DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IMMOBILIZATION OF CATALASE ONTO CLAYS

by
Sevilay CENGİZ

January, 2007
İZMİR

IMMOBILIZATION OF CATALASE ONTO CLAYS

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of Dokuz Eylül University
In Partial Fulfillment of
the Requirements for the Degree of Master of Science
in Chemistry

by
Sevilay CENGİZ

January, 2007
İZMİR

M. Sc. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"IMMOBILIZATION OF CATALASE ONTO CLAYS "** completed by **SEVİLAY CENGİZ** under supervision of **Prof. Dr. Kadir YURDAKOÇ** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

.....

Prof. Dr. Kadir YURDAKOÇ

Supervisor

....

(Jury Member) (Jury Member)

Prof. Dr. Cahit HELVACI

Director

Graduate School of Natural and Applied Science

ACKNOWLEDGMENTS

I would like to thank my supervisor, Prof. Dr. Kadir YURDAKOÇ, for his encouragement, guidance and support.

Also, I would like to thank to Assistant Prof. Dr. Levent ÇAVAŞ for his precious suggestions and discussions. I am deeply grateful to him.

In addition, I wish to express my gratefulness to all my friends for their continuous contributions and valuable supports.

Additionally, I owe great thanks my all teachers.

Finally, I would like to thank my family for bringing me in this situation with their unique patience and supports.

Sevilay CENGİZ

IMMOBILIZATION OF CATALASE ONTO CLAYS

ABSTRACT

The immobilization characteristics of catalase onto bentonite and sepiolite were investigated and the relative activities of free and immobilized catalases were examined under varying experimental conditions. The effects of various factors such as concentration of catalase solution, pH, temperature and ionic strength were observed on both immobilization ratio and its relative activity. Experiments were done on free and immobilized catalases concerning the determination of thermal stability, storage stability, operational stability and kinetic parameters. It was determined that optimum temperature, pH and ionic strength for free and immobilized catalases are 35ºC, pH 7 and 50 mM (for free catalase) and 35ºC, pH 8 and 50 mM (for immobilized catalases), respectively. The increased resistance of the immobilized catalase against the changes of temperature, pH and ionic strength indicates that immobilizing catalase onto bentonite and sepiolite is useful. It was found K_m=116.8 mM, V_{max}=30.1 μmol/min.mg protein for free catalase, K_m=55.6 mM, V_{max}=6.6 μmol/min.mg protein for bentonite supported catalase and K_m=49.2 mM, V_{max}=8.7 μmol/min.mg protein for sepiolite supported catalase, respectively. It was observed that the storage stabilities of immobilized catalases were better than free catalase, and immobilized catalases showed an operational stability. By using Langmuir equation, maximum adsorption capacities (q_m) of bentonite and sepiolite were obtained. In conclusion, clay materials can be alternative and low cost material for catalase immobilization.

Keywords: immobilization, adsorption, catalase, bentonite, sepiolite, clay, adsorption isotherm

ÇEŞİTLİ KİLLER ÜZERİNE KATALAZ İMMOBİLİZASYONU

ÖZ

Bentonit ve sepiolit üzerine katalaz immobilizasyon karakteristikleri incelenerek, değişen deney koşulları altında serbest ve immobilize katalazın bağıl aktivitesi belirlenmiştir. İmmobilizasyon oranı ile bağıl aktivite üzerine katalaz konsantrasyonu, pH, sıcaklık ve iyon şiddeti gibi değişkenlerin etkisi incelenmiştir. Serbest ve immobilize katalaz için termal ve operasyonel dayanıklılık ile depolama kararlılığı incelemeleri ve kinetik değerlendirmeler yapılmıştır. Optimum koşullar; serbest katalaz için 35ºC, pH=7 ve 50 mM ve immobilize katalaz için ise 35ºC, pH= 8 ve 50 mM iyon şiddeti olarak belirlenmiştir. İmmobilize katalazın sıcaklık, pH ve iyon şiddeti değişimlerine karşı daha dayanıklı olması, bentonit ve sepiolit üzerine katalaz immobilizasyonunun uygun olduğunu göstermektedir. K_m ve V_{max} değerleri serbest katalaz, bentonit immobilize katalaz ve sepiolit immobilize katalaz için sırasıyla 116,8 mM, 55,6 mM ve 49,2 mM ile 30,1 μmol/min.mg protein, 6,6 μmol/min.mg protein ve 8,7 μmol/min.mg protein olarak bulunmuştur. Bununla birlikte immobilize katalazın depolama kararlılığının, serbest katalazdan daha yüksek olduğu ve operasyonel kararlılık gösterdiği belirlenmiştir. Langmuir eşitliği kullanılarak, bentonit ve sepiolit için maksimum adsorpsiyon kapasiteleri (q_m) mg g^{-1} olarak elde edilmiştir. Sonuç olarak, kil materyali katalaz immobilizasyonu için düşük maliyetli, alternatif bir materyal olarak önerilebilir.

Anahtar sözcükler : immobilizasyon, adsorpsiyon, katalaz, bentonit, sepiolit, kil, adsorpsiyon izotermleri

CONTENTS

Page

THESIS EXAMINATION RESULT FORM ii
ACKNOWLEDGEMENTS iii
ABSTRACT iv
ÖZ v

CHAPTER ONE – INTRODUCTION 1

1.Immobilization 1
1.1 The Adsorption-Desorption Processes 3
1.1.1 What is Adsorption 3
1.1.2 How do Molecules Bond to Adsorbent? 5
1.1.3 Which Factors Influence Adsorption? 7
1.1.4 Adsorption Isotherms 7
1.1.4.1 Langmuir Isotherm 8
1.1.4.2 Freundlich Isotherm 11
1.1.4.3 Dubinin – Radushkevich (DR) Isotherm 12
1.1.5. The Desorption Process 13

CHAPTER TWO – CLAYS 14

2. Introduction to Clays 14
2.1 Description of Clay 14
2.2 Classification of the Clay Minerals 15
2.3 Structure of the Clay Minerals 16
2.4 Uses of Clays 21

2.5 Properties of Clays 22
2.5.1 Ion Exchange 22
2.5.2 Cation- Exchange Capacity (CEC) 23

2.6 Identification of Clay Minerals 24
2.6.1 Thermal Analysis 24
2.6.2 Surface Area Determination 24
2.6.3 XRD Analysis 24

CHAPTER THREE – ENZYMES 26

3.1 Enzymes 26
3.1.1 Apoenzyme 26
3.1.2 Cofactors 26
3.1.3 Final Enzyme 27

3.2 The Michaelis-Menten Mechanism of Enzyme Catalysis 28

3.3 Enzyme Nomenclature and Classification 30
3.3.1 Oxidoreductase 30
3.3.2 Transferase 30
3.3.3 Hydrolase 31
3.3.4 Lyase 31
3.3.5 Isomerase 31
3.3.6 Ligase 31

3.4 Enzyme Activity 32

3.5 Enzyme Immobilization 32

CHAPTER FOUR – CATALASE 38

4.1 Catalase 38

4.2 Where is Catalase found? 38

4.3 Catalase Structure 40

4.4 Catalase Reaction Mechanism 41

4.5 Catalase Activity 41

4.5.1 UV Method 42

4.6 Which Factors Affect the Activity of Catalase 43

4.6.1 Substrate Concentration 43

4.6.2 Temperature 44

4.6.3 pH 44

4.6.4 Salt Concentration 44

4.6.5 Presence of Inhibitors 44

4.6.6 Presence of Activators 46

4.7 Immobilized Catalase in Industrial Field 46

4.8 Why do We Choose Clay for Immobilization 47

CHAPTER FIVE - MATERIAL AND METHOD 50

5.1 Clay and Catalase 50

5.2 Surface Area Measurement 51

5.3 FTIR Study 51

5.4 SEM Measurements 51

5.5 Adsorption from Solution Experiments: Determination of Equilibrium Concentration 52

CHAPTER SIX – RESULTS 54

6.1 Determination of Optimum Conditions for Free and Immobilized Catalase 54

6.2 Determination of Optimum Conditions for Immobilization 56

6.3 Km and Vmax Values for Free and Immobilized Catalase 58

6.4 Thermal Stability of Free and Immobilized Catalase 60

6.5 Operational Stability of Immobilized Catalase 63

6.6 Storage Stability of Free and Immobilized Catalase 64

6.7 The Effect of Contact Time on The Immobilization of Catalase onto Bentonite and Sepiolite 66

6.8 Immobilization of Catalase onto Bentonite and Sepiolite 68

6.9 Adsorption Kinetics 75

6.10 Thermodynamic Parameters of Adsorption 78

6.11 SEM Analysis 81

CHAPTER SEVEN – CONCLUSION..... 84

7. Conclusion..... 84

REFERENCES..... 89

CHAPTER ONE
INTRODUCTION

1. Immobilization

Immobilization means limiting the motions.The various immobilization protocols used with enzymes have been extensively studied (Kennedy & Cabral, 1983). Immobilization of enzyme consists of four different methods: adsorption of the enzyme on a carrier, entrapment of the enzyme within an insoluble gel matrix, containment of enzyme within porous hollow fibers or microcapsules, binding of the enzyme to dried mycelia and ion exchange between the enzyme and support (Malcata, Reyes, Garcia, Hill, & Admundson, 1990). Physical adsorption of an enzyme onto a solid is probably the simplest way of preparing immobilized enzymes. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. A major advantage of adsorption is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap and easily carried out.

Confining enzymes within the lattices of polymerized gels is another method for immobilization. This allows the free diffusion of low molecular weight substrates and reaction products. The usual method is to polymerize the hydrophilic matrix in an aqueous solution of the enzyme and break up the polymeric mass to the desired particle size. As there is no bond formation between the enzyme and the polymer matrix, occlusion provides a generally applicable method that, in theory, involves no disruption of the protein molecules. However, free radicals generated on the course of the polymerization may affect the activity of entrapped enzymes. Another disadvantage is that only low molecular weight substrates can diffuse rapidly to the enzyme, thus making the method unsuitable for enzymes that act on macromolecular substrates.

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Cross-linking an enzyme to it is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support, resulting in relatively low enzyme activity.

The most intensely studied of the insolubilization techniques is the formation of covalent bonds between the enzyme and the support matrix. When trying to select the type of reaction by which a given protein should be insolubilized, the choice is limited by the fact that the binding reaction must be performed under conditions that do not cause loss of enzyme activity, and the active site of the enzyme must be unaffected by the reagents used.

When immobilizing an enzyme on a surface it is most important to choose a method of attachment aimed at reactive groups outside the active catalytic and binding site of that enzyme. Considerable knowledge of active sites of particular enzymes will enable methods to be chosen that would avoid reaction with the essential groups therein. Alternatively, these active sites can be protected during attachment as long as the protective groups can be removed without loss of enzyme activity. In some cases, this protective function can be fulfilled by a substrate of the enzyme or a competitive inhibitor; this also contributes towards retention of tertiary structure of the enzyme.

Among the several methods of immobilization, adsorption (resulting from both hydrophobic and hydrophilic interactions) is the method which induces fewer modifications on the active conformation of the enzymes. Consequently, adsorption is one of the preferred methods for enzyme immobilization (Fuentes, Viseras, Ubiali, Terreni, & Alcántara, 2001). In this method, the forces between a support and the enzymes include hydrogen bonding, van der Waals forces and hydrophobic interactions (Burns, 1986).

1.1 The Adsorption – Desorption Process

1.1.1 What is Adsorption?

The use of solids for removing substances from either gaseous or liquid solutions has been widely used since biblical times. This process, known as *adsorption,* involves nothing more than the preferential partitioning of substances from the gaseous or liquid phase onto the surface of a solid substrate. From the early days of using bone char for decolourization of sugar solutions and other foods, to the later implementation of activated carbon for removing nerve gases from the battlefield, to today's thousands of applications, the adsorption phenomenon has become a useful tool for purification and separation (Marczewski, 2002).

Adsorption phenomena are operative in most natural physical, biological, and chemical system, and adsorption operations employing solids such as activated carbon and synthetic resins are used widely in industrial applications and for purification of waters and wastewaters (Marczewski, 2002).

The interface of two different phases is always anisotropic. That is, in the interface the molecular interactions in one side differ from interactions in the other side. If the molecules in a phase are mobile (e.g., gas – solid or liquid – solid interfaces), then these different molecular interactions cause a concentration difference between the interface and bulk phase of mobile molecules. This type of change in concentration taking place in the interface is called *adsorption*. This is the reason why the interface is often referred to as the adsorption space or adsorbed phase. If the mobile molecules can penetrate into the bulk of the other phase, then this process is called *absorption*. It is sometimes difficult or impossible to distinguish between adsorption and absorption; it is then convenient to use the wider term *sorption*. The name of the phase with localized (non-mobile) molecules is the *adsorbent*, and the mobile molecules bound on the adsorbent surface called *adsorbate*. The phase with mobile molecules before the adsorption, i.e., the phase that is capable of being adsorbed is named adsorptive. So the whole process of

adsorption can be expressed with the following simple and symbolic relationship (Adamczyk, 2002).

