular proteins generally show a finite initial slope and well-defined plateau-values not far from the close-packed monolayer of native molecules. Contrary to the belief that the equilibrium behavior of lipase adsorption would generally conform to the Langmuir model with good correlation, it can change depending on the properties of the support and the enzyme and also the enzyme loading and the working conditions. Therefore, insight analysis of the equilibrium behavior may be resulted in that the lipase distribution on the support surface could be described by the Freundlich or Linear models (Duri et al., 2000; Gitlesen et al., 1997).

Also, it should be noted that at low loadings, there is a large excess of surface area that the enzyme can occupy and the lipase attempts to maximize its contact with the surface, which results in a loss of conformation and consequently in a reduction of activity. As the loading increased, less area is available for the lipase to spread itself, more of its active conformation is retained, and the loss in activity is reduced (Akova and Üstün, 2000). Therefore, working in the most suitable concentration range has a positive effect on the enzyme activity.

Bastida et al. (1998) had reported that commercial octyl-sepharose 4B is able to load up to 20-30 mg/ml medium size protein (e.g. 60,000 Daltons molecular mass). However, in their study, 1 g protein was immobilized on 1 ml of octyl-sepharose. This occasion was explained by the achievement of a high level of purification. In this study, maximum concentration worked was 75 mg enzyme/ml solution which was finally resulted in an immobilization yield of 53 mg protein/ml octyl-sepharose. This result shows that the support was loaded with protein higher than its capacity and can be explained in the same manner with those of Bastida et al. (1998) (Lafunte et al., 1998).

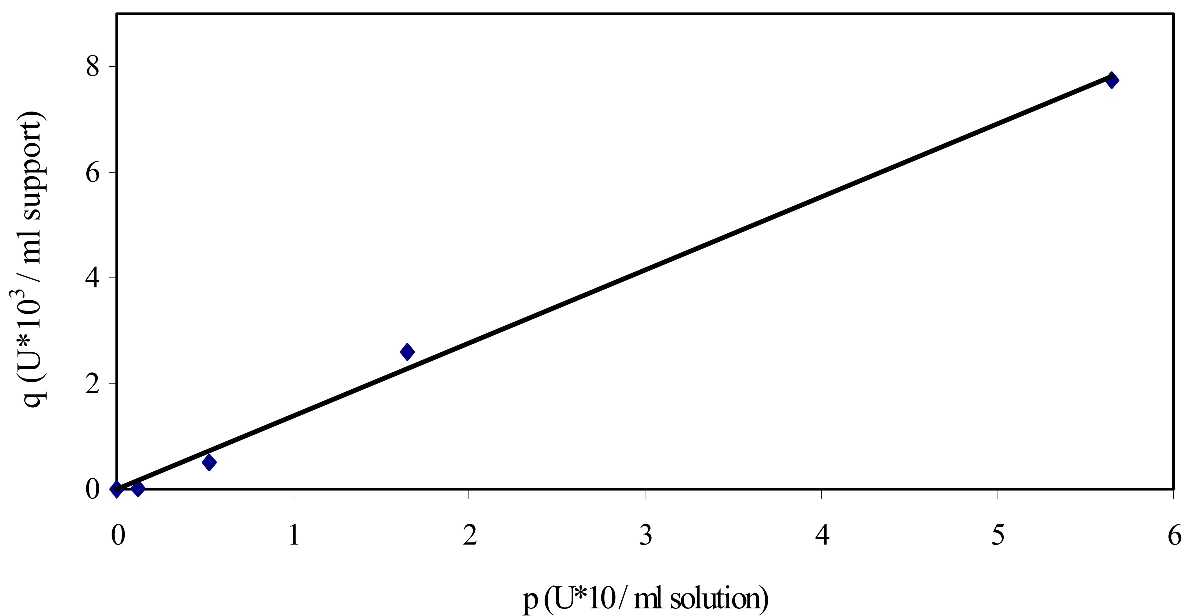


Figure 5.12. Effect of enzyme loading on immobilization of lipase on octyl-sepharose at 20 ^{0}C (Working conditions: 1 h. incubation, pH 7.0, 1.0 M NaCl).

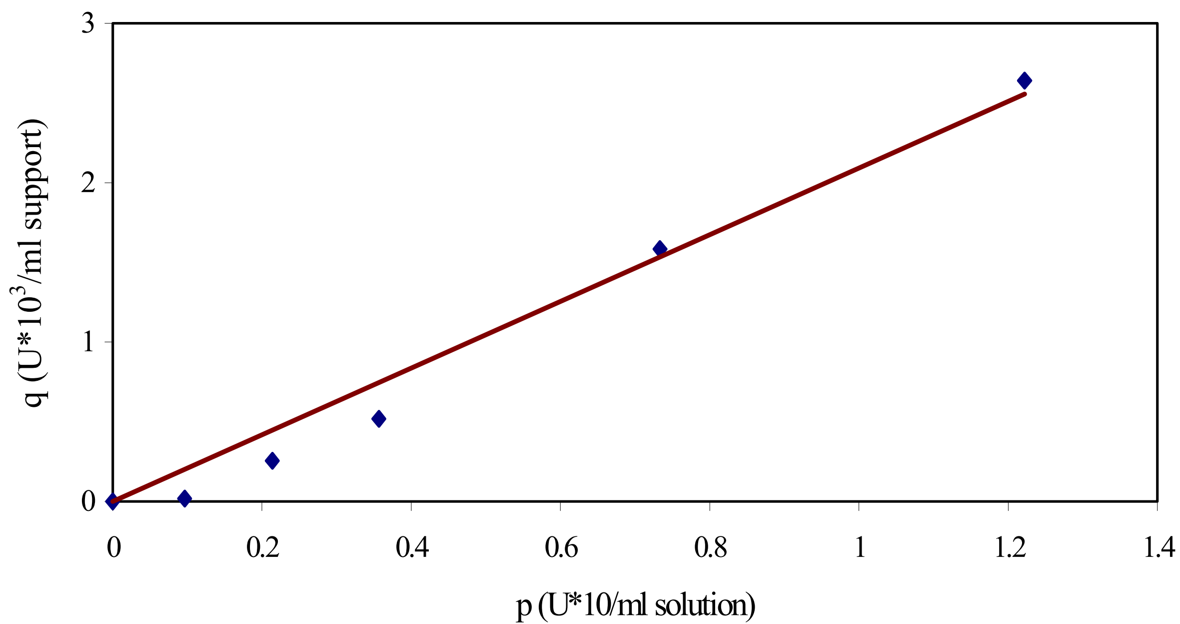


Figure 5.13. Effect of enzyme loading on immobilization of lipase on octyl-sepharose at 4 ^{o}C (Working conditions: 1 h. incubation, pH 6.0, 1.0 M NaCl).

Equilibrium behavior of lipase/octyl-sepharose system was investigated also at 4 ^{0}C. This study showed that there was a rapid increase in immobilization yield up to 305.5 U/ml. As at 20 ^{0}C, a plateau value could not be reached and further analysis could not be carried out again because of the increasing viscosity at higher enzyme concentrations and consequently difficulties in having a homogeneous mixture. These experimental data fitted well with linear isotherm with the C value of 209.11. An additional experiment was also conducted to investigate the behavior of lipase/octyl-sepharose system at higher enzyme concentrations. However, these analyses showed that data again fitted to linear isotherm, but in this case, the correlation coefficient was decreased. This result suggested that the equilibrium behavior was changed at high enzyme concentrations.

Table 5.1. Isotherm constants for the lipase/octyl-sepharose system.

Temperature (^{0}C)	Isotherm Type - R^2 value	IsothermType- R^2 value	IsothermType- R^2 value	Selected isotherm	C
20	Langmuir - 0.9713	Freundlich- 0.9551	Linear - 0.9959	Linear	138.22
4	Langmuir - 0.8668	Freundlich- 0.9429	Linear - 0.9766	Linear	209.11

Table 5.1 also shows the temperature dependency of C value. As seen in the table, as temperature increased, C value also increased.

Gitlesen et al. (1997) had investigated the adsorption behavior of *Candida rugosa* and *Pseudomonas fluorescence* during immobilization on polypropylene powder. The concentration range studied was between 10-100 mg enzyme/g carrier. The behavior of each system was different from each other; P. Fluorescence/PP system did not reach to the plateau value in the concentration range given while the carrier was saturated with the lipase from *C. rugosa*. However, all systems fitted to Langmuir isotherms.