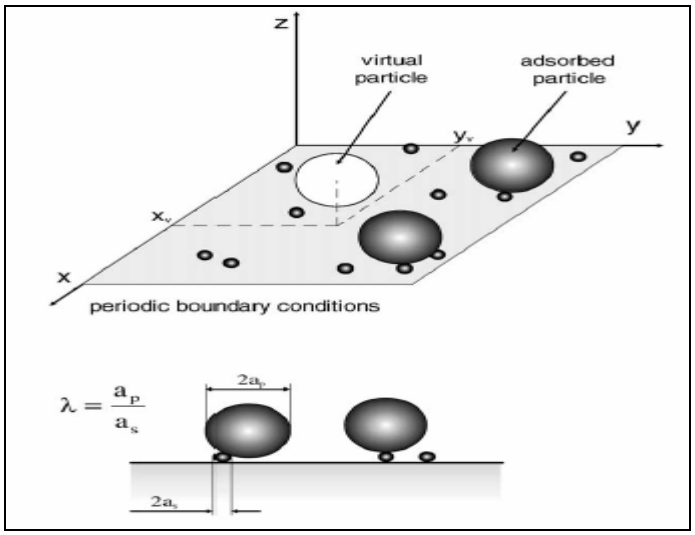


Figure 1.1 A schematic representation of particle adsorption modeling over heterogeneous surfaces bearing spherically shaped adsorption sites. (Adamczyk, Siwek, Weroński, & Musiał, 2002).

Adsorbents are often characterized first by their surface properties such as specific surface area and pore size distribution. The pore size distribution provides information on the size and amount of pores present in the solid adsorbent: Macropores are pores with widths exceeding about 50 nm. Mesopores are pores widths between 2 nm and 50 nm. Micropores are pores with widths not exceeding about 2 nm (Toth, 2002).

A large specific surface area is preferable for providing large adsorption capacity, but the creation of a large internal surface area in a limited volume inevitably gives rise to large numbers of small sized pores between adsorption surfaces. The size of the micropores determines the accessibility of adsorbate molecules to the internal adsorption surface, so the pore size of the micropores is another important property for characterizing adsorptivity of adsorbents. Especially materials such as zeolite and

carbon molecular sieves can be specifically engineered with precise pore size distributions and hence turned for a particular separation (Marczewski, 2002).

Surface polarity corresponds to affinity with polar substances such as water or alcohols. Polar adsorbents are thus called “hydrophilic” and aluminosilicates such as zeolites, porous alumina, silica gel or silica-alumina are examples of adsorbents of this type. On the other hand, nonpolar adsorbents are generally “hydrophobic”. Carbonaceous adsorbents, polymer adsorbents and silicate are typical nonpolar adsorbents. These adsorbents have more affinity with oil or hydrocarbons than water (Marczewski, 2002).

The average thickness of the adsorbate is often expressed with names mono- and multilayer adsorption. In monolayer adsorption, all the adsorbed molecules are in contact with the surface of the adsorbent. In the multilayer adsorption, the adsorption space accommodates more than one layer or molecules so that not all adsorbed molecules are in direct contact with the molecules of the adsorbent (Toth, 2002).

1.1.2 How do Molecules Bond to Adsorbent?

There is two type of adsorption:

- Physical Adsorption (Physisorption)
- Chemical Adsorption (Chemisorption)

Physical Adsorption: Physical adsorption is caused mainly by van der Waals forces and electrostatic forces between adsorbate molecules and the atoms which compose the adsorbent surface.

There is no significant redistribution of electron density in either the molecule or at the substrate surface.

Chemical Adsorption: A chemical bond, involving substantial rearrangement of electron density, is formed between the adsorbate and substrate. The nature of this

bond may lie anywhere between the extremes of virtually complete ionic at complete covalent character. This is called as chemical adsorption (Blatt, Middleton, & Murray, 1980; Hillier, 1995; Velde, 1995). Typical characteristics of adsorption processes are shown in Table 1.1.

Table 1.1 Typical characteristics of adsorption process.

Properties	Chemisorption	Physisorption
Binding Force	Due to chemical forces or bonding, thus this process is also called as activated adsorption.	Due to physical force of attraction, thus this process is also called as van der Waals adsorption
Temperature Range (over which adsorption occurs)	Adsorption can take place even at higher temperatures. When temperature increases adsorption increases.	Adsorption is appreciable at lower temperature below boiling of adsorbate. When temperature increases adsorption decreases
Adsorption Enthalpy	50-100 kCal/mole	1 kCal/mole
Nature of Adsorption	Often dissociative may be irreversible	Non-dissociative. Reversible
Saturation Uptake	Limited to one monolayer	Multilayer uptake possible
Crystallographic Specificity	Marked variation between crystal planes	Virtually independent of surface atomic geometry
Kinetics of Adsorption	Very variable-often an activation process.	Fast- since it is a non–activation process

The most definitive method for establishing the formation of a chemical bond between the adsorbing molecule and the substrate (i.e. chemisorption) is to use an appropriate spectroscopic technique IR to observe the vibration frequency of the substrate –adsorbent bond.

1.1.3 Which Factors Influence Adsorption?

Adsorption can be expressed as the separation of substances of one phase accompanied by its application or concentration on the surface of adsorbing phase. These transfer process takes place until equilibrium conditions have been reached (Arslanoğlu, Kar, & Arslan, 2005).

Adsorption from solution is a competitive process with equilibrium determined by many factors resulting from adsorbent, adsorbate and solvent properties. The porous structure of a solid, its energetic heterogeneity (depending also on a solute) and surface chemical properties (functional groups) are the main factors influencing adsorption equilibria. For adsorbate and solvent the adsorption phenomenon depends on the differences between their chemical properties, structure and interactions (Mutual and with solid surface) (Marczewska & Marczewski, 2002).

The pore properties (i.e., surface area and total pore volume) play a significant role in determining adsorption capacity based on the results of the adsorption equilibrium and adsorption kinetics experiments (Tsai, Lai, & Hsien, 2003).

Because hydrogen and hydroxide ions are adsorbed quite strongly, the adsorption of other ions is influence by the pH of the solution. In general, adsorption of typical organic pollutant from water is increased with decreasing pH. We should also take care of the ionization equilibrium of the adsorbate.

Adsorption reaction are normally exothermic, thus the extent of adsorption generally increases with decreasing temperature.

1.1.4 Adsorption Isotherms

Adsorption isotherms are important for the description of how adsorbates will interact with an adsorbent and are critical in optimizing the use of adsorbent (Hamdaoui, 2006). Adsorption equilibrium is established when the amount of solute

being adsorbed onto adsorbent is equal to the amount being desorbed. At this point, the equilibrium solution concentrations remain constant. The correlation of equilibrium data using either a theoretical or empirical equation is essential for adsorption data interpretation and prediction. Several models have been published in the literature to describe experimental data of adsorption isotherms (Hamdaoui, 2006).

1.1.4.1 Langmuir Isotherm

The Langmuir isotherm theory assumes monolayer coverage of adsorbate over a homogenous adsorbent surface (Langmuir, 1918). Langmuir isotherm was originally derived from adsorption kinetics by equating the rates of adsorption and desorption onto a flat surface.

The Langmuir equation is based on the following assumptions:

1- Adsorption of adsorbate molecules takes place at well-defined adsorption sites and each site can contain only one molecule.

2- The surface is homogeneous; the energy of adsorption is equal for all adsorption sites.

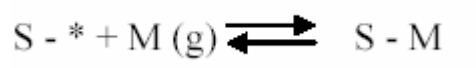
Whenever a gas is in contact with a solid there will be an equilibrium established between the molecules in the gas phase and the corresponding adsorbed species (molecules or atoms) which are bound to the surface of the solid.

As with all chemical equilibrium, the position of equilibrium will depend upon a number of factors:

1. The relative stability of the adsorbed and gas phase species involved
2. The temperature of the system (both the gas and surface)
3. The pressure of the gas above the surface

In general, factor (2) and (3) exert opposite effects on the concentration of adsorbed species. We can say that the surface coverage may be increased by raising the gas pressure but will be reduced if the surface temperature is raised.

We may derive the Langmuir isotherm by treating the adsorption process as we would any other equilibrium process - except in this case the equilibrium is between the gas phase molecules (M), together with vacant surface sites, and the species adsorbed on the surface. Thus, for a non-dissociative (molecular) adsorption process we consider the adsorption to be represented by the following chemical equation:



S - *, represents a vacant surface site

In writing this equation we are making an inherent assumption that there are a fixed number of localized surface sites present on the surface.

We may now define an equilibrium constant (K) in terms of the concentrations of "reactants" and "products"

$$K = \frac{[S-M]}{[S-^{*}][M]}$$

We may also note that:

- [S-M] is proportional to the surface coverage of adsorbed molecules, i.e. proportional to θ
- [S -*] is proportional to the number of vacant sites, i.e. proportional to (1-θ)
- [M] is proportional to the pressure of gas, P

Hence, it is also possible to define another equilibrium constant, b, as given below:

$$b = \frac{\theta}{(1-\theta)P}$$

Rearrangement then gives the following expression for the surface coverage

$$\theta = \frac{bP}{1+bP}$$

This is the usual form of expressing the Langmuir Isotherm.

Sorption is assumed to take place at specific homogeneous sites within the adsorbent. It is believed that once the adsorbate occupies a site, no further adsorption take place at that site. Moreover, it is based on the assumption that the adsorbent are structurally homogeneous where all the sites are identical and energetically equivalent (Senthilkumaar, Kalaamani, Porkodi, Varadarajan, & Subburaam, 2006). In Eq. (1), K_L and a_L are the Langmuir isotherm constants; C_e and C_s are the liquid phase concentration and solid phase concentration of adsorbate at equilibrium.

$$C_s = \frac{K_L}{1+a_L C_e} C_e \qquad (1)$$

The Langmuir constants, K_L and a_L are evaluated through linearization of Eq. (1):

$$\frac{C_e}{C_s} = \frac{1}{K_L} + \frac{a_L}{K_L} C_e \qquad (2)$$

Hence by plotting C_e / C_s against C_e it is possible to obtain the value of K_L from the intercept which is $(1/K_L)$ and the value of a_L from the slope, which is (a_L/K_L). The theoretical monolayer capacity is C_m and is numerically equal to (K_L/a_L). So Eq. (2) is rearranged as the below.

$$\frac{C_e}{C_s} = \frac{1}{C_m\, a_L} + \frac{C_e}{C_m} \quad (3)$$

The essential characteristics of Langmuir equation can be expressed in terms of a dimensionless separation factor R_L, which is defined by Hall, Eagleton, Acrivos, & Vermeulen, (1966), as

$$R_L = 1 / (1+bC_0) \quad (4)$$

where C_0 is the initial concentration of the adsorbate in solution and b is the Langmuir constant (Senthilkumaar et al., 2006).

The shapes of the isotherms for $0< R_L < 1$, $R_L > 1$, $R_L = 1$ and $R_L = 0$ are favourable, unfavourable, linear and irreversible, respectively (Weber & Chakkravorti, 1974).

1.1.4.2 Freundlich Isotherm

The Empirical Freundlich expression (Eq. (5)) is an exponential equation and therefore, assumes that as the adsorbate concentration increases so too does the concentration of adsorbate on the adsorbent surface. Theoretically, using this expression, an infinite amount of adsorption can occur (Freundlich, 1906).

Freundlich equation is based on a monolayer adsorption by the adsorbent with a heterogeneous energy distribution of active sites (Aksu & Kabasakal, 2004).

$$C_s = K_f\, C_e^{\,n_f} \quad (5)$$

In this equation K_f and n_f are the Freundlich constants characteristic on the system. K_f and n_f are indicators of adsorption capacity and heterogeneity factor, respectively. The value of heterogeneity factor ranges between 0 and 1; the more heterogeneous the surface, the closer n_f value is to 0 (Hoda, Bayram, & Ayranci, 2006).

The Freundlich equation aggress well with the Langmuir expression, it does not reduce to the linear isotherm (Henry's Law) at low surface coverage and provides no information on the monolayer adsorption capacity (Allen, Gan, Matthews, & Johnson, 2003).

To determine the constants K_f and n_f , the linear form of the equation shown below may be used to produce a graph of $\ln C_s$ against $\ln C_e$ (Allen et al., 2003).

$$\ln C_s = \ln K_f + n_f \ln C_e \quad (6)$$

1.1.4.3 Dubinin – Radushkevich (DR) Isotherm

Langmuir and Freundlich isotherms do not give any idea about adsorption mechanism. In order to understand the adsorption type, equilibrium data is applied to following linear form of DR isotherm:

$$\ln C_{sorb} = \ln X_m - \beta\varepsilon^2 \quad (7)$$

where C_{sorb} is the amount of ions sorbed onto the adsorbent ($molg^{-1}$), X_m represents DR monolayer capacity of the sorbent ($molg^{-1}$), β is a constant related to sorption energy (mol^2/kJ^2) and ε Polanyi sorption potential, the amount of energy required to pull a sorbed molecule from its sorption site to infinity which is equal to:

$$\varepsilon = RT \ln (1+1/C_e) \quad (8)$$

where R is the gas constant in $kJmol^{-1}K^{-1}$; T is the temperature in Kelvin and C_e is the equilibrium concentration in solution ($moldm^{-3}$). The Polanyi adsorption theory postulates fixed volume of sorption site close to sorbent surface and existence of sorption potential over these sites. The sorption potential is related to an excess of sorption energy over the condensation energy and is independent of temperature. The plot of $\ln C_{sorb}$ versus ε^2 follows linearity. The value of X_m is determined from the

intercept and the value of β is derived from the slope. The sorption energy, E for ions onto adsorbent calculated using the expression:

$$E = -(2\beta)^{1/2} \quad (9)$$

is the range of 8-16 $kJmol^{-1}$ designated for ion exchange mechanism (Malik, Hasany, & Subhani, 2005). If the value of E is smaller than 8 $kJ\ mol^{-1}$, this shows physical adsorption due to weak van der Waals forces (Singh & Pant, 2004).

1.1.5 The Desorption Process

An adsorbed species present on a surface at low temperatures may remain almost indefinitely in that state. As the temperature of the substrate is increased, however, there will come a point at which the thermal energy of the adsorbed species is such that one of several things may occur:

1. A molecular species may decompose to yield either gas phase products or other surface species.
2. An atomic adsorbate may react with the substrate to yield a specific surface compound, or diffuse into the bulk of the underlying solid.
3. The species may desorb from the surface and return into the gas phase.

The last of these options is the desorption process. In the absence of decomposition the desorbing species will generally be the same as that originally adsorbed but this is not necessarily always the case.

CHAPTER TWO
CLAYS

2. Introduction to Clays

2.1 Description of Clay

Clay has two definitions. According to the clay mineralogist, a clay mineral is a layer silicate mineral (also called a phyllosilicate) or other mineral which imparts plasticity and which hardens upon drying or firing (Guggenheim & Martin, 1995).

The term “clay” is often (e.g. in soil mechanics) used in the restricted sense of a particle size classification term for the natural constituents of soils and sediments. In this usage, clays describe a chemically very heterogeneous and structurally complex assemblage of colloidal particles, having a diameter less than 0.002 mm (2 microns) (Giese & Oss, 2002).

In general the term clay implies a natural, earthy, fine-grained material which develops plasticity when mixed with a limited amount of water. By plasticity is meant the property of the moistened material to be deformed under the application of pressure, with the deformed shape being retained when the deforming pressure is removed (Grim, 1968).

Chemical analyses of clays show that alkalies such as sodium, lithium and potassium and alkaline earths such as magnesium, calcium and barium are often present in the molecular structure of clays and have a significant effect in the physical and chemical properties.

2.2 Classification of The Clay Minerals

The Grim's classification of the clay minerals which based distinctions on the shape of the clay minerals and the expandable or nonexpendable character of the 2:1 and 1:1 layer silicates are given below.

I. Amorphous

- Allophane group

II. Crystalline

A. Two – layer type (sheet structure composed of units of one layer of silica tetrahedrons and one layer of alumina octahedrons)

1. Equidimensional

- Kaolinite group

- Kaolinite, nacrite, etc.

2. Elongate

- Halloysite group

B. Three- layer types (sheet structures composed of two layers of silica tetrahedrons and one central dioctahedral or trioctahedral layer)

1. Expanding lattice
    a. Equidimensional
    - Montmorillonite group
    - Montmorillonite, saucinite, etc.
    - Vermiculite
    b. Elongate
    - Montmorillonite group
    - Nontronite, saponite, hectorite
2. Nonexpanding lattice
    Illite group

C. Regular mixed – layer types (ordered stacking of alternate layers of different types)

- Chlorite group

D. Chain structure types (hornblende – like chains of silica tetrahedrons linked together by octahedral groups of oxygen and hydroxyls containing Al and Mg atoms)

- Attapulgite
- Sepiolite
- Palygorskite (Grim, 1968).

2.3 Structure of The Clay Minerals

Two structural units are involved in the atomic lattices of most of the clay minerals (Grim, 1968). The manner in which these units are combined and the type of exchangeable atoms that are present will play a very significant role in the type of clay mineral that forms (Juma, 1998).

The first unit consists of two sheets of closely packed oxygens or hydroxyls in which aluminium, iron or magnesium atoms are embedded in octahedral coordination, so that they are equidistant from six oxygens or hydroxyls (Grim, 1968). If the layer contains mainly trivalent aluminium ions which occupy two thirds of the available octahedral interstices, this is known as a gibbsite type or dioctahedral layer. Conversely, if the layer contains mainly divalent magnesium ions, this will occupy all of the octahedral sites, and the layer is called a brucite – like or trioctahedral layer (Wilson, 1987).

❖

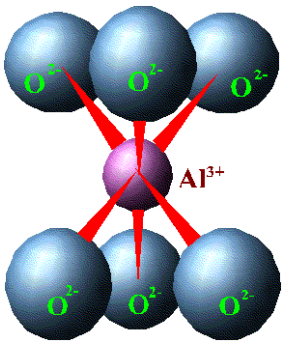


Figure 2.1 Octahedral unit

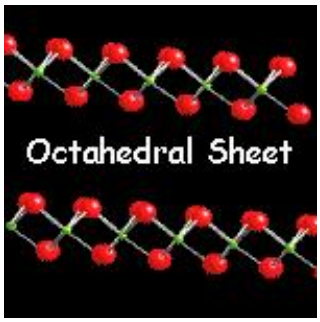


Figure 2.2 Octahedral sheet

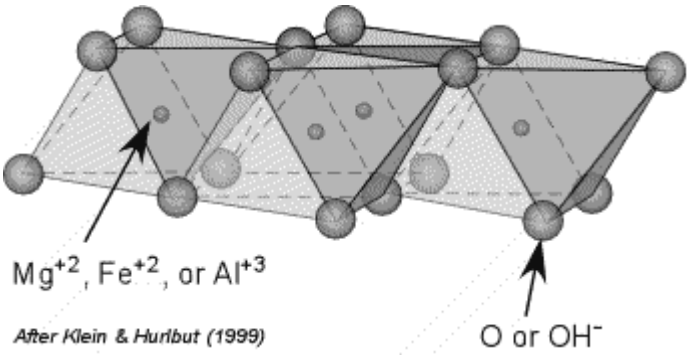


Figure 2.3 Octahedral sheet

The second unit is built of silica tetrahedrons. In each tetrahedron a silicon atom is equidistant from four oxygens, or hydroxyls it needed to balance the structure, arranged in the form of a tetrahedron with a silicon atom at the centre. The silica tetrahedral groups are arranged to form a hexagonal network. Silicon ion shares its charge equally between the four oxygen ions, leaving each oxygen with an excess charge of negative one. We now have the orthosilicate anion, which could at least theoretically be neutralised by four protons. This anion tends to react readily with alkali and alkali earth ions.

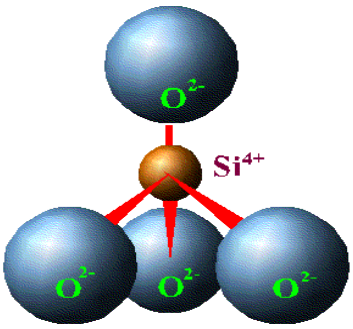


Figure 2.4 Silica tetrahedral units

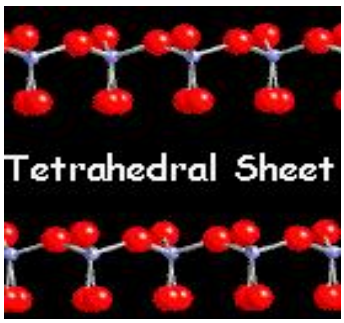


Figure 2.5 Tetrahedral sheet

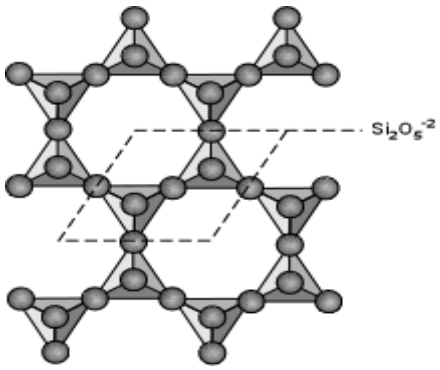


Figure 2.6 A basic structural unit of $Si_2O_5^{2-}$

The structural units of clays therefore consist of either alternating tetrahedral (T) and octahedral sheets (O) (OT OR 1:1 structure), as in the kaolinite group of clay minerals; tetrahedral sheets (TOT or 2:1 structure), as in illite and the smectite clay minerals, of which the most common member is montmorillonite; or an arrangement in which the layer TOT units alternate with a brucite layer (2:1:1 structure) as in chlorite. The basic structural arrangements of the more common clay minerals are illustrated schematically in figure 2.7 (Wilson, 1987).

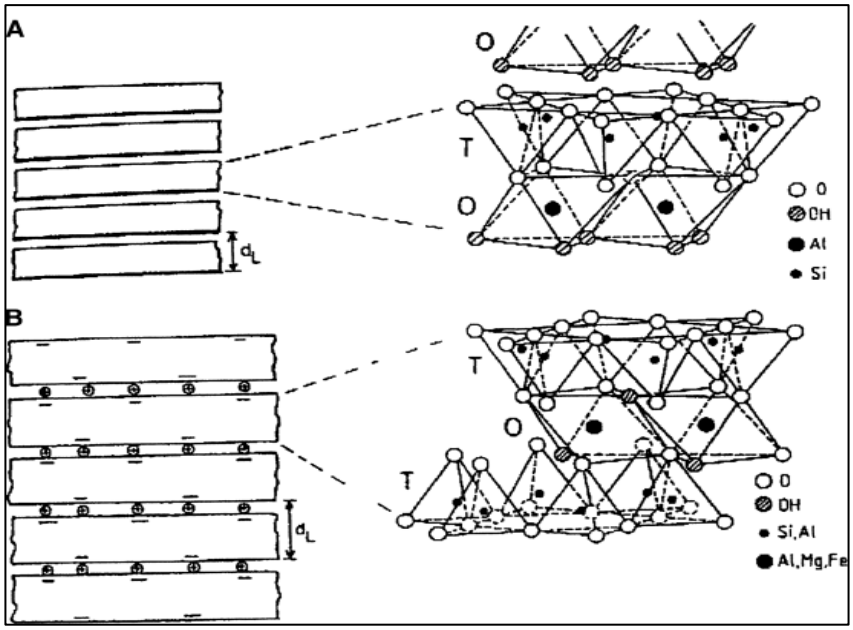


Figure 2.7 Layer structures of two-layer minerals (1:1 clay minerals) (A) and of three-layer minerals (2:1 clay minerals) (B); T, O=tetraeder and octaeder layers, respectively; d_L=layer distance.

Clay minerals are generally classified into three layer types based upon the number and arrangement of tetrahedral and octahedral sheets in their basic structure. These are further separated into five groups that differ with respect to their net charge (Table 2.1).

Table 2.1 Clay minerals and their net charge

Group	Layer Type	Net Negative Charge ($Cmolkg^{-1}$)	Surface Area (m^2/g)	Basal Spacing (nm)
Kaolinite	1:1	2-5	10-30	0.7
Fine-grained mica	2:1	15-40	70-100	1.0
Smectite	2:1	80-120	600-800	1.0-2.0
Vermiculite	2:1	100-180	550-700	1.0-1.5
Chlorite	2:1:1	15-40	70-100	1.4

Sepiolite is a hydrated magnesium-silicate type clay mineral. Its chemical formula, for a half unit cell, is given as $[(Si_{12})(Mg_8)O_{30}\ (OH)_4\ (OH_2)_4\ 8H_2O]$ by (Brauner & Preisinger, 1956). Typically, sepiolite has a fibrous morphology with micro-channels along the fiber axis. The cross section of such a channel is 13.4Å by 6.7Å . Each macro-fiber consists of tens of hundreds of sub-nanometer fibers. The parallel `piped` fibers are bundled together along the *c*-axis to form microscopically fibrous structure, which is porous in its crystalline form. The generic molecular structure of sepiolite is given in Fig. 2.8.

The crystal structure of sepiolite consists of two tetrahedral sheets that are continuous along the *b*- and *c*-axes and an octahedral layer that lies in one dimension is sandwiched between the two tetrahedral sheets. Micro-channels exist along the *c*-axis and contain free water (zeolitic water) molecules attached to the edges of the octahedral layer. This gives sepiolite molecular-sieving properties since only certain size molecules can either pass through or be trapped in these channels. In industry, sepiolite and its derivatives have been widely used for adsorption and molecular sieving applications in chemical industries. It is a well-known application to exchange some Mg^{2+} for other cations and with that of heavy metals in order to increase sepiolite`s sorptive and catalitic properties (Alvarez, 1984; Bilgiç, 2005; Santaren, Sanz, & Ruiz-Hitzky, 1990).

The use of phyllosilicates, mainly sepiolite, to immobilize enzymes was described some years ago; nevertheless, silicate-type materials have been usually used in a two-step covalent immobilization method, which involves the previous activation of the clay and a posterior covalent binding of the enzyme (Fuentes et al., 2001).

The employ of silicates as carriers presents numerous advantages, such as the high specific surface available (between 200 and 800 m^2/g), the facility of water dispersion/recuperation, the high water uptake capacity, and the excellent mechanical resistance of these materials. Last, but not least, their natural origin and their low cost make them even more attractive from an applied point of view (Fuentes et al., 2001).