During immobilization, concentration not the amount, was the critical factor since excess protein was available in all cases. At high enzyme concentration the support material becomes saturated and little additional protein can be adsorbed even at

higher concentrations. It is apparent that simply adsorbing a large amount of enzyme on a support material is not sufficient to produce a high activity derivative (Pitcher, 1980).

At this point, it should be noted that support hydrophobicity effects the extent of the enzyme binding. Among the ligands that can be bound to sepharose, octyl has the highest hydrophobicity, whereby, immobilization of hydrophobic *Candida rugosa* lipase on octyl-sepharose results in higher yields. However, at the same time, it is difficult to desorb lipase from octyl-sepharose; which may lead to denaturation of enzyme (Sabuquillo, 1998).

5.4. Immobilization of Lipase on Hydrophilic Support (Chitosan)

The free amine groups of chitosan contribute polycationic property to this molecule. On the basis of this property, it was assumed that ionic interactions might take place between the chitosan and lipase. However, there were only a few studies related with this subject in the literature. Thus, experiments were carried out to investigate the interaction between these molecules. At the end of this study, it was also possible to compare the behavior of lipase against a hydrophilic and a hydrophobic support.

In this section, effect of environmental conditions on the immobilization of *Candida rugosa* lipase on hydrophilic support, time course of immobilization and effect of enzyme loading was presented. During the investigation of the effect of environmental conditions, the optimum values achieved by each experiment are used in subsequent experiments.

5.4.1. Effect of Solid-Liquid Ratio

The effect of solid-liquid ratio on the amount of lipase immobilized is given in Figure 5.14. The experiments were carried out in a range of 4-24 ml. solution phase where the total activity of lipase in the solution was 29 Units.

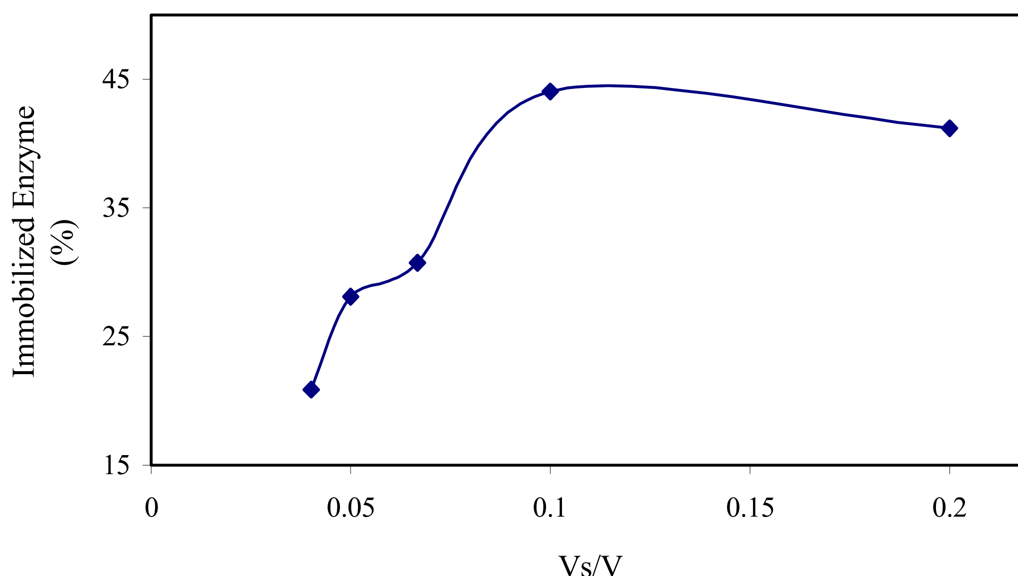


Figure 5.14. Effect of solid-liquid ratio for immobilization on chitosan beads (Working Conditions: pH 6.0, no salt addition, 20 ^{0}C/24 h. incubation).

Effect of solid-liquid ratio on immobilization on chitosan beads gave similar results with the experiments conducted with hydrophobic support, octyl-sepharose. It is observed that the ratio of volume of solid to volume of solution phase affects the immobilization ratio. When the Vs/V = 0.1 was used, maximum amount of enzyme (44 %) was immobilized and in the case of Vs/V = 0.2, immobilization ratio was similar to the results of Vs/V = 0.1. Support concentrations smaller than 0.1 led to a decrease in the immobilization amount. The lowest immobilization ratio of 20 % was found for the Vs/V = 0.05.

The point where the amount of free enzyme was minimized should be selected for further experiments. Therefore, Vs/V = 0.1 was selected as the optimum solid to liquid ratio and was used in further experiments.

5.4.2. Effect of pH on Immobilization

Figure 5.15 shows the relationship between the immobilized amount of enzyme and pH of medium.

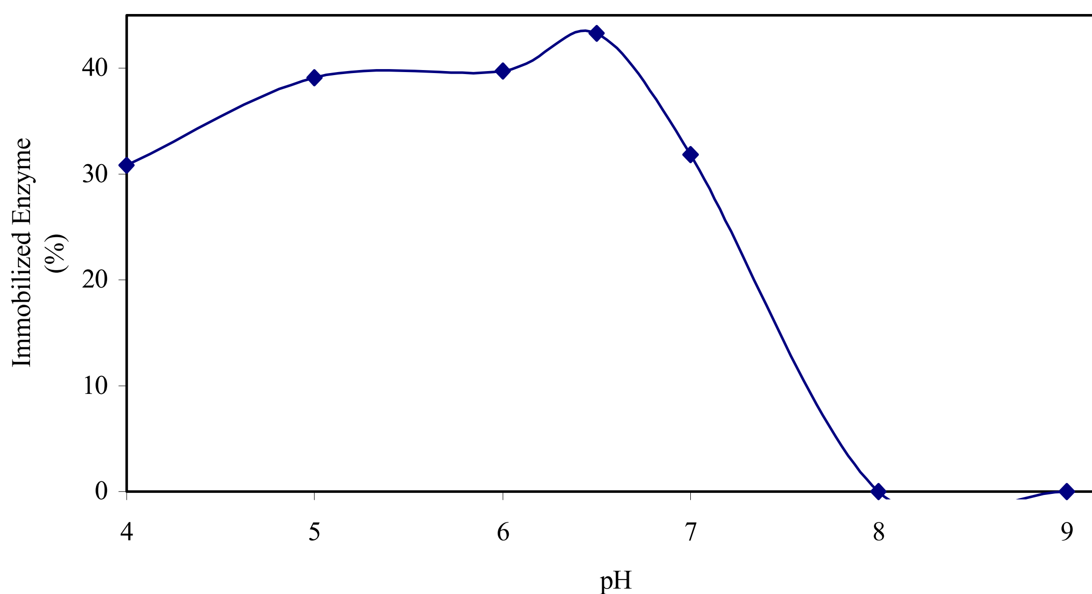


Figure 5.15. Effect of pH on immobilization of lipase on chitosan beads (Working Conditions: 0,5 M NaCl, 20 ^{0}C/24 h. incubation).

At pH 6.5, immobilization ratio was found to be around 44 % with the immobilized lipase activity of 170 U/ml support. However, interestingly, usage of pH 8.0 and 9.0 buffers prevented the binding of the lipase to the chitosan. At the pH values of 4.0-5.0 which corresponds to the pI of lipase, an immobilization yield around 40 % was achieved. As seen, around the pI, there was not a significant change in immobilization yield but as the pH value is elevated, changes become significant.

Briefly, in lipase/chitosan system, immobilization of lipase is accelerated at low pH values while elevated pH values led to sharp decreases in immobilized enzyme ratio.

Because of the ionic and hydrophilic nature of chitosan, it can be thought that interactions between the proteins and this support may be governed by ionic interactions. Ionic binding can greatly be affected by the pH value used for the operation since capacity of binding depends on the ionized groups on the matrix and the number of charges that the enzyme molecule carries. Enzyme binds to the matrix when they carry a charge opposite to that of the supports. This binding is electrostatic and reversible. However, since enzyme activity also changes with pH, choice of the working pH also depends on the optimum working pH of the enzyme. Therefore, in order to maximize the immobilization yield, to work in a suitable pH range is essential.