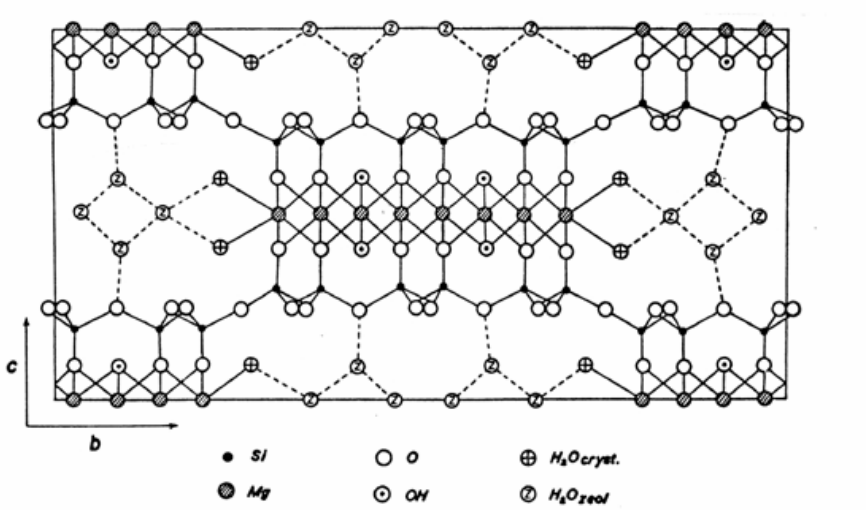


Figure 2.8 Structure of sepiolite

Bentonite that is also known as montmorillonite derives from deposits of weathered volcanic ash. It often contains substantial amounts of magnesium and a wide variety of rare trace minerals. (Önal, Sarıkaya, & Alemdaroğlu, 2001; Murray, 1991; Grim & Güven, 1978). Bentonite is a clay known as a 2/1 type aluminosilicate. Its crystalline structure presents an alumina octahedral between two tetrahedral layers of silica. The isomorphous substitution of Al^{3+} for Si^{4+} in the tetrahedral layer and Mg^{2+} for Al^{3+} in the octahedral layer results in a net negative surface charge on the clay. This charge imbalance in offset by exchangeable cations, denominated exchange cations (typically Na^+ and Ca^{2+}) to compensate the negative charges at the surface. The parallel layers in these structures are held together by weak electrostatic

forces and can expand by penetration of polar species between the clay layers (Bouberka, Kacha, Kameche, Elmaleh, & Derriche, 2005). Montmorillonite is acidic in nature; the acid sites can serve as centres of binding through the -NH_2 group of enzymes. Hence, simple adsorption of enzymes onto clays is enough to hold them on the surface and permit heterogeneous catalysis (Sanjay & Sugunan, 2006). Its advantages over other minerals, have been accepted for a long time, and they account for most of the current uses of bentonite (Alkan, Ceylan, & Arslan, 2005; Robertson, 1973)

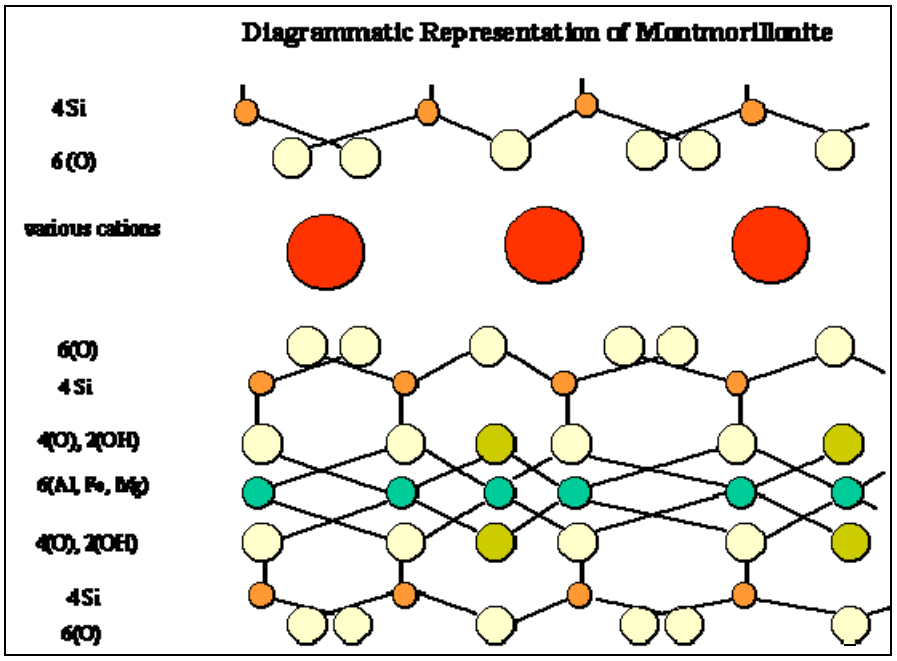


Figure 2.9 Structure of montnorillonite (smectite) (Juma, 1998).

2.4 Uses of Clay

The physical and chemical properties of clays make their usage possible for industrial processes. Their important uses are listed as below;

As a natural scavenger by filtering out pollutants from water.

In the paper industry as a coating and filling agent.

In foundries, as binding agent in the preparation of moulding sands for metallurgical casting.

As an effective adsorbent for a large number of inorganic and organic contaminants. It is also used for decolorizing vegetables oils, clarifying wine, and in water filtration and purification.

In producing of bricks, tiles and other building materials.

In pharmacology, as a drug carrier.

In spas and cosmetic products.

2.5 Properties of Clays

2.5.1 Ion Exchange

The clay minerals have the property of sorbing certain anions and cations and retaining them in an exchangeable state. These ions are exchangeable for other anions or cations by treatment with such ions in a water solution. The exchangeable ions are held around the outside of the silica-alumina clay mineral structural unit, and the exchange reaction generally does not affect the structure of the silica-alumina packet (Grim, 1968). Except under extremely acidic conditions, the layer charge is predominantly negative, a fact which may be verified by investigating the electrochemical properties of clay. This means that clays tend to sorb cations, and are said to have a certain cation exchange capacity, or CEC, which can expressed as a certain number of chemical equivalents per unit weight of dry clay (normally quoted in milliequivalents per hundred grams).

The negatively charged clay surfaces do not have an equal affinity for all cations, but in fact exhibit a selectivity which depends upon such factors as the ionic charge, size and state of hydration of the cations. In general, cation-exchange selectivity increases with ionic radius. Thus, the order of increasing affinity for the alkali-metal ions are Li, Na, K, Rb, and Cs, and for the alkaline-earth ions Mg, Ca, Sr, Ba. The both series, increasing adsorption selectivity of corresponds with decreasing polarizability and energy of hydration in aqueous solution, i.e. the cations which hold on to their hydration shells most firmly are the least strongly adsorbed by clays. The relative adsorption selectivity of monovalent and bivalent ions varies somewhat with

clay type and layer charge. Bivalent ions and polyvalent ions are in general quite strongly adsorbed. Also some monovalent ions, e.g. ammonium or the organic alkylammonium ions, are very strongly adsorbed and permit only limited swelling on account of their large sizes and relatively low hydration energies (Stephen, 2001).

2.5.2 Cation- Exchange Capacity (CEC)

The cation exchange capacity (CEC) is defined as the total amount of positively charged cations that can exchange. The property of exchange capacity is measured in terms of miliequivalents per gram or more frequently per 100 g. Exchange capacity is determined at neutrality, that is pH 7 (Grim, 1968).

There is no single capacity value that is characteristic of a given group of clay minerals. A range of capacities must be shown for each group. The large range shown for the cation exchange capacity is due to the large variation in particle size, crystallinity and time of treatment. Cation exchange capacity of some clay minerals is shown in Table 2.2(Grim, 1968).

Table 2.2 CEC values of the some samples

Clay Mineral	CEC (milliequivalents per 100 g, pH =7)
Kaolinite	3-15
Halloysite. $2H_2O$	5-10
Halloysite. $4H_2O$	40-50
Smectite	80-150
Illite	10-40
Vermiculite	100-150
Chlorite	10-40
Sepiolite	3-15

2.6 Identification of Clay Minerals

The basic methods used for clay mineral identification and quantification involve X-ray diffraction (XRD) and thermal analyses (TA). Electron microscopy (EM) is also employed for complementary mineral characterizations.

2.6.1 Thermal Analysis

Thermal methods are based on characteristic temperature regions in which specific minerals lose their structural OH water (thermogravimetric analysis or TGA) and the enthalpy associated with the dehydroxylation reaction (differential scanning calorimetry, DSC). Mineral quantities are estimated from comparisons of the OH- or enthalpy fractions that have been measured and standard quantities of representative pure minerals.

In the case of the clay minerals, differential thermal analyses show characteristic endothermic reactions due to the dehydration and to loss of crystal structure, and exothermic reactions due to the formation of new phases at elevated temperatures (Grim, 1968).

2.6.2 Surface Area Determination

The specific surface area of powders can be determined using nitrogen (alternatively krypton) adsorption. The specific surface area plays an important role in determining soil physical and chemical properties and therefore has a great influence on processes related to soil conversation, fertility and pollution (İnel & Tümsek, 1999).

2.6.3 XRD Analysis

In XRD analysis the basal spacing (or d-spacing) is measured by using Bragg's Law:

$n\lambda = 2d \sin \theta$

This equation allows calculating basal spacing if the wavelength, λ, of the X- rays is known, and the diffraction angle θ is known.

Table 2.3 shows the d spacing for the {001} plane as measured for various clay-type minerals. Untreated is for the minerals in their natural state, Ethylene Glycol values are obtained after treating the minerals in a solution of Ethylene Glycol, and the last column show the effect if the mineral is heated to 550°C after the Ethylene Glycol treatment.

Table 2.3 d-spacing values for various clay minerals.

d Spacing on {001} for Clay-Type Minerals (Å)			
Mineral	Untreated	Ethylene Glycol	Heated to 550°C
Kaolinite	7.1	No Change	Destroyed
Montmorillonite	14 – 15	17	9.5
Illite	10	No Change	Little Change
Chlorite	7	No Change	13.2
Mixed Layer	11	12	10

CHAPTER THREE
ENZYMES

3.1 Enzymes

Enzymes are very large and complex organic molecules that are synthesized by the cell to perform very specific functions. A living system controls its activity through enzymes. An enzyme is a protein molecule that is a biological catalyst with three characteristics. First, the basic function of an enzyme is to increase the rate of a reaction (by lowering the activation energy). Most cellular reactions occur about a million times faster than they would in the absence of an enzyme. Second, most enzymes act specifically with only one reactant (called a substrate) to produce products. The third and most remarkable characteristic is that enzymes are regulated from a state of low activity to high activity and vice versa. Gradually, you will appreciate that the individuality of a living cell is due in large part to the unique set of some 3,000 enzymes that it is genetically programmed to produce. If even one enzyme is missing or defective, the results can be disastrous (Ophardt, 2006).

The activity of an enzyme depends, at the minimum, on a specific protein chain. In many cases, the enzyme consists of the protein and a combination of one or more parts called cofactors. This enzyme complex is usually simply referred to simply as the enzyme (Ophardt, 2006).

3.1.1 Apoenzyme: The polypeptide or protein part of the enzyme is called the apoenzyme and may be inactive in its original synthesized structure. The inactive form of the apoenzyme is known as a proenzyme or zymogen. The proenzyme may contain several extra amino acids in the protein which are removed, and allows the final specific tertiary structure to be formed before it is activated as an apoenzyme.

3.1.2 Cofactors: A cofactor is a non-protein substance which may be organic, and called a coenzyme. The coenzyme is often derived from a vitamin. Another type of cofactor is an inorganic metal ion called a metal ion activator. The inorganic metal

ions may be bonded through coordinate covalent bonds. The major reason for the nutritional requirement for minerals is to supply such metal ions as Zn^{+2}, Mg^{+2}, Mn^{+2}, Fe^{+2}, Cu^{+2}, K^{+1}, and Na^{+1} for use in enzymes as cofactors.

3.1.3 Final Enzyme: The type of association between the cofactor and the apoenzymes varies. In some cases, the bonds are rather loose and both come together only during the course of a reaction. In other cases, they are firmly bound together by covalent bonds. The activating role of a cofactor is to either: activate the protein by changing its geometric shape, or by actually participating in the overall reaction (Ophardt, 2006).

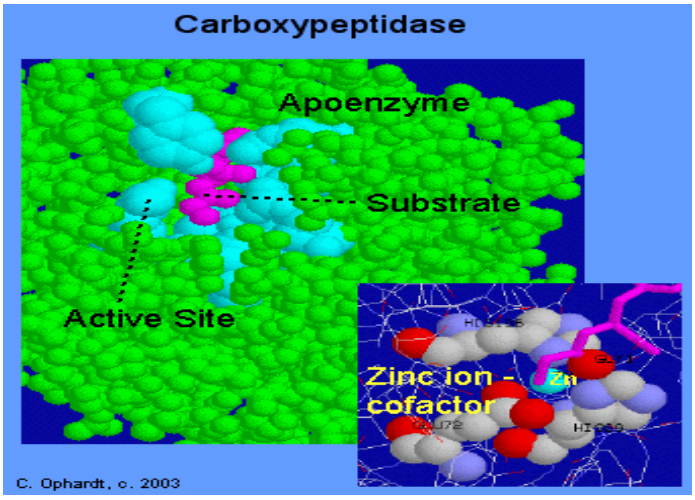


Figure 3.1 Three dimensional structure ofcarboxypeptidase (Ophardt, 2006).

The overall enzyme contains a specific geometric shape called the active site, which is responsible for binding the substrates, the reactants, and processing them into products. As is true of any catalyst, the active site returns to its original state after the products are released. There are two models that explain the binding of a substrate to the active site of an enzyme. In the lock and key model, the active site and substrate have complementary three-dimensional structures and dock perfectly without the need for major atomic rearrangements. In the induced fit model, binding of the substrate induces a conformational change in the active site. The substrate fit well in the active site after the conformational change has taken place (Atkins & Paula, 2006).

The structure of the active site is specific to the reaction that is catalyzes, with groups in the substrate interacting with groups in the active site via intermolecular interactions, such as hydrogen bonding, electrostatic, or van der Waals interactions (Atkins & Paula, 2006).

3.2 The Michaelis- Menten Mechanism of Enzyme Catalysis

Because enzyme-controlled reactions are so important in biochemistry, we need to build a model of their mechanism. The simplest approach proposed by Michaelis and Menten is our starting point (Atkins & Paula, 2006).

The principal features of many enzyme-catalyzed reactions are as follows:

1. For a given initial concentration of substrate, $[S]_0$, the initial rate of product formation is proportional to the total concentration of enzyme, $[E]_0$.
2. For a given $[E]_0$ and low values of $[S]_0$, the rate of product formation is proportional to $[S]_0$.
3. For a given $[E]_0$ and high values of $[S]_0$, the rate of product formation becomes independent of $[S]_0$, reaching a maximum value known as the maximum velocity, v_{max} (Atkins & Paula, 2006).