Investigations were performed with lipase from *Candida rugosa* which was dissolved in buffers of pH 4.0-9.0 and these pH values correspond to positive, electroneutral and negative net charge of the lipase (Gitlesen et al., 1997). The findings of this study support the theory that electrostatic interactions are the major driving force in immobilization of lipase on chitosan.

Different from the results of this study, He et al. (2001) has immobilized porcine pancreatic lipase on porous silica beads and it was found that optimum pH value for immobilization medium was 8.0. Therefore, it is obvious that the pH value that would optimize the immobilization yield depends on the type of support and source of lipase.

5.4.3. Effect of Temperature on Immobilization

In this study, effect of temperature on immobilization of lipase on chitosan was investigated in a temperature range between 4-30 ^{0}C. Temperatures such as 37 ^{0}C, 40 ^{0}C and 50 ^{0}C were also examined but the polymer structure changed at these temperature values and it was impossible to carry out an activity assay, therefore, the results were not presented here.

As seen in Figure 5.16, the optimum operating temperature for immobilization was 20 ^{0}C where 44 % of the enzyme was found in bound form with the immobilized enzyme activity of 153 U/ml support. Incubation at 4 and 30 ^{0}C did not change the immobilization ratio significantly, 38 % and 43 % of the enzyme was bound on chitosan at 4 ^{0}C and 30 ^{0}C, respectively. As indicated in Section 5.2.2., lipase was stable in this temperature range.

Adsorption is an exothermic process, therefore, the adsorptivity is expected to decrease with increasing temperature. However, when the ionic interactions are concerned, the standard enthalpy change is very small, so, that the temperature dependence of the equilibria is expected to be minor (Harsa, 1995). This hypothesis also supports the results of this study in which temperature dependency of immobilization process found to be on a small extend.

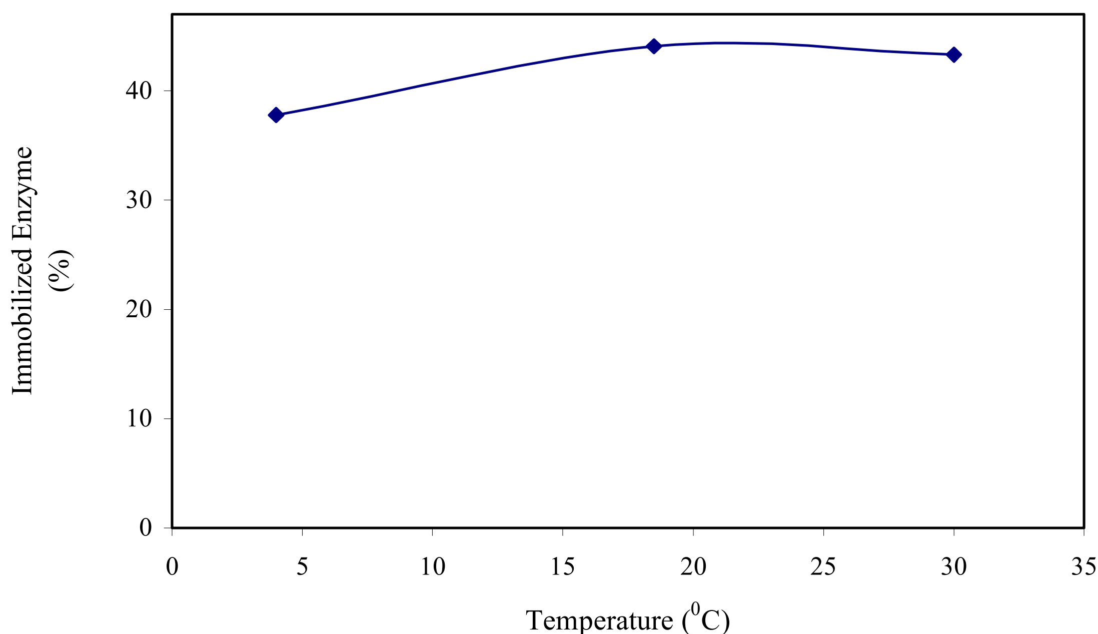


Figure 5.16. Effect of incubation temperature on immobilization of lipase on chitosan beads (Working Conditions: 24 h. incubation, no salt addition, pH 6.0).

Similar studies on the effect of temperature were given in the literature. Porcine pancreatic lipase was immobilized on porous silica beads by He et al. (2001) and in this study, it was concluded that the immobilized lipase prepared at 30 °C had the highest activity. Below 30 °C, the immobilization reaction could not performed effectively. On the other hand, the native enzyme lost its activity before the immobilization reaction when the temperature was above 30 °C.

5.4.4. Effect of Ionic Strength

Figure 5.17 shows the influence of salt concentration on the immobilization process. As seen in the figure, increase in the concentration of the salt led to the decreased immobilization yield. At zero salt concentration, 44 % of the lipase was bound on chitosan while the addition of 2.0 M NaCl to the immobilization medium decreased this ratio to the 33 %. At zero salt concentration, activity of immobilized lipase was calculated as 305 U/g support.

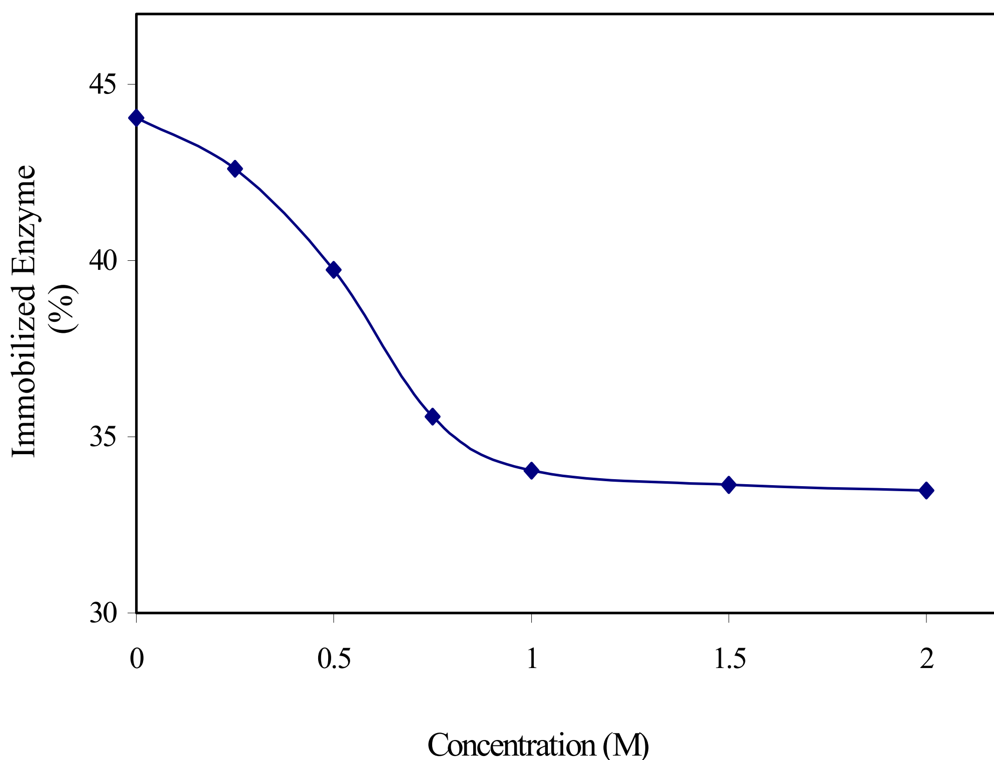


Figure 5.17. Effect of NaCl concentration on immobilization of lipase on chitosan beads. (Working Conditions: pH 6.0, 20 ^{0}C/24 h. incubation)

At low ionic strength, competition for charged groups on the carriers is at a minimum and substances are bound strongly. Increasing the total number of ions present in the solution decreases the strength of binding of each individual ion by increasing the competition between ions for binding sites. An increase in ionic strength therefore decreases interactions between the enzyme molecules and the support and this leads to reduced immobilization yield (Harsa, 1995).

5.4.5. Time Course of Lipase Immobilization on Hydrophilic Support (chitosan)

Figure 5.18 shows the time course of the immobilization of lipase on chitosan beads. These experiments were carried out with 5 mg/ml lipase enzyme in 0.025 M phosphate buffer of pH 6.0.

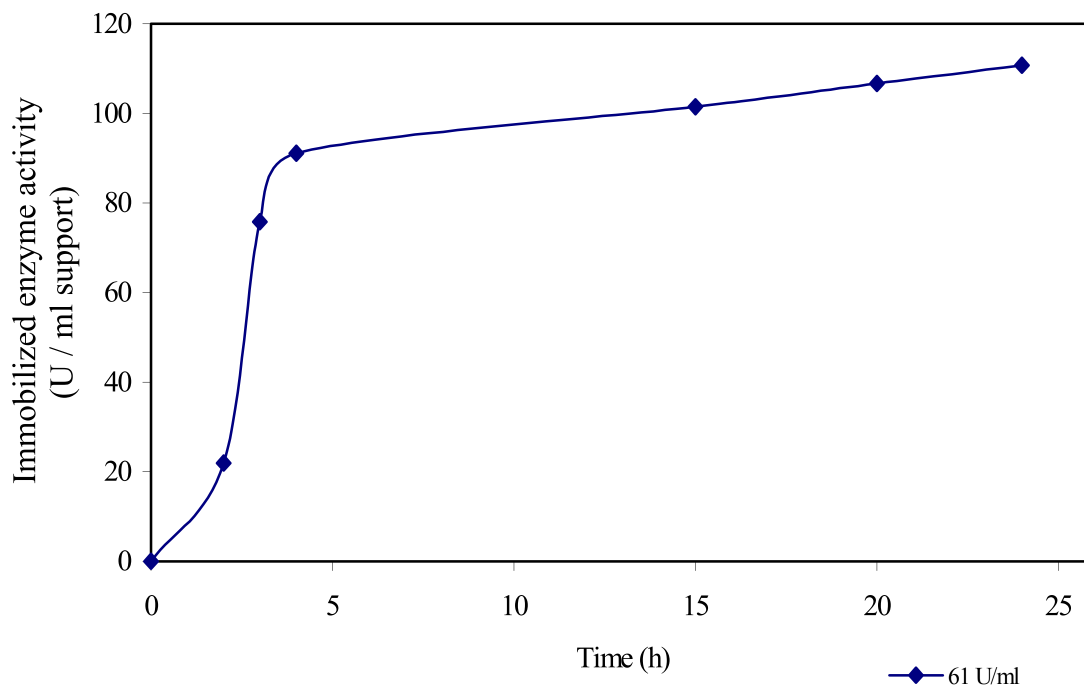


Figure 5.18. Time course of lipase immobilization on chitosan (Working Conditions: 20 ^{0}C, pH 6.0, 0.75 M NaCl).

As seen in Figure 5.18, after 4 hours of incubation, nearly 30 % of the lipase was immobilized and at the prolonged incubation times, rate of immobilization was decreased. At the end of 24 hours, 35 % of the lipase was immobilized on chitosan under given working conditions and prolonged incubation time did not change this ratio. The activity of immobilized lipase was found to be 110 U/ml support.

5.4.6. Effect of Enzyme Loading

The effect of enzyme loading was investigated in the concentration range between 0.25-75 mg lipase/ml solution, which corresponds to 1.1-327 U/ml solution. The optimum working conditions obtained in previous studies were used in these experiments and the results of this study can be seen in Figure 5.19. At low concentrations, there was a linearization while it reached to plateau value at high concentrations. At concentrations higher that 25 mg/ml, amount of the enzyme remained constant at a value of around 160-180 U/ml support.

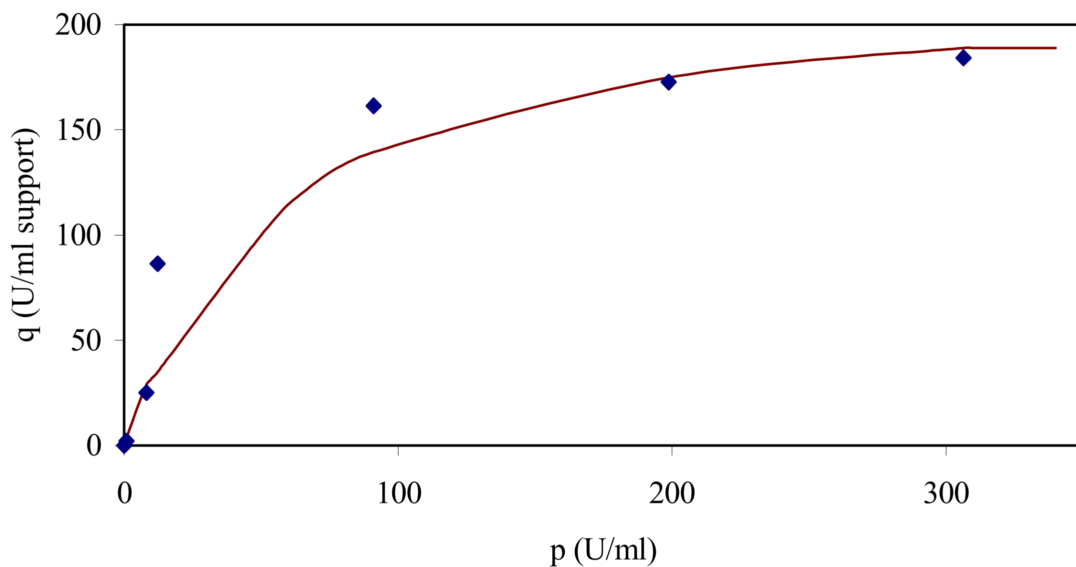


Figure 5.19. Effect of enzyme loading on immobilization of lipase on chitosan beads (Working Conditions: 20 ^{0}C/24 h. incubation, pH 6.0, no salt addition), (Lines correspond to the Langmuir isotherm).

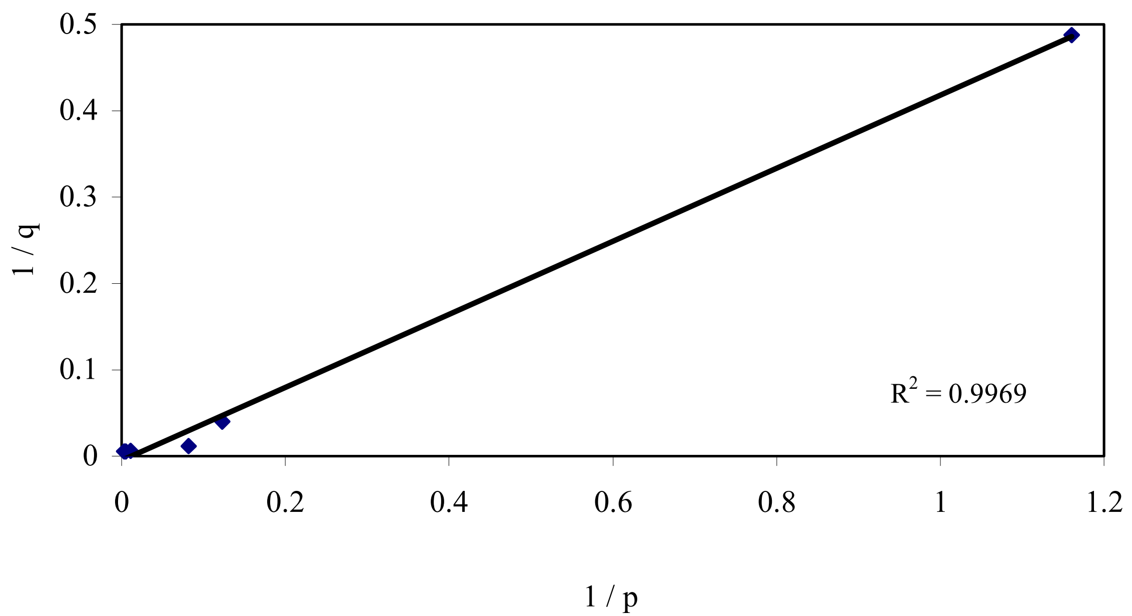


Figure 5.20. Reciprocal plot of the Langmuir isotherm.

As seen in Figure 5.20, data obtained for the lipase/chitosan system fitted well to the Langmuir isotherm where K=72.5 U/ ml solution and q_m=200 U/ml support. The correlation coefficients obtained for the different isotherms considered can be seen in Table 5.2.

Table 5.2. Isotherm constants for the lipase/chitosan system (20 ^{0}C).