According to this mechanism, an enzyme-substrate complex, ES, is formed in the first step and either the substrate is released unchanged or after modification to form products:

$$E + S \longrightarrow ES \qquad v = k_a[E][S]$$

$$ES \longrightarrow E + S \qquad v = k_a'[ES]$$

$$ES \longrightarrow P \qquad v = k_b[ES]$$

This mechanism implies that the rate of product formation is given by the Michaelis-Menten equation:

$$v=k_b.[E]_0 / 1+K_M / [S]_0 \quad (1)$$

where $K_M = (k_a^{'} + k_b)/ k_a$ is the Michaelis constant, characteristic of a given enzyme acting on a given substrate.

When $[S]_0 << K_M$, the rate is proportional to $[S]_0$:

$$v = (k_a / K_M). [S]_0[E]_0 \quad (2)$$

When $[S]_0 >> K_M$, the rate reaches its maximum value and is independent of $[S]_0$:

$$v = v_{max} =k_b[E]_0 \quad (3)$$

Substitution of the definition of v_{max} gives

$$v = v_{max} / (1+ K_M / [S]_0) \quad (4)$$

We can rearrange this expression into a form that is amenable to data analysis by linear regression:

$$1/v = 1/ v_{max} + (K_M / v_{max}).1/[S]_0 \quad (5)$$

A Lineweaver-Burk plot is a plot of 1/v versus $1/[S]_0$ and, according to last equation, it should yield a straight line with slope of K_M / v_{max}, a y-intercept at $1/ v_{max}$, and an x-intercept at $1/K_M$ (Atkins & Paula, 2006).

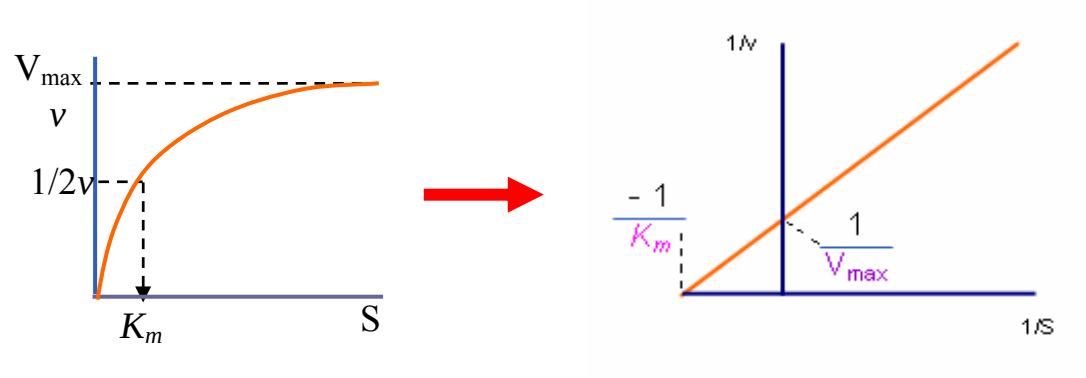


Figure 3.2 Michaelis-Menten and Lineweaver-Burk plots for enzyme-catalyzed reactions.

3.3 Enzyme Nomenclature and Classification

Enzymes are commonly named by adding a suffix "-ase" to the root name of the substrate molecule it is acting upon. For example, Lipase catalyzes the hydrolysis of a lipid triglyceride. Sucrase catalyzes the hydrolysis of sucrose into glucose and fructose (Ophardt, 2006).

A few enzymes discovered before this naming system was devised are known by common names. Examples are pepsin, trypsin, and chymotrypsin which catalyzes the hydrolysis of proteins (Ophardt, 2006).

The latest systematic nomenclature system known as the International Union of Biochemistry and Molecular Biology (IUBMB) system is based upon the type of reaction catalyzed (Ophardt, 2006).

3.3.1. Oxidoreductase: catalyze a variety of oxidation-reduction reactions. Common names include dehydrogenase, oxidase, reductase and catalase.

3.3.2. Transferase: catalyze transfers of groups (acetyl, methyl, phosphate, etc.). Common names include acetyltransferase, methylase, protein kinase and polymerase. The first three subclasses play major roles in the regulation of cellular processes. The polymerase is essential for the synthesis of DNA and RNA.

3.3.3. Hydrolase: catalyze hydrolysis reactions where a molecule is split into two or more smaller molecules by the addition of water. Common examples are given below.

Proteases splits protein molecules. Examples: HIV protease and caspase. HIV protease is essential for HIV replication. Caspase plays a major role in apoptosis.

Nucleases splits nucleic acids (DNA and RNA). Based on the substrate type, they are divided into RNase and DNase. RNase catalyzes the hydrolysis of RNA and DNase acts on DNA. They may also be divided into exonuclease and endonuclease. The exonuclease progressively splits off single nucleotides from one end of DNA or RNA. The endonuclease splits DNA or RNA at internal sites.

Phosphatase catalyzes dephosphorylation (removal of phosphate groups). Example: calcineurin. The immunosuppressive drugs FK506 and Cyclosporin A are the inhibitors of calcineurin.

3.3.4. Lyase: catalyze the cleavage of C-C, C-O, C-S and C-N bonds by means other than hydrolysis or oxidation. Common names include decarboxylase and aldolase.

3.3.5. Isomerase: catalyze atomic rearrangements within a molecule. Examples include rotamase, protein disulfide isomerase (PDI), epimerase and racemase.

3.3.6. Ligase: catalyze the reaction which joins two molecules. Examples include peptide synthase, aminoacyl-tRNA synthetase, DNA ligase and RNA ligase.

The IUBMB committee also defines subclasses and sub-subclasses. Each enzyme is assigned an EC (Enzyme Commission) number. For example, the EC number of catalase is EC1.11.1.6. The first digit indicates that the enzyme belongs to oxidoreductase (class 1). Subsequent digits represent subclasses and sub-subclasses (Classification of enzymes, 2006).

Table 3.1 Enzyme classification. (Ophardt, 2006).

IEC Classification of Enzymes	
Group Name	**Type of Reaction Catalyzed**
Oxidases or Dehydrogenases	Oxidation-reduction reactions
Transferases	Transfer of functional groups
Hydrolases	Hydrolysis reactions
Lyases	Addition to double bonds or its reverse
Isomerases	Isomerization reactions
Ligases or Synthetases	Formation of bonds with ATP cleavage

3.4 Enzyme Activity

An unit of enzyme activity is expressed as certain amount of enzyme that is catalyses the transformation of 1 micromole of substrate per minute in certain conditions such pH, temperature and ionic strength (Eliot & Eliot, 2001).

3.5 Enzyme Immobilization

The resistance of the catalytically active protein structure toward high temperatures, pHs and other denaturing influences is one of the most important criteria for commercialization and for industrial application of the enzymes (Costa et al., 2002).

Compared with conventional chemical catalysts, enzymes as biocatalysts exhibit a number of features that make their use advantageous (Ye, Xu, Che, Wu, & Seta, 2005). Enzymes are valuable biocatalysts of nanometer scale that regulate the chemistry of cells and organisms (Kim et al., 2006). The specific characteristics presented by enzymes are high level of catalytic efficiency and high degree of specificities including substrate specificity, region specificity and stereo-specificity (Ye et al., 2005). These properties of enzymes offers great promises in various applications such as chemical conversions, biosensing and bioremedication (Kim et

al., 2006) and to catalyze series biotransformation reactions, which yield a wide range of useful biological and chemical compounds for pharmaceutical, food, agrochemical derivatives (Ye et al., 2005) and as ligands in clinical and chemical analysis (Li, Hu, & Liu, 2004).

With the unquestionable advantages, there exist a number of practical problems in the application of enzymes. Foremost among them are high cost and the instability of their structures once they are isolated from their natural environments (Ye et al., 2005).

The limited stability of enzymes during long-term storage has been attributed to the deleterious effects of environmental moisture and microbial contamination. Enzymes may be easily denatured by slight change of the environmental conditions such as temperature, pressure, pH and ionic strength (Costa et al., 2002). And also organic solvent in the reaction media affect enzymatic performance by protein denaturation (Manoj, Lakner, & Hager, 2000), enzyme inhibition (Liu, Bo, Cheng, Lin, & Dong, 1998) and stabilization of the substrate ground state, which limits substrate accessibility to the enzyme (Gorjup, Lampic, Penca, Perdih A., & Perdih M., 1999). In general, stabilization of the enzymes could be achieved in several ways: screening for more stable ones (thermophiles, extremophiles), chemical modification, protein engineering, and immobilization or using stabilizing additives (Costa et al., 2002).

To exploit the technical and economical advantages of enzymes efficiently, it is recommended to use them in an immobilized state to reduce the cost and to enhance the stability (Ye et al., 2005).

The various immobilization protocols used with enzymes have been extensively studied (Kennedy & Cabral, 1983). Immobilization of enzyme consisted four different methods: adsorption of the enzyme on a carrier, entrapment of the enzyme within an insoluble gel matrix, containment of enzyme within porous hollow fibers or microcapsules, binding of the enzyme to dried mycelia and ion exchange between

the enzyme and support (Malcata et al., 1990). Among the several methods of immobilization, adsorption (resulting from both hydrophobic or hydrophilic interactions) is the method which induces less modifications on the active conformation of the enzymes. Consequently, adsorption is one of the preferred methods for enzyme immobilization (Fuentes et al., 2001).

Most globular proteins, such as enzymes, have a strong tendency to accumulate at interfaces. This reactive behaviour has a key role in enzyme immobilization and stability. Enzymes are able to be sorbed and immobilized by clay minerals and humic colloids in soil environments. Adsorption of enzymes can result in inactivation due to conformational changes and kinetic enhancement due to the increased concentration of enzyme and substrate at the solid–water interface. Staunton and Quiquampoix (1994) found that positively charged enzymes (i.e., below their isoelectric points, IEP) were subject to electrostatic interactions with clay minerals and thereby lost their activity. At pH values above the IEP the enzymes were mainly dissolved (Safari Sinegani, Emtiazi, & Shariatmadari, 2005).

There has been tremendous interest in the adsorption of enzymes from aqueous solutions due to their practical applications. For preparing bioreactor, immobilization of enzymes by physical interaction is considered to be the easiest way. The major objectives of immobilization are the economic application of enzyme systems in various industrial and technological processes. Immobilized enzymes are currently the subject of considerable research because of their advantages over soluble enzymes especially in industrial preparations. Because the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization on solid support, which offers advantages over free enzymes in the possibility of running enzymatic reactions continuously, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture (thus saving time of a purification step for removing enzyme from the product system), adaptability to various engineering designs, prolonged activity, and obtainability of a concerted or sequential reaction of several enzymes by the use of mixed or stratified beds (Bajpai & Sachdeva, 2002).

This method ensures the reusability of supports after inactivation of immobilized enzyme. Nevertheless, as the interactions between enzyme and support is not very strong some of the adsorbed protein will desorb during washing and other operation steps (Akgöl et al., 2001).

In this method, the forces between a support and the enzymes include hydrogen bonding, Van der Waals forces and hydrophobic interactions (Burns, 1986). The main disadvantage of these methods is weak binding of enzyme. The stability of the adsorbed enzyme derivative will depend on the strength of the non-covalent bonds formed between the support and the amino acid residues on the surface of the protein (Yeşiloğlu, 2005).

Enzymes are often immobilized onto or into solid support to increase their thermostability, operational stability, and recover (Çetinus & Öztop, 2000).

Obviously, the support used in enzyme immobilization is important since its interaction with the enzyme may have an influence on the stability and kinetics (Ye et al., 2005). The supports used for immobilizing enzyme should possess mechanical strength, microbial resistance, thermostability, chemical durability, chemical functionality, low cost, hydrophylicity, regenerability, and a large surface area suitable for enzyme reaction, and substrate and product transport with the least-diffusional restriction (Kilara, 1981; Li et al., 2004).

There are various supports available for enzyme immobilization, which can be classified into three general types: inorganic supports, synthetic polymers and natural macromolecules. The inorganic supports, such as porous glass, silica and magnetic iron oxide, normally are with good mechanical stability, rigidity and regeneratability, but are expensive and their biocompatibilities are poor. Thus, so far the application of inorganic supports for enzyme immobilization is relatively rare. The natural macromolecules, including chitosan, cellulose, agarose and carrageenan with excellent biocompatibility and hydrophilicity, are non-toxic, biodegradable and inexpensive. However, the weak mechanical stabilities of these natural materials

have limited their applications greatly. The synthetic polymers such as polypropylene, polyacrylonitrile and nylon can be used for enzyme immobilization, since they are with good mechanical stability, availability of reactive functional groups for direct reactions with enzymes and ease of preparation in different geometrical configurations. Nevertheless, there are also some disadvantages for them, including imperfect biocompatibility and hydrophobicity (Ye et al., 2005).

The choice of supports is often limited by some other factors related to their structure: one is the pore shape and size. The widths of micropores range from 0.3 to 2.0 nm, mesoporous substances have pore sizes from 2 up to 50 nm and macropores range from widths of 50 up to 105 nm. Silica and other inorganic materials with pore diameter of around 8 nm, allow only the immobilization of small enzymes within the pores. The larger ones can only be placed on the external surface of the support particles. The use of meso- or macroporous supports decreases these unwanted effects and the derivatives usually retain higher activities and have higher enzyme loading. According to some authors, a convenient pore size is around 100 nm. A disordered network of pores and channels, may only allow the smallest substrates to penetrate while the bigger substrates would clog the channels, slowing down the reactions. Thus, a heterogeneous pore size distribution interferes not only with the optimization of enzyme load on the internal surfaces, but also with the diffusion of substrates and/or products (Blanco, Terreros, Fernández-Pérez, Otero, & Díaz-González, 2004).