Isotherm Type-R^2 value	Isotherm Type-R^2 value	Isotherm Type-R^2 value	Selected Isotherm	q_m (U/ml support)	K (U/ml solution)
Linear 0.569	Freundlich 0.8647	Langmuir 0.9969	Langmuir	200	72.5

5.5. Storage Stability of Immobilized Lipase

Stability of immobilized enzyme preparation is important to enhance the usability in a longer period of time. In order to find the storage stability, immobilized lipase was kept at 4 ^{0}C in 4 ml. 0.025 M phosphate buffer, pH 6.0 and also in dry conditions, being kept in aluminum papers.

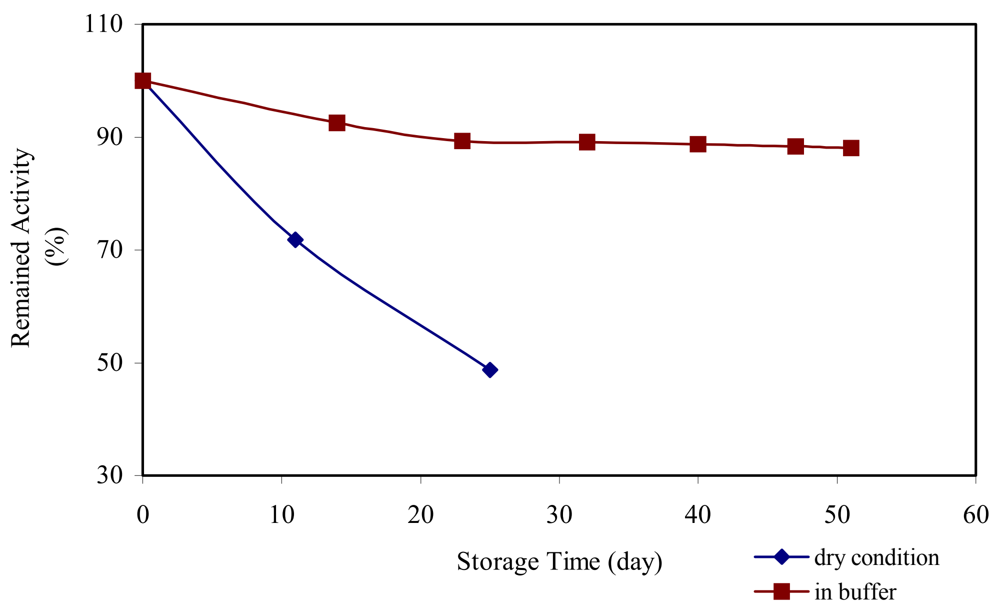


Figure 5.21. Storage stability of chitosan immobilized lipase.

As seen in Figure 5.21, immobilized enzyme was more stable when kept in buffer solutions. After a month storage, nearly 90 % of its original activity was maintained while this value was only 40 % when immobilized enzyme preparations were stored in dry state.

Bastida et al. (1998) investigated the stability of lipase/octyl-sepharose system at 25 ^{0}C. Stability varied depending on the type of lipase source while the most stable system expressed 70 % of the initial activity after an incubation period of 15 days in aqueous media. Variation in stability was explained by the differences in the stability of soluble forms of enzyme.

The storage stability of *Candida rugosa* lipase immobilized on polymer beads of varying hydrophobicity was investigated by Basri et al. (1994a, 1994b). All lipase forms were stored in benzene at room temperature and finally, it was found that soluble lipase lost its activity completely in 8 days time. However, Amberlite XAD2 (most hydrophobic) immobilized lipase kept 50 % of its original activity at the same period. These results prove the fact that immobilization process increases the stability of enzymes.

Arıca et al. (2001) investigated the storage stability of immobilized lipase in 0.1 M phosphate buffer, pH 7.0 at 4 ^{0}C. After a period of 56 days, immobilized preparation lost only 24% of its original activity.

In another study conducted by Fadıloğlu (1996), celite immobilized *Candida rugosa* lipase retained about 56 % of the initial activity during the course of 6 months.

Consequently, it can be said that stability of soluble or immobilized lipases depends on the enzyme source, support type and environmental conditions such as pH, temperature and presence/absence of buffer solutions.

5.6. Hydrolysis Reaction

It is known that activity of enzymes depends on the substrate type and also its concentration in substrate solution. Therefore, in this section, results of corresponding experiments are presented.

5.6.1. Effect of substrate type

Three different type of substrates, namely olive, sunflower and corn oils were tested for hydrolysis efficiency of free and chitosan immobilized lipases.

As seen in Figure 5.22, free lipase showed the highest activity when olive oil is used as substrate and showed the lowest activity against corn oil among the substrates investigated. These results can be explained with the chain length specificity of lipases. As mentioned before, specificity of *Candida rugosa* lipase can be listed as oleic > lauric > palmitic > myristic > stearic acids. The fatty acid compositions of different oils are listed in Table 5.3.

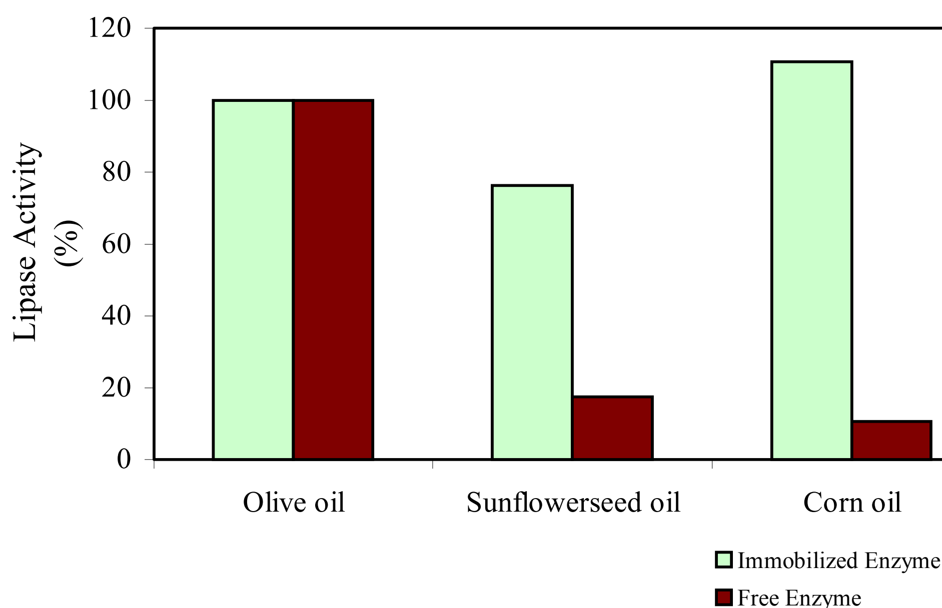


Figure 5.22. Effect of substrate type on free and chitosan immobilized lipase activity (Hydrolysis reaction carried out at 20 ^{0}C).

Table 5.3. Fatty acid composition of some oils (Telefoncu, 1993; Watson et al., 1987; Gümüşkesen, 1993).

Type of oil	Oleic acid	Palmitic acid	Stearic acid	Linoleic acid	Palmitoleic acid	Linoleic + Oleic acids
Sunflower oil	29 %	11 %	6 %	52 %	-	81 %
Olive oil	64 %	14 %	2 %	16 %	2 %	80 %
Corn oil	24.1 %	11 %	2 %	61.9 %	-	86 %

If the oleic acid contents of these oils were compared, it was seen that olive oil had the highest oleic acid content, which was followed with the sunflower and corn oils. Since *Candida rugosa* lipase firstly attached to oleic acid, it was natural to obtain a higher yield of activity towards the olive oil. However, comparison of fatty acid composition of these oils is not enough to state the conclusion since the position of fatty acid in the structure primarily affects its hydrolysis. It is known that the most stable form is at β-position. It is a general characteristic property of vegetable oils that the saturated fatty acids are located at α-position while the monounsaturated and polyunsaturated fatty acids are dominant at β-position (Hui, 1996). The distribution of oleic acid in α- and β- positions of triglycerides in sunflower, olive and corn oils can be seen in Table 5.4.

Table 5.4. The distribution of oleic acid in α-, β- and α^1- positions of triglyceride structure in sunflower, olive and corn oils (Tekin, 1997)

Position	Sunflower Oil	Corn Oil	Olive Oil
α	16.49	22.26	38.6
β	33	36.2	33.46
α^1	50.51	41.54	27.9
$\alpha+\alpha^1$	67	63.8	66.5

In this table, it is seen that oleic acid can be located in both positions of triglyceride molecule. The comparison of the distribution of oleic acid among the

positions available shows that oleic acid is mainly located at the position of α and α^1 which are the less stable forms, therefore, enzyme can easily break these bonds down. The oleic acid content of sunflower and olive oil at these positions are nearly equal while it was less than others in the case of corn oil. If the Table 5.3. and 5.4. are examined together, the following conclusions can be stated:

- The oleic content: olive oil > sunflower oil > corn oil.
- The percentage of oleic acid at the α and α^1 positions: sunflower oil> olive oil> corn oil.

The reason for the achievement of relatively low hydrolysis yield with corn oil can clearly be seen from the conclusions listed above. However, in the case of sunflower oil, the statement was somewhat complicated. Although the distributions of oleic acid in available positions were nearly equal with olive oil, the hydrolysis ratio was obviously less than olive oil. This may have been resulted from the low oleic acid content of sunflower oil than olive oil.

In the case of immobilized lipase, highest activity was obtained with corn oil and lowest with sunflower oil. When the percentages of hydrolysis were compared, it was found that sunflower oil had 76.2 % and corn seed oil had 110.753 % activity of that of olive oil's. These results were in contrast with the fatty acid compositions of oils investigated. However, it was known that immobilization process leads to changes in enzyme structure. Therefore, it can be concluded that immobilization lead to changes in fatty acid specificity of *Candida rugosa* lipase.

5.6.2. Effect of Substrate Concentration

In order to find the optimum substrate concentration, different amounts of olive, sunflower and corn oil was added to hydrolysis medium.

In Figure 5.23, effect of olive oil concentration can be seen with comparison of free and chitosan immobilized lipase activity. Optimum substrate concentrations for maximum hydrolysis for free enzyme and for immobilized enzymes were 3 ml and 1 ml, respectively.

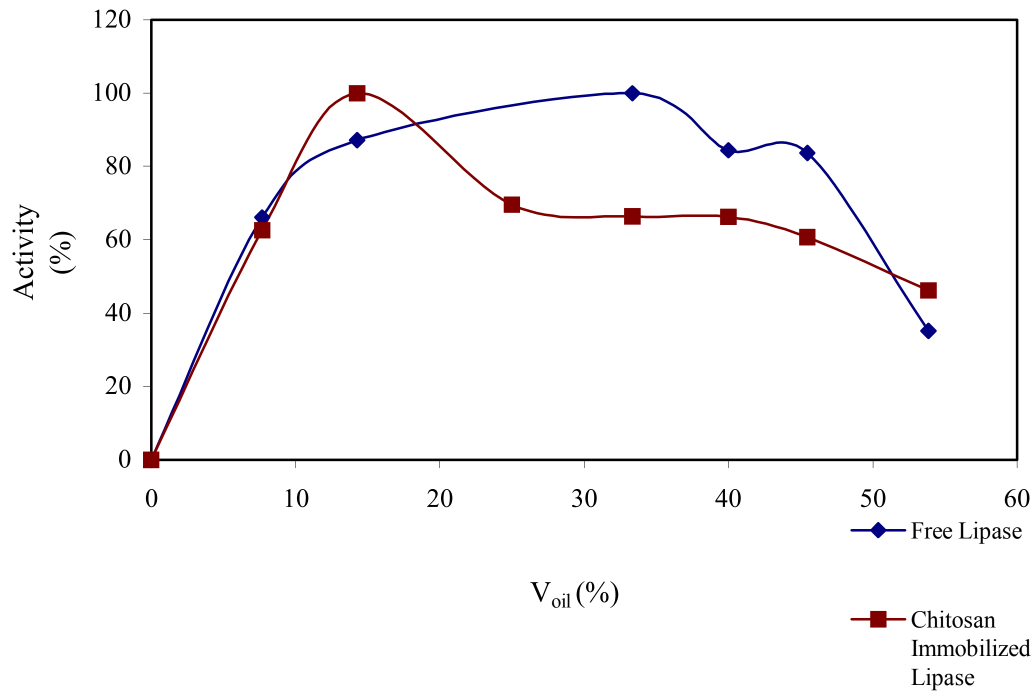


Figure 5.23. Effect of substrate (olive oil) concentration on free and chitosan immobilized lipase activity (Hydrolysis reaction carried out at 20 ^{0}C).

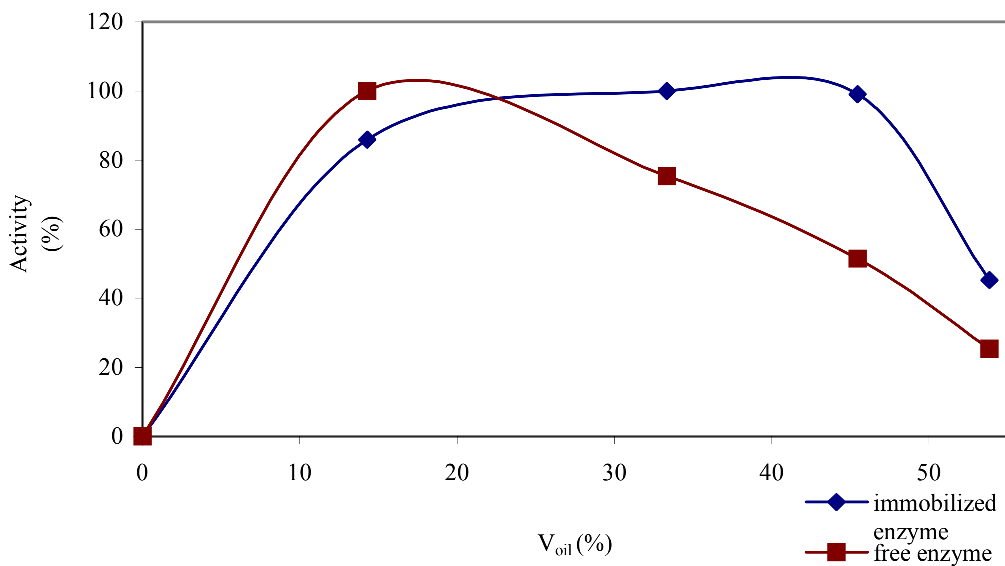


Figure 5.24. Effect of substrate (sunflower oil) concentration on free and chitosan immobilized lipase activity (Hydrolysis reaction carried out at 20 ^{0}C).

In Figures 5.24 and 5.25, effects of sunflower and corn oil concentration can be seen, respectively. In sunflower oil hydrolysis, optimum substrate concentration was found to be 3 ml for immobilized lipase and 5 ml for free enzyme. Maximum hydrolysis of corn oil with immobilized lipase was enhanced with 5 ml substrate addition for immobilized and free enzyme.

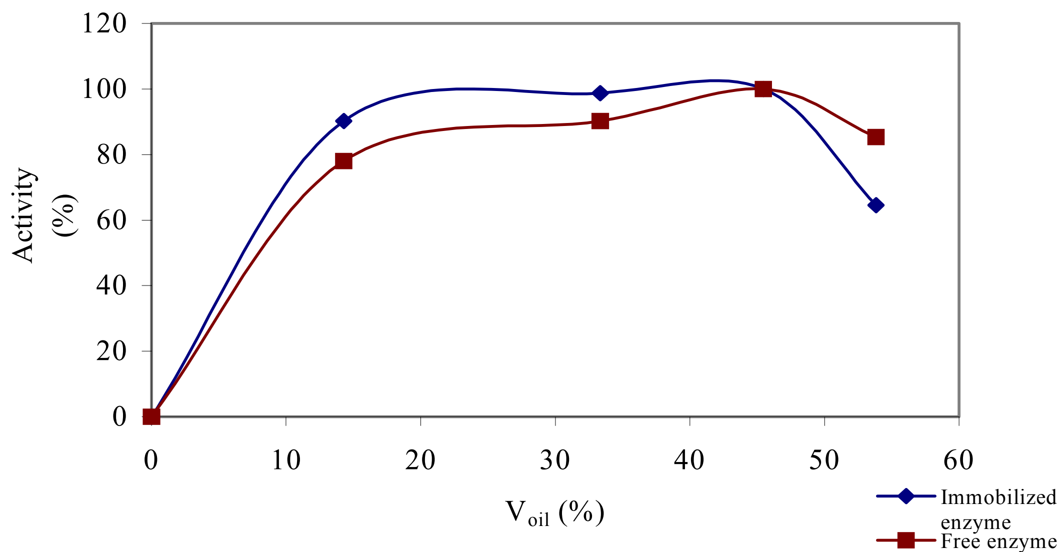


Figure 5.25. Effect of substrate (corn oil) concentration on free and chitosan immobilized lipase activity (Hydrolysis reaction carried out at 20 ^{0}C).