The main advantages of the new periodic mesoporous silica materials are the uniform pore diameters (2–40 nm), the high specific surface area (300–1500 m^2/g) and the high pore volume (≈1 cc/g). Additionally, these materials are inert and stable at elevated temperatures (Moelans, Cool, Baeyens, & Vansant, 2005).

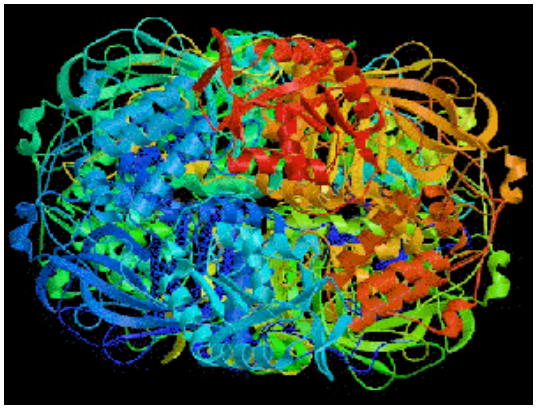
The main problems of using the enzymes industrially are the difficulty of their separation from the solution and their de-activation by using organic solvent and extreme pH or temperature (Moelans et al., 2005). There are several reasons to use immobilized enzymes. In addition to the convenient handling of enzyme

preparations, the two main targeted benefits are: (i) easy separation of enzyme from the product. Easy separation of the enzyme from the product simplifies enzyme applications and permits reliable and efficient reaction technology. (ii) reuse of the enzyme (Bajpai & Sachdeva, 2002). Novel designs with immobilised enzymes and without need of separation are of major concern. This also reduces the loss of enzymes and offers the opportunity to use a continuous reactor with a re-use of the enzyme for many reaction cycles (and thus lowering the total production cost of enzyme mediated reactions). Since most enzymes are normally active in the presence of water but most reactions occur in organic solvents, there is the problem of their de- activation. A major concern hence goes to increasing the chemical stability of the enzymes in organic solvents. Their thermal stability should moreover be examined. Enhancing both stabilities can be achieved by immobilisation and enzyme engineering (modification of enzyme structure) (Moelans et al., 2005).

CHAPTER FOUR
CATALASE

4.1 Catalase

Catalase is widely distributed in nature. It is found in all aerobic microorganisms, in plant and animal cells (Aebi, 1974).



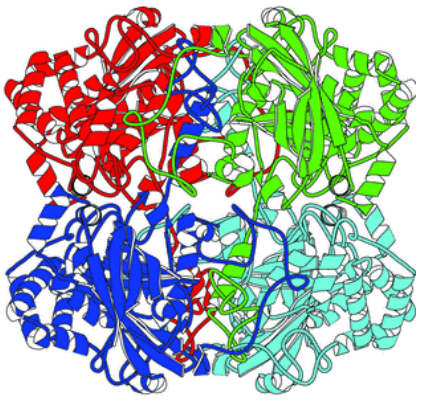


Figure 4.1 Three dimensional structure of catalase

4.2 Where is Catalase Found?

Most aerobic cells contain catalase activity, although a few do not, such as the bacterium *Bacillus popilliae, Mycoplasma pneumoniae*, the green alga *Euglena,* several parasitic helminths (e.g. the liver fluke) and the blue-green alga *Gloeocapsa.* A few anaerobic bacteria, such as *Propionibacterium shermanii,* contain catalase, but most do not. In animals catalase is present in all major body organs (Halliwell & Gutteridge, 2003).

The catalase activity of mammalian tissues varies greatly: it is highest in liver and kidney and low in connective tissues. (Aebi, 1974). The brain, heart and skeletal muscle contain lower levels of catalase than liver although the activity varies between cell types (Halliwell & Gutteridge, 2003

Tablo 4.1 Catalase activities in human tissues (Halliwell & Gutteridge, 2003).

Tissue	Catalase Activity (mg^{-1} protein)
Liver	1300
Erythrocytes	990
Kidney cortex	430
Adrenal gland	300
Kidney medulla	700
Spleen	56
Pancreas	100
Lung	210
Heart	54
Skeletal muscle	36
Brain (grey matter)	11
Brain (white matter)	20

In these cells it is mainly particle-bound (in mitochondria and peroxisomes) whereas in erythrocytes it exists in a soluble state (Aebi, 1974). Peroxisomes in animal cells are involved in the oxidation of fatty acids, and the synthesis of cholesterol and bile acids. Hydrogen peroxide is a byproduct of fatty acid oxidation. White blood cells produce hydrogen peroxide to kill bacteria. In both cases catalase prevents the hydrogen peroxide from harming the cell itself (Catalase, 2006).

Catalase exerts various physiological functions. On one hand the enzyme located in organelles acts as a regulator of H_2O_2 levels and as a specific peroxidase. On the other hand, in erythrocytes, catalase and glutathione peroxidase jointly exert a protective function for haemoglobin and other SH- proteins (Aebi, 1974). Catalase in erythrocytes may help protect them against H_2O_2 generated by dismutation of $O_2^{\bullet-}$ generated by haemoglobin autoxidation (Halliwell & Gutteridge, 2003).

4.3 Catalase Structure

Animal catalases consist of four protein subunits, each of which contains a ferric haem group bound to its active site. The haem groups are buried in non-polar pockets, connected to the surface by narrow channels, thus preventing most molecules larger than H_2O_2 from gaining access (Halliwell & Gutteridge, 2003). The haem group is responsible for catalase's enzymatic activity. Catalase has one of the highest turnover rates for all enzymes: one mol of catalase is able to decompose 500 million mol of H_2O_2 to water and oxygen in one minute (Costa et al., 2001). Each subunit usually has one molecule of NADPH bound to it. Dissociation of catalase into its subunits, which easily occurs on storage, freeze-drying or exposure of the enzyme to acid or alkali, causes loss of catalase activity (Halliwell & Gutteridge, 2003).

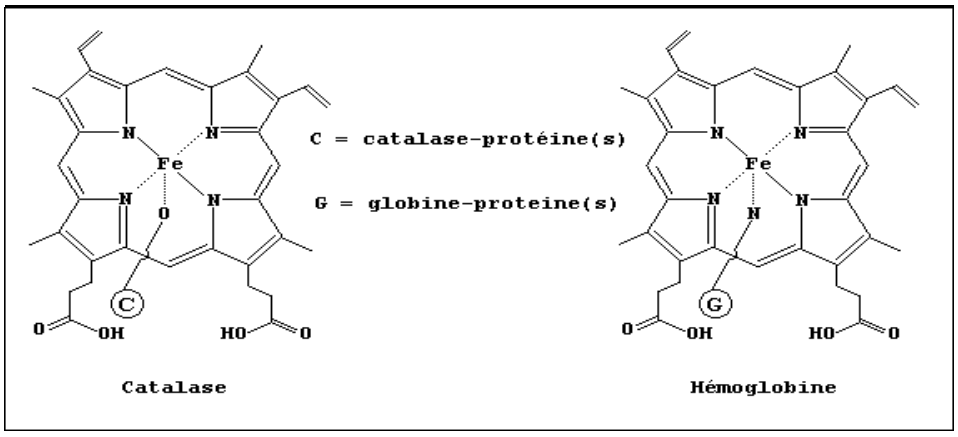


Figure 4.2 Comparison of catalase structure with hemoglobin.

The three-dimensional structures of several catalases have been determined by X-ray crystallography. For example, each subunit of bovine liver catalase consists of a large antiparallel β-pleated sheet domain with helical insertions, followed by a smaller domain containing four α-helices. The haem group is buried at least 20 Å below the molecular surface, only accessible by a channel lined with hydrophobic residues (Halliwell & Gutteridge, 2003).

4.4 Catalase Reaction Mechanism

Catalase is an enzyme showing unusual features in several respects. First, it exerts a dual function because it catalyzes the following reactions:

- decomposition of H_2O_2 to give H_2O and O_2 (catalase activity)
- oxidation of H donors, e.g. methanol, ethanol, formic acid, phenols, with the consumption of 1 mole of peroxide ("peroxidase-like" activity)

The predominating reaction depends on the concentration of H donor and the steady-state concentration or rate of production of H_2O_2 in the system. In both cases the active catalase- H_2O_2 –complex I is formed first. The decomposition of H_2O_2, in which a second molecule of H_2O_2 serves as H donor for complex I, proceeds exceedingly rapidly (rate constant $k \sim 10^7$ L/mol.s), whereas peroxidative reactions proceed relatively slowly ($k \sim 10^2$-10^3 L/mol.s) (Aebi, 1974).

Second, the kinetics of catalase do not obey the normal pattern. On one hand it is not possible to saturate the enzyme with "substrate" within the feasible concentration range (up to 5 mol/L), and on the other there is a rapid inactivation of catalase at H_2O_2 concentrations above 0.1 mol/L, when the active enzyme- H_2O_2–complex I is converted to the inactive complexes II or III. Measurement of enzyme activity at substrate saturation or determination of the K_s is therefore impossible. In contrast to reactions proceeding at substrate saturation, the enzymatic decomposition of H_2O_2 is a first order reaction, the rate of which is always proportional to the peroxide concentration. Consequently, to avoid a rapid decrease in the initial rate of the reaction, the assay must be carried out with relatively low concentrations of H_2O_2 (ca. 0.01 mol/L) (Aebi, 1974).

4.5 Catalase Activity

Measurements of catalase activity are widely performed in food sciences and industrial food production. Owing to the considerable heat stability of catalase (and

other peroxidatic agents), the detection of residual catalase activity in processed and preserved food is important with regard to changes in colour, taste and odour brought about by peroxidatic reactions (Aebi, 1974).

In the dairy industry catalase activity is a sensitive and easily detectable indicator of contamination of milk by neutrophil granulocytes, i.e., it is a reliable test to detect any kind of inflammation or infection process. In biomedical research the study of catalase in liver, leukocytes and erythrocytes has received increased attention owing to its role in oxidative metabolism (e.g. peroxidation of methanol, formate) as well as its protective function by acting as a H_2O_2-scavenger. Furthermore, in genetics the pronounced heterogeneity pattern of plant catalase (e.g. in maize) as well as the rare but puzzling situation in murine and human acatalasaemia has prompted a number of investigations of general relevance (Aebi, 1974).

Catalase activity can be measured by following either the decomposition of H_2O_2 or the liberation of O_2. Accordingly, the remaining substrate concentration at a given moment of the reaction can be determined either by UV-spectrophotometry or – at the end of the reaction period – by simple titration. Alternatively, O_2 –production can be followed by means of the oxygen electrode, polarography (Aebi, 1974) manometric techniques or by gas chromatography (Trasar-Cepeda, Camiña, Leirós, & Gil-Sotres, 1999). However, the method of choice for biological material is UV-spectrophotometry (Aebi, 1974).

There is no official reference method at present. However, UV-spectrophotometry has been and still is widely used. Since it is quick, specific and sensitive is has to be regarded as the generally preferred method (Aebi, 1974).

4.5.1 UV- Method

In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm (ε_{240}= 0.00394 ± 0.0002 L $mmol^{-1}$ mm^{-1}). The

difference in absorbance per unit time is a measure of the catalase activity (Aebi, 1974).

Optimized Conditions for Measurement

To avoid inactivation of the enzyme during the assay (usually 30s) or formation of bubbles in the cuvette due to the liberation of O_2, it is necessary to use a relatively low H_2O_2 concentration (10 mmol/L).The H_2O_2 concentration is critical in that there is direct proportionality between the substrate concentration and the rate of decomposition. The dependence of H_2O_2 decomposition on temperature is small (Q_{10}<1.1) so that measurement can be carried out between 0-37°C; however, 20°C is recommended. The pH activity curve relative to v_0 has a fairly broad pH optimum (pH 6.8 to7.5); measurements are made at pH=7.0 (Aebi, 1974).

4.6 Which Factors Affect the Activity of Catalase?

The rate at which an enzyme works is influenced by several factors including the concentration of substrate, temperature, pH, salt concentration and the presence of inhibitors or activators. Every enzyme has an optimal range for each of these factors. Activity decreases when an enzyme is exposed to conditions that are outside the optimal range.

4.6.1 Substrate Concentration

If all other conditions are held constant, the rate of the reaction should increase with increasing concentrations of substrate. At very low values of substrate the reaction rate will increase very rapidly. At higher substrate concentrations the rate begins to level off. Eventually the maximum rate for that reaction will be achieved and further increases in substrate concentration will have no effect.

4.6.2 Temperature

In general, chemical reactions speed up as the temperature is raised. When the temperature increases, more of the reacting molecules have the kinetic energy required to undergo the reaction. Enzyme catalyzed reactions also tend to go faster with increasing temperature until a temperature optimum is reached. Above this value the conformation of the enzyme molecule is disrupted. Changing the conformation of the enzyme results in less efficient binding of the substrate. Temperatures above 40-50°C denature many enzymes.

4.6.3 pH

pH is a measure of the acidity or hydrogen ion concentration of a solution. It is measured on a scale of 0-14 with pH values below 7 being acidic, values above 7 being basic and a value around 7 is neutral. As the pH drops into the acidic range an enzyme tends to gain hydrogen ions from the solution. As the pH moves into the basic range the enzyme tends to lose hydrogen ions to the solution. In both cases the changes produced in the chemical bonds of the enzyme molecule result in a change in conformation that decreases enzyme activity.

4.6.4 Salt Concentration

Every enzyme has an optimal salt concentration in which it can catalyze reactions. Too high or too low a salt concentration will denature the enzyme.