Amount of substrate required for the expression of maximum activity was in small extend for immobilized enzyme than that required for soluble enzyme except corn oil. The reason for the decreased amount of olive oil for immobilized enzyme may be the change in the configuration of enzyme upon immobilization. It was also observed that the amount of substrate required changes with respect to the substrate type.

Chapter 6

CONCLUSION

The aim of this study was to investigate the optimum immobilization conditions of lipase from *Candida rugosa* on a hydrophobic and a hydrophilic support and also to investigate the effect of different substrates on the hydrolysis reaction. Results showed that immobilization conditions change with the enzyme structure and also by the type of the support. Moreover, hydrolysis ratio of substrate depends on the substrate type and the concentration in substrate solution.

Prior to the immobilization studies, the stability ranges of soluble enzyme was investigated since the stability of *Candida rugosa* lipase provides key information for choosing the operating conditions for immobilization process. pH stability tests showed that lipase is very stable in the pH range of 6.0-7.0 and the full activity is remained after 24 hours. Below and above this pH range, the activity decreases and nearly 70 % of enzyme activity was lost at the pH values of 4.0 and 9.0. The reason of such a loss is the denaturation of enzyme at extremes of pH range. It was indicated that for most of the enzymes the variation of activity with pH, within a range of two or three pH units on each side of the isoelectric points, is normally a reversible process. However, extremes of pH would cause irreversible denaturation.

The temperature stability experiments showed that, lipase was stable when stored at temperature range of 4-37 ^{0}C over a period of 24 hours in pH 7.0 buffers with 0.5 M NaCl. However, 5 % and 70 % of the activity was lost at 40 and 50 ^{0}C, respectively. This is again mainly due to the denaturation of the enzyme structure at high temperatures.

Another factor that affects the enzymatic activity is ionic strength. In spite of long incubation period, 100 % of lipase activity was maintained in the solutions containing 1.0 M NaCl, while lower and higher salt concentrations led to about 60 % activity loss. This can be explained by the activating effect of Na^+ ions on the lipase enzyme and therefore as Na^+ ions increases in the solution up to 1.0 M, higher activity of lipase remains.

After the establishment of the stability ranges of soluble *Candida rugosa* lipase, immobilization studies were carried out in order to find out the optimum conditions for immobilization.

Acidity of the solution is an important parameter for immobilization. Maximum yield was obtained with 0.025 M phosphate buffer with pH 7.0 for hydrophobic support and with pH 6.5 for hydrophilic support where the free enzyme had a pH optimum of 6.5. Therefore, it was concluded that optimum pH for maximum yield of immobilized enzyme was related with the optimum pH of soluble enzyme. In addition to this fact, hydrophobic and ionic interactions change with respect to the pH. At highly basic solutions, hydrophobic interaction is decreased. Also, it is known that a protein with no net charge (at pI) will have maximum hydrophobicity, but at pH where protein and adsorbent have similar charges, repulsion may occur, which will lead to a reduction in interaction. However, the ionic and hydrophilic nature of chitosan suggests that adsorption of lipase is governed by ionic interactions. As such, these interactions are greatly affected by changes in the pH. Also, binding of the enzyme to the positively or negatively charged resin of the matrix occurs via electrostatic forces between opposite charges of the ligand and enzyme respectively. Therefore, optimum pH for maximum immobilization yield on hydrophobic or hydrophilic support depends on the support and the enzyme characteristics.

It should also be noted that results obtained for lipase/chitosan system were different from lipase/octyl-sepharose system. At pH 8.0 and 9.0, there was no lipase bound to the chitosan while around 70 % immobilization yield was achieved for octyl-sepharose at indicated pH values. Therefore, it can be said that lipase/chitosan system is more sensitive to pH changes than lipase/octyl-sepharose system. This is mainly because the hydrophobic interactions are not greatly influenced by changes in the pH of solution. However, if electrostatic forces are important for the adsorption, changes in pH over the isoelectric point of the protein will have a large impact on the protein binding constant (Gitlesen et al., 1997).

Optimum temperature was found to be 37 ^{0}C for hydrophobic and 20 ^{0}C for hydrophilic support. Soluble enzyme was fully active up to 37 ^{0}C and immobilized enzyme ratio had not changed on a great extend up to this temperature. Therefore, as in the case of the pH effect, it was observed that optimum temperature for immobilization on octyl-sepharose and chitosan depended to the properties of the enzyme. It should also be noted that chitosan changed its structure by drying at higher temperature than

30 ^{0}C and it was not possible to work at these temperatures. In fact, hydrophobic interaction is an entropy-driven process and, thus, it increases with temperature. Therefore, it can be said that, hydrophobic interaction was considerably enhanced at high temperatures causing rapid adsorption of the enzyme. However, even higher temperatures lead to the denaturation of the enzyme. Adsorption is an exothermic process; therefore, the adsorptivity is expected to decrease with increasing temperature. It should also be noted that the temperature can influence adsorption, but this is usually not readily apparent in systems of interest where adsorption is normally strong and temperature ranges are relatively small.

Salt concentration was especially important to define the type of interaction between the enzyme and the support. In this study, maximum yield was obtained with the usage of 0.5 M NaCl solution in immobilization media for hydrophobic support and higher concentrations did not lead to sharp decreases in immobilized enzyme ratio. However, increased salt concentration decreased the immobilization of lipase on chitosan and maximum immobilized enzyme yield was obtained when there was no salt in the immobilization media. This meant that this support was not hydrophobic in character. Additionally, for maximum activity, soluble lipase required 1.0 M NaCl. The presence of the salts increases the free energy of the proteins and enzyme molecules and, thus, these molecules try to minimize the increase in free energy by decreasing the hydrophobic contact area of the enzyme with the polar solvent media. Therefore, as the ionic strength increases, binding of the enzyme to hydrophobic support increases because it minimizes the surface contact area of enzyme and support with the salt solvent medium and produces a minimum increase in free energy. So, in a medium of high salt concentration the bound form of enzyme is thermodynamically more stable than the unbound protein. This explains the enzyme binding to hydrophobic surfaces at high salt concentration. However, experimental results showed that interaction between lipase/octyl-sepharose is very special since it is elevated at low ionic strength, therefore, it is concluded that adsorption of lipases on Octyl-Sepharose at low ionic strength seems to be very selective and also follow a very specific mechanism of adsorption. In the case of ionic interactions, effect of salt is quite different. Increasing the total number of ions present in the solution decreases the strength of binding of each individual ion by increasing the competition between ions for binding sites. An increase in ionic strength, therefore, decreases interactions between the enzyme molecules and the support and this leads to reduced immobilization yield.

Consequently, it was concluded that the most effective parameter on immobilization process was ionic strength, which was also concluded by various investigators.

Ratio of the amount of enzyme solution to the support added resulted that when the gel to liquid ratio is 0.1, immobilized enzyme yield reached to maximum for all supports.

Kinetic studies showed that affinity of lipase towards hydrophobic support is higher than that of hydrophilic. Immobilization of lipase on octyl-sepharose reached to a plateau value maximum in 10 minutes time, while it took 24 hours for chitosan. This result also led to a conclusion that the loading of lipase on octyl-sepharose was initially localized near the outer surface of the support instead of being uniformly distributed throughout the pores of the support. Affect of temperature on enzyme kinetics was not very clear in this case but, generally, it was observed that, increasing temperature decreased the time required to reach the equilibrium. The amount of the activity adsorbed by octyl-sepharose increased very rapidly in a few minutes and reached a plateau after a longer period of incubation. Time required to reach the saturation is related with the affinity of the support towards the support. Octyl- sepharose is a highly hydrophobic support and it is known that affinity of lipase is high towards hydrophobic supports. Therefore, immobilization on hydrophobic support was completed in a shorter period of time with respect to the hydrophilic support.