4.6.5 Presence of Inhibitors

A molecule that interacts with the enzyme and decreases its activity is an inhibitor. Enzyme activity can be affected in different ways. Competitive inhibition occurs when the inhibitor has a similar structure as the substrate, allowing it to compete for the active site on the enzyme molecule. In the case of catalase the active site is the haem group. Noncompetitive inhibition occurs when the inhibitor binds

somewhere other than the active site of the enzyme. This causes a change in the shape of the enzyme molecule so that the substrate molecule can no longer bind to the active site (Catalase, 2006).

Among the many substances affecting catalase activity in vitro are inhibitors, H-donor substances acting as substrates in peroxidatic catalase activity, and catalysts imitating the H_2O_2 decomposing activity of catalase. A selection of substances interfering with catalase activity is listed in Table 4.2. However, in most instances, particularly if the sample has been adequately diluted before analysis, these effectors do not play a significant role and are only of theoretical interest (Aebi, 1974).

Table 4.2. Catalase inhibitors (acting in vitro)

Azide	Fluoride
Cyanide	Acetate (acetic acid)
Cyanogenbromide	Formate (formic acid)
Hydroxylamine	Methanol
Ascorbate	Ethanol
Nitrite	

Many of these inhibitors are non-specific: they inhibit many other enzymes. A more useful inhibitor is aminotriazole (Fig. 4.3) which inhibits catalase when administered to animals or plants. Aminotriazole will only inhibit catalase if H_2O_2 is present. (Aebi, 1974).

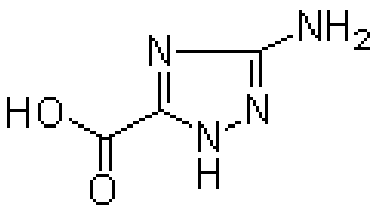


Figure 4.3. Structure of aminotriazole. The full name of this compound is 3-amino-1,2,4-triazole.

4.6.6 Presence of Activators

A molecule that interacts with an enzyme and increases its activity is an activator.

In living systems the optimal ranges of temperature, pH and salt concentration for a given enzyme are the ranges found in that system. When determining various optimum conditions for a catalase solution it is important to consider the source of the catalase. Catalase derived from a potato or from yeast might "prefer" slightly different condition than catalase derived from beef liver. For example, mammalian enzymes have a temperature optimum of about 40°C, but there are enzymes that work best at very different temperatures, e.g. enzymes from the arctic snow flea work at -10°C, and enzymes from thermophilic bacteria work at 90°C. The optimal pH range is about 7-8 (physiological pH of most cells), but a few enzymes can work at extreme pH, such as protease enzymes in animal stomachs, which have an optimum of pH 1 (Catalase, 2006).

4.7 Immobilized Catalase in Industrial Field

Immobilized catalase has useful applications in various industrial fields in the removal of hydrogen peroxide (Tukel & Alptekin, 2004).

Hydrogen peroxide is used in many industrial processes such as textile, food and treatment of waste waters. Moreover, measuring of hydrogen peroxide in various field of chemistry is also important topic in chemistry because of harmful effects of hydrogen peroxide. For example, unscavenged of hydrogen peroxide in aerobic metabolism can be transformed other more harmful substance such hydroxyl radical and superoxide radical anion. Likewise it is also important to determine the hydrogen peroxide content in various commercial products such as contact-lenses solutions, cosmetics and pharmaceutical formulations. In recent years, hydrogen peroxide and peracetic acid have been increasingly used for disinfection purposes since they are more potent oxidizing agents than many of the routinely used halogenated

disinfectants. In addition, hydrogen peroxide readily decomposes into water and oxygen and therefore poses no serious threat to the environment (Choi & Yiu, 2004).

Several reports deal with the use of H_2O_2 to eliminate chemical bleaching before dyeing in cotton textile process (Eberhardt, Pedroni, Volpe, & Ferreira, 2004). Even a trace amount of hydrogen peroxide could cause dyeing problems such as unevenness of color, insufficient dyeing, and shade changes between batches (Costa et al., 2002)

As a result, immobilization strategies for enzymes are of paramount importance to preserve their biological activity (Choi & Yiu, 2004).

4.8 Why do We Choose Clay for Immobilization?

The clay minerals, an important constituent of all soils, are a group of microcrystalline secondary minerals consisting of hydrous aluminum silicates with sheet like structures (Bhattacharyya & Gupta, 2006).

They are effective natural adsorbents due to their small particle sizes, lamellar structures (Tsai et al., 2003), high specific surface area, chemical and mechanical stability, high cation exchange capacity, etc. Presence of both Bronsted and Lewis types of acidity in clays boosts the adsorptive capacities of clays (Gupta & Bhattacharyya, 2006).

Treatment of clay minerals with concentrated inorganic acids usually at high temperature is known as acid activation. Acid treatments of clay minerals are an important control over mineral weathering and genesis. Such treatments can often replace exchangeable cations with H ions and release Al and other cations out of both tetrahedral and octahedral sites, but leaving the SiO_4 groups largely intact. It was reported that acid activation followed by thermal treatment increases the adsorbent capacity to a good extent.

The swelling phyllosilicate minerals, known as smectite clays, constitute a naturally occurring class of inorganic catalysts. These ubiquitous minerals are components of many soils and sediments and often are found as large mineralogically pure deposits. Due to their small size and unusual intercalation properties, they afford an appreciable surface area for adsorption of organic and inorganic moieties (Pinnavaia, 1983). Montmorillonite, a well-studied smectite clay, is a 2:1 dioctahedral clay and has been widely used as a catalyst as well as a support. The speciality of montmorillonite (smectites in general) is that the properties can be tailor made to suit the need by simple methods, such as acid activation, ion-exchange, pillaring and intercalation with organics. Montmorillonite is acidic in nature; the acid sites can serve as centres of binding through the $-NH_2$ group of enzymes. Hence, simple adsorption of enzymes onto clays is enough to hold them on the surface and permit heterogeneous catalysis. Also, the clay can be suitably activated with amino silane and coupled with glutaraldehyde which can covalently bind the enzyme (Sanjay & Sugunan, 2006).

Expandable clays such as smectites have a high affinity for protein adsorption. Fusi, Ristori, Calamai, & Stotzky, (1989) reported that montmorillonite may adsorb enzyme molecules on both external and internal surfaces. Naidja & Huang (1996) found that the large molecules of aspartase (MW 180,000) were intercalated with the montmorillonite layers. Garwood, Mortland, & Pinnavaia, (1983) observed an expansion of 35 Å for the complex glucose oxidase (MW 153,000)–Na-montmorillonite. On the other hand, Harter & Stotzky, (1973) reported that the adsorption of catalase (MW 238,000) onto Ca-montmorillonite did not result in expansion of the mineral structure. They concluded that the adsorption is entirely external (Safari Sinegani et al., 2005).

We choose clay because these low cost materials have high specific surface area, chemical, mechanical and thermal stability, porous structure and high adsorption capacity. And also these materials are not toxic. Catalase was chosen because of its industrial using.

Goal of the study is to understand the immobilization process of catalase onto some natural clays. The effect of the immobilization conditions such as initial catalase concentration, contact time, pH, ionic strength and temperature were investigated with the aim to achieve maximum recovery in the activity of the immobilized enzymes. The kinetic parameters, thermal stability, storage stability and operational stability of free and immobilized enzymes were determined for comparison.

CHAPTER FIVE
MATERIAL AND METHOD

5.1 Clay and Catalase

Bentonite and sepiolite were purchased from Edirne and Eskişehir, respectively. They were utilized for adsorption experiments as an adsorbent. The properties of the natural clays are summarized in Table 5.1 and 5.2.

Table 5.1 Chemical composition of bentonite

Component	%
SiO_2	57.78
Al_2O_3	19.24
Fe_2O_3	3.58
MgO	2.13
CaO	4.21
Na_2O	2.73
K_2O	1.35
Sulphate	-
Ignition Loss	7.48

Table 5.2 Chemical composition of sepiolite

Component	%
SiO_2	48.00
Al_2O_3	0.50
Fe_2O_3	0.40
MgO	24.00
CaO	4.00
Na_2O	0.02
K_2O	0.03
Sulphate	0.02
Ignition Loss	23.03

Catalase (Sigma Cat. No. C 9322) was purchased from Sigma and used without further purification and treatment. In the preparation of catalase solution, phosphate buffer was used.

5.2 Surface Area Measurements

BET specific surface area of bentonite and sepiolite were determined as 77 and 292 m^2/g respectively by adsorption of N_2 at 77K with Quantachrome Autosorb Automated Gas Sorption System. Characterization data of the samples are given below.

Table 5.3 The properties of bentonite and sepiolite.

Sample	CEC (meg/100g clay)	BET Specific Surface Area (m^2/g)	d_{001} (Å)	Total Pore Volume (cm^3/g)	Average Pore Diameter (Å)
Bentonite	97	76.84	7.63	0.203	105.8
Sepiolite	82	292	12.56	0.575	80.04

5.3 FTIR Study

FTIR spectroscopy was used to obtain the information about the interactions between catalase and clay samples. Bentonite, sepiolite, and catalase loaded bentonite and sepiolite were brought to constant weight in a drying oven at 50 ºC for 24 h. The samples were kept in the desiccators. 100 mg of fine KBr powder was dried at 110 ºC and mixed with 1 mg of samples. For pellets, the infrared spectra in the range 4000-400 cm^{-1} were recorded on a Perkin Elmer FTIR spectrophotometer (Spectrum BX-II). The background spectrum of each KBr pellet was obtained by subtracting it from the sample spectra.

5.4 SEM Measurements

Scanning electron microscopy, SEM was carried out in a Philips FEG-XL30 in Darmstadt Technical University/Germany. The surface morphologies of the samples

were studied using a scanning electron microscope at an accelerating voltage of 15 kV. The samples that were used were dried and coated with gold before scanning. For SEM, photographs were taken at different magnifications (between 1000X and 5000X).

5.5 Adsorption from Solution Experiments: Determination of Equilibrium Concentration

In the experimental studies, the effects of pH, temperature and ionic strength, on the free enzyme activity, on the immobilization and on the immobilized enzyme activity were studied. And also thermal and storage stabilities of free and immobilized enzymes and operational stabilities for immobilized enzymes were determined. The pH of the immobilization medium was varied from 3 to 12 by using different buffer systems (0.05 M CH_3COOH for pH 3-5; 0.05 M KH_2PO_4 - K_2HPO_4 for pH 7-9 and 0.05 M NaOH – KCl for pH 12). The temperature and the ionic strength of the immobilization medium were varied from 15 °C to 65 °C and from 0.01 to 1 M , respectively.

For immobilization of catalase onto clays, 5 mg of bentonite and sepiolite were added into 1mL catalase solutions. Concentrations of catalase in aqueous solutions were between 30 and 200 µg/mL. The solutions were shaken at 105 rpm in a temperature controlled shaking water bath (Memmert) for 1 hour, which is proven to reach equilibrium. At the end of this period, the immobilized catalase was separated from the residual aliquot by centrifuging (5000 rpm for 5 min at +4°C) (Hettich, R21) and the amount of free catalase was determined by measuring the activity of the free catalase in the supernatant. The concentration of catalase was determined spectrophotometrically (Shimadzu UV-Visible 1601) at 240 nm with Aebi method using a comparison sample prepared previously. The amount of catalase immobilized by clays was calculated by measuring the initial and equilibrium concentrations of catalase in the medium. The same procedures were performed for different temperature, pH and ionic strength values to find the effects of these parameters on immobilization.

To determine the thermal stability of free and immobilized catalases, free and immobilize enzymes in phosphate buffer (pH=7) were incubated at various temperatures (15 – 65°C) for 1 hour and then their activities were found at 25°C.

To determine the storage stability of free and immobilized catalases, free and immobilize enzymes in phosphate buffer (pH=7) were incubated at various temperatures (-18°C, 4°C and 25°C) for days and then their activities were found at 25°C.

Operational stability was determined by using immobilized enzymes several times.

Immobilization procedures mentioned above were performed at solution temperatures of 25ºC and 45ºC to find thermodynamic parameters. Experiments were also carried out at various time intervals to determine kinetic parameters.

In this study, immobilization of catalase onto bentonite and sepiolite has been studied. The Langmuir and Freundlich equations were used to fit the equilibrium isotherm. The kinetic and thermodynamic parameters were calculated to find out adsorption rate constants and adsorption mechanism.

CHAPTER SIX
RESULTS

6.1 Determination of Optimum Conditions for Free and Immobilized Catalase

The temperature dependence of the activities of the free and immobilized catalases were studied in 0.05 M phosphate buffer (pH=7.0) in the temperature range 15 - 65°C. Relative activities of free and immobilized catalases as a function of temperature were shown in Figure 6.1. A maximum activity for free enzyme was found at 35°C. And it was found for immobilized enzymes at about 35 – 40°C.

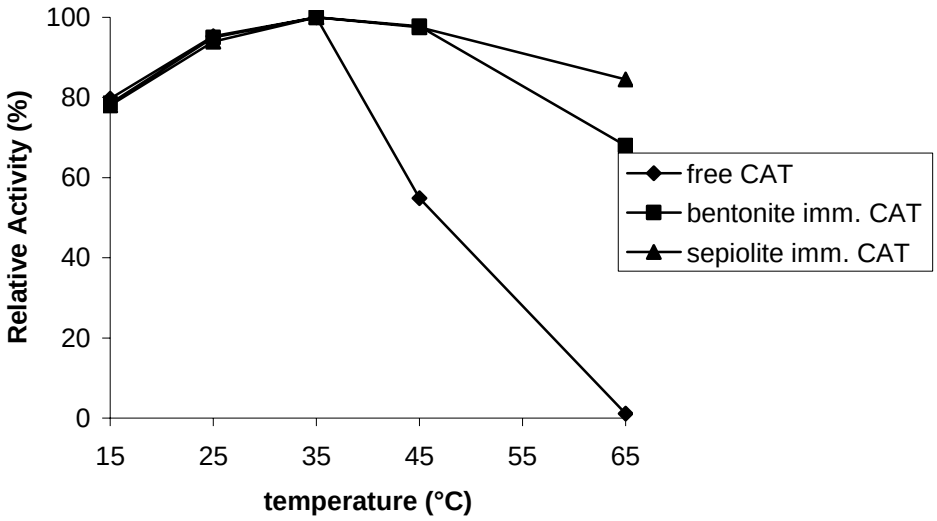


Figure 6.1 Effect of temperature on activity for free and immobilized catalases. Reactions were performed in phosphate buffer pH (7.0, 50 mM) of various temperature values.