Effect of enzyme loading on lipase immobilization on octyl-sepharose was investigated at two different temperatures. At 20 ^{0}C, in the studied range of enzyme concentration (3.05-916.5 U/ml), the experimental data well fitted to the Linear isotherm with the C value of 138.2. Also, at 4 ^{0}C, results were similar and the experimental data fitted to the Linear isotherm with the C value of 209.1. In the case of chitosan, data fitted to the Langmuir isotherm where the constants were calculated as q_m=200 U/ml support and K= 72.5 U/ml solution. The Langmuir model implied the formation of lipase monolayer on the support surface. Thermodynamically, it indicated an energetically homogeneous surface where all sites were identical. In Table 6.1., the isotherms and constants obtained in this work can be seen together.

The equilibrium behavior of the lipase/support in the immobilization systems depends on the physical and structural properties of the support, and the physical and chemical properties of the lipase. Contrary to the belief that the equilibrium behavior of lipase adsorption would generally conform to the Langmuir model, an insight analysis

of the equilibrium behavior showed that the lipase distribution on the support surface could be described by the Freundlich or Linear models.

Table 6.1. Isotherm constants for the immobilization systems studied.

Support Type	Temperature (^0C)	Isotherm Type	R^2 value	C	q_m (U/ml sup.)	K (U/ml sol.)
Chitosan	20	Langmuir	0.9969	-	200	72.5
Octyl-Sepharose	20	Linear	0.9952	138.22	-	-
	4	Linear	0.9566	209.11	-	-

The maximum immobilization ratios and equilibrium times of different lipase solution concentrations are briefly listed in Table 6.2. Under optimum conditions indicated above, 88.5 % of lipase can be immobilized on octyl-sepharose with the activity of 410 U/ml support and 44 % of lipase can be bound to chitosan with the enzymatic activity of 190 U/ml support.

Storage stability of immobilized lipase was investigated in two different conditions. When the chitosan immobilized lipase was kept at 4 ^0C under dry conditions, it lost 50 % its original activity in 25 days time. However, after 60 days of storage, it lost only 10 % of its activity when kept in pH 6.0, 0.025 M phosphate buffer at 4 ^0C. Therefore, it can be concluded that the stability of immobilized lipases depends on the enzyme source, support type and environmental conditions such as pH, temperature and presence/absence of buffer solutions.

In addition to immobilization studies, effect of substrate type and concentration was also in the scope of this study. Olive, sunflower and corn oils were tested for hydrolysis efficiency and hydrolysis ratio of free lipase was highest when olive oil was used as substrate. It was followed with sunflower and corn oil. These results may have been resulted from the fatty acid specificity of *Candida rugosa* lipase. This lipase firstly attaches to the oleic acid and oleic acid content of tested oils can be listed as olive>sunflower>corn oils. However, the results were different from soluble; highest activity was observed against corn oil and it was followed with olive and sunflower oils. This difference may have been resulted from the change in the enzyme structure upon immobilization. Substrate concentrations for maximum hydrolysis for free enzyme were

3 ml olive oil, 5ml sunflower and corn oil. In the case of chitosan immobilized lipase, these values changed as 5 ml for corn oil, 3 ml sunflower oil and 1 ml. for olive oil.

Table 6.2. Comparison of equilibrium times and maximum immobilization ratios of different concentrations of lipase solutions during immobilization.

OCTYL-SEPHAROSE	Concentrations and temperatures								
Time	0.25 mg/ml (4 ^{0}C)	0.25 mg/ml (20 ^{0}C)	2.5 mg/ml (4 ^{0}C)	5 mg/ml (4 ^{0}C)	5 mg/ml (20 ^{0}C)	15 mg/ml (4 ^{0}C)	25 mg/ml (4 ^{0}C)	25 mg/ml (20 ^{0}C)	75 mg/ml (20 ^{0}C)
20 sec.	20 %	45.2 %	47 %	49.3 %	80 %	79 %	79 %	88 %	88 %
2 min.		60.27 %						94.6 %	
4 min.					91 %				
5 min.				94.2 %		96 %			
7 min.									93 %
8 min.							96 %		
10 min.	68.5 %	60.3 %	93 %	94.2 %	91 %	96 %	96 %	94.6 %	93 %
CHITOSAN	Concentration and temperature								
Time	5 mg/ml								
24 h.	40 %								

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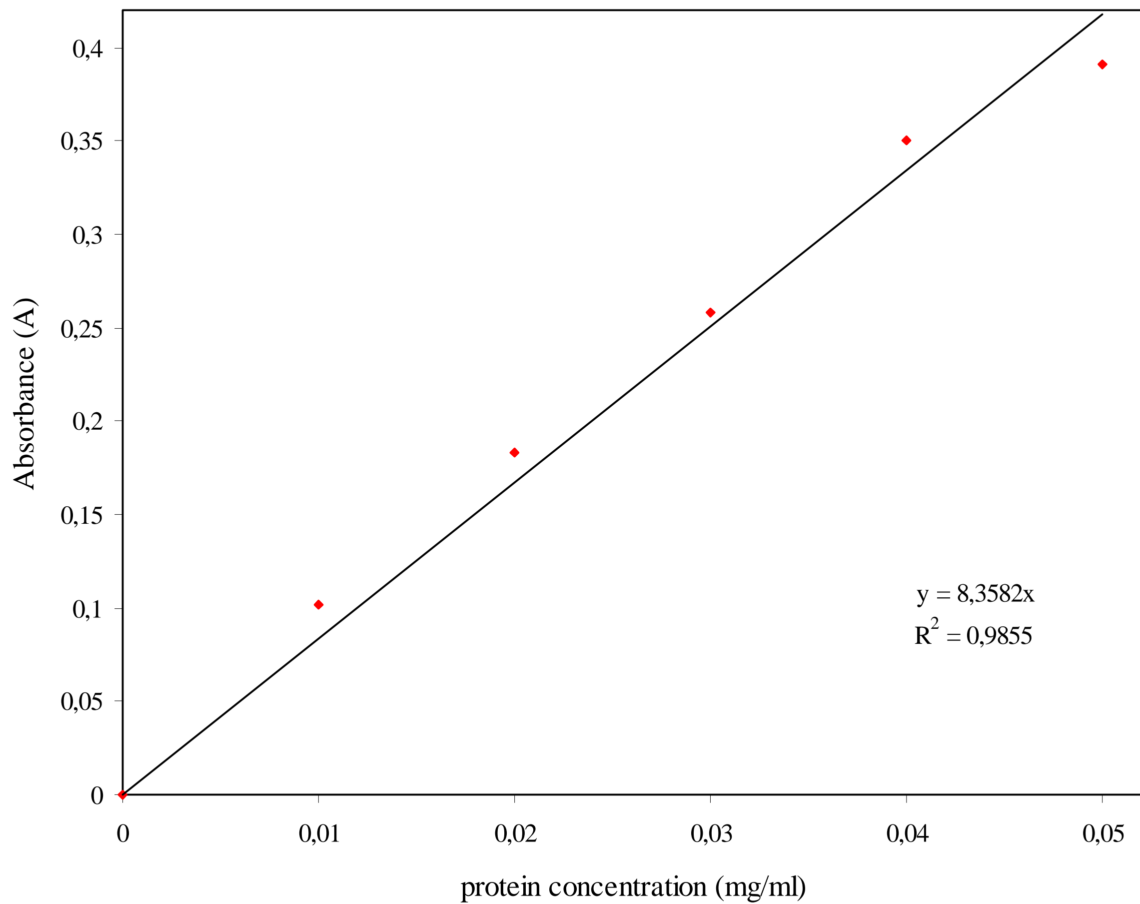
APPENDIX

A.1. Protein Determination Calibration Curve

During the determination of protein content of *Candida rugosa* lipase, following calibration was used with the regression coefficient of 0.9855.



A.2. Enzyme Activity Calculations

As mentioned in Section 4.2.2., enzyme activity was calculated based on following formula:

$$A = \frac{\text{Normality of NaOH} * 1000 * \text{Amount of NaOH expended (ml)}}{\text{Amount of Enzyme (mg)}}$$

As an example; if our enzyme solution contained 5 mg enzyme and amount of NaOH expended was 0.77 ml;

$$A = \frac{0.05 * 1000 * 0.77}{5}$$

A = 7.7 U / mg enzyme

It should be noted that during analysis, normality of NaOH was 0.05 in each case.