Figure 6.1 presents that immobilized catalases show lower activity loss than free catalase at high temperature values in which enzyme deactivation takes place. It might have been caused because of the protecting effect of support. At high temperatures, immobilized enzyme is not able to move like free enzyme therefore it can be said that inhibition of enzyme activity of free catalase was higher than that of immobilised catalase onto clay material (Çetinus & Öztop, 2003; Akgöl et al., 2001).

The effect of pH on the activity of free and immobilized catalases were studied in the pH range 3.0 – 12.0. To indicate pH effects on activity, relative activity versus pH was plotted and the results were shown in Figure 6.2. The data shows that optimum pH is shifted from 7.0 for free catalase to 8.0 for sepiolite and bentonite immobilized catalase.

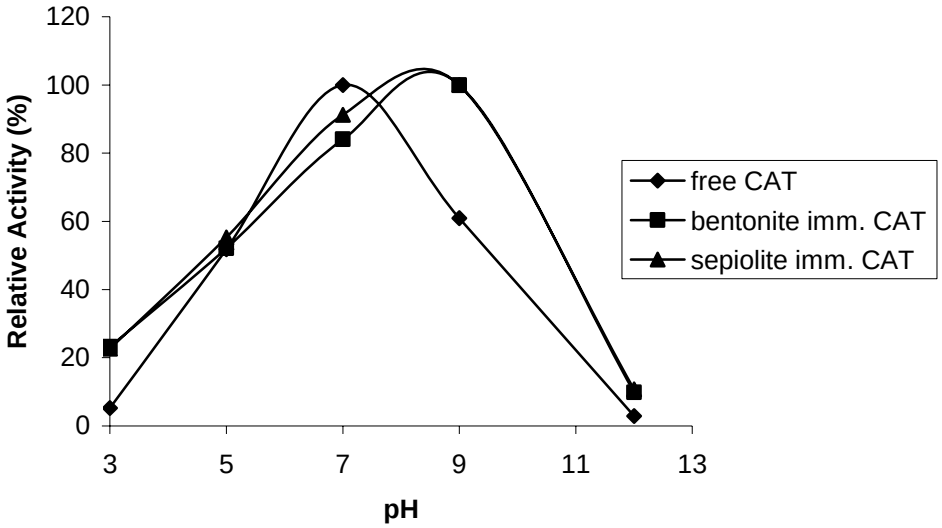


Figure 6.2 Effect of pH on activity for free and immobilized catalases. Reactions were performed at 35ºC in buffers (50 mM) of various pH values.

Immobilized catalases have a broader pH range of activity than free one. Higher activity than free catalase at alkaline pH's may be caused by the change of microenvironment of immobilized enzyme which is formed by binding support.

The effect of ionic strength on the activity of free and immobilized catalases were studied in the ionic strength range 0.01 – 1 M. The results were presented in Figure 6.3.

At low ionic strength values, free catalase is more active than immobilized ones. The activities of free and immobilized catalases were decreased with increasing ionic strength. And also bentonite immobilized catalase activity was more affected than the free catalase and sepiolite immobilized catalase.

At 1 M concentration; free catalase retained 52.6 % of its maximum activity, bentonite immobilized catalase retained 51.5 % of its maximum activity and sepiolite immobilized catalase retained 62 % of its maximum activity.

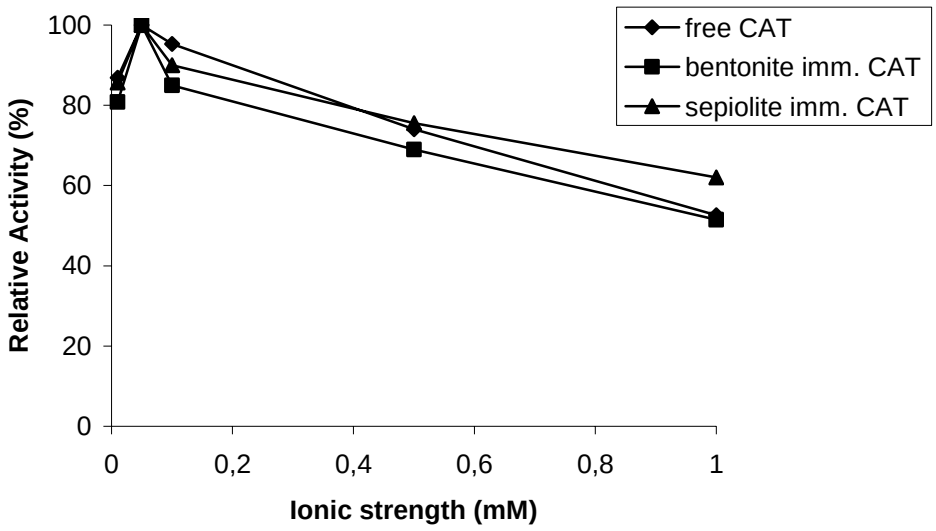


Figure 6.3 Effect of ionic strenght on activity for free and immobilized catalases. Reactions were performed at 35ºC in phosphate buffers pH 7.0 of various ionic strenght values.

6.2 Determination of Optimum Conditions for Immobilization

The effects of temperature on catalase immobilization onto clays were researched in the temperature range 15 – 65°C, and the data were presented in Figure 6.4. As seen in this figure, immobilized catalase is reached a maximum at 45°C. When the temperature is increased, the adsorption capacities of clays are decreased. There is no significant difference to sepiolite's adsorption capacity at low temperature values. But lower adsorption capacities were obtained at lower temperature values for bentonite.

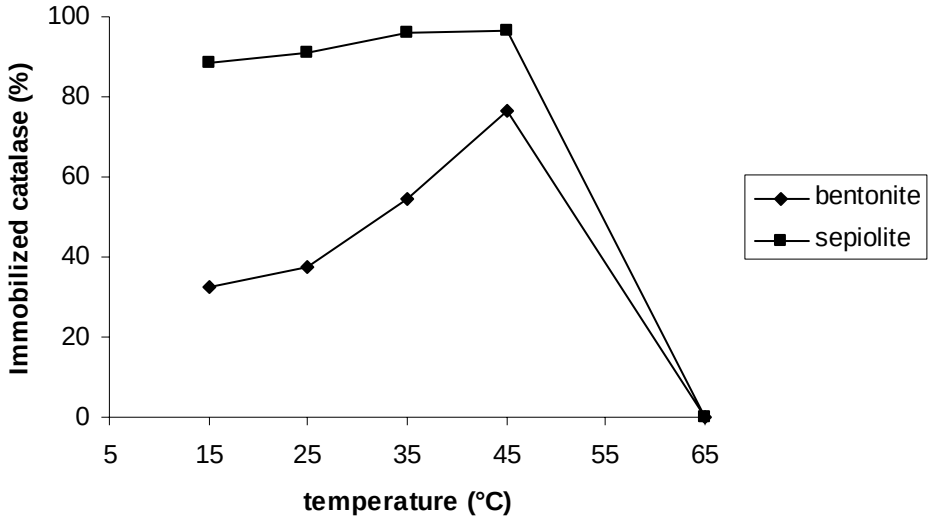


Figure 6.4 Effect of temperature on catalase immobilization onto clays. Reactions were performed in phosphate buffer (pH 7.0, 50 mM) of various temperature values.[CAT] = 100µg/ml, adsorbent dose = 5 mg.

The effects of pH on catalase immobilization onto clays were studied in the pH range 3.0 – 12.0. The results were presented in Figure 6.5.

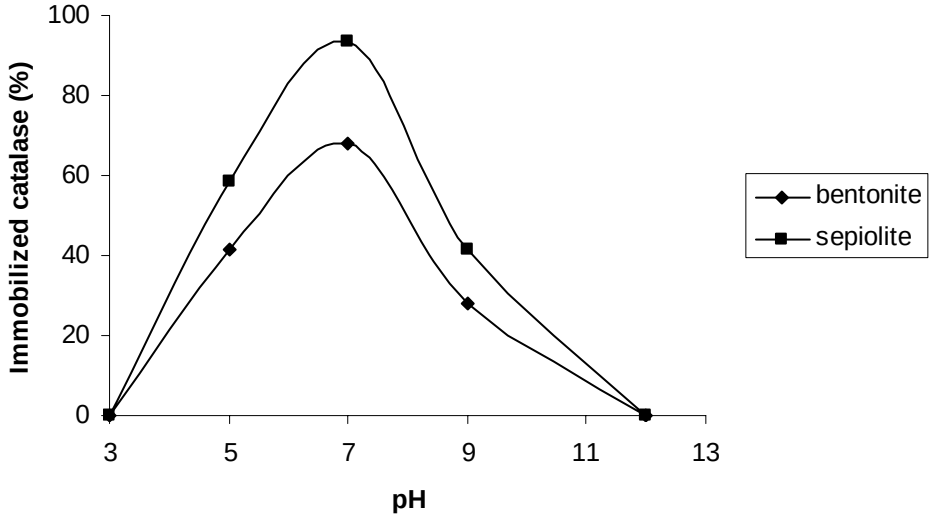


Figure 6.5 Effect of pH on catalase immobilization onto clays. Reactions were performed at 35ºC in buffers (50 mM) of various pH values.[CAT] = 100µg/ml, adsorbent dose = 5 mg.

Figure 6.5 shows that maximum adsorption capacities were obtained at pH 7.0 for bentonite and sepiolite. Lower adsorption capacities were determined at lower and higher pH values than pH 7.0. These results showed that pH of the medium is an important parameter for adsorption.

The effects of ionic strength on catalase immobilization onto clays were studied in the ionic strength range 0.01 – 1 M. To indicate ionic strength effects on adsorption, the amount of catalase immobilized by the clays (%) versus equilibrium ionic strength was plotted. The results were presented in Figure 6.6. As seen in this figure, maximum immobilization was obtained at 0.05 M. And when the ionic strength is increased, immobilized catalase is decreased. It might have been caused by the interactions between enzyme and ions in the medium, or interactions between support and ions in the medium.

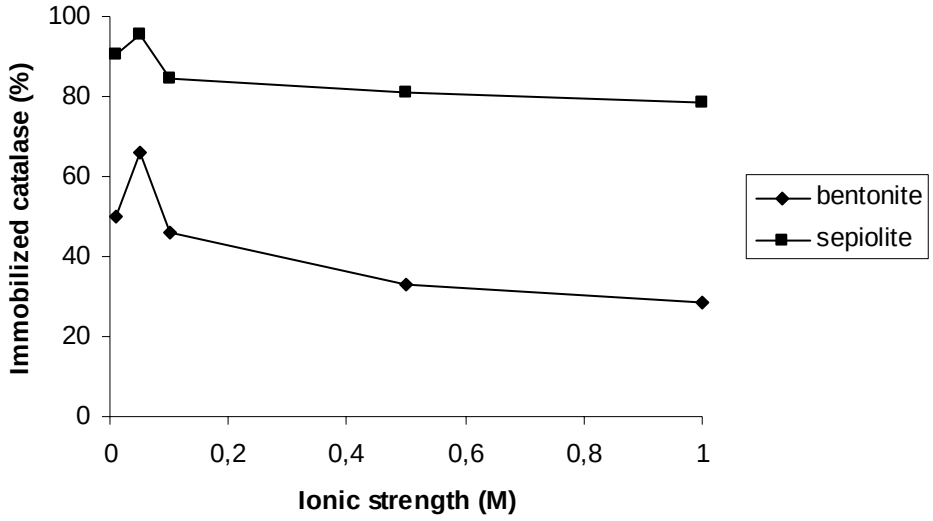


Figure 6.6 Effect of ionic strenght on catalase immobilization onto clays. Reactions were performed at 35ºC in buffers pH 7.0 of various ionic strenght values.[CAT] = 100μg/ml, adsorbent dose = 5 mg.

6.3 K_m and V_{max} Values for Free and Immobilized Catalase

Various levels of hydrogen peroxide (H_2O_2) solutions (from 4 mM to 30 mM) were prepared to find the initial rates of H_2O_2 decomposition. To determine the rate

of reactions, free or immobilized catalases were added into H_2O_2 solutions, and the rate of reaction was determined by spectrophotometrically. These data were plotted according to the method of Lineweaver-Burk and presented in Figure 6.7 and Figure 6.8 for free and immobilized catalases, respectively. Michaelis constants and maximum rate values for free and immobilized catalases were calculated from Lineweaver-Burk plots and were given in Table 6.1.

Table 6.1 Adsorption kinetics values for free and immobilized catalase.

Adsorption Kinetics	**Free catalase**	**Bentonite immobilized catalase**	**Sepiolite immobilized catalase**
K_m (mM)	116.8	55.6	49.2
V_{max} (µmol/min.mg)	30.1	6.6	8.7

These data showed that kinetic parameters of enzymes were affected by immobilization. K_m is a marker for the affinity of enzyme to substrate. The order for substrate affinity can be written like below:

sepiolite immobilized catalase > bentonite immobilized catalase > free catalase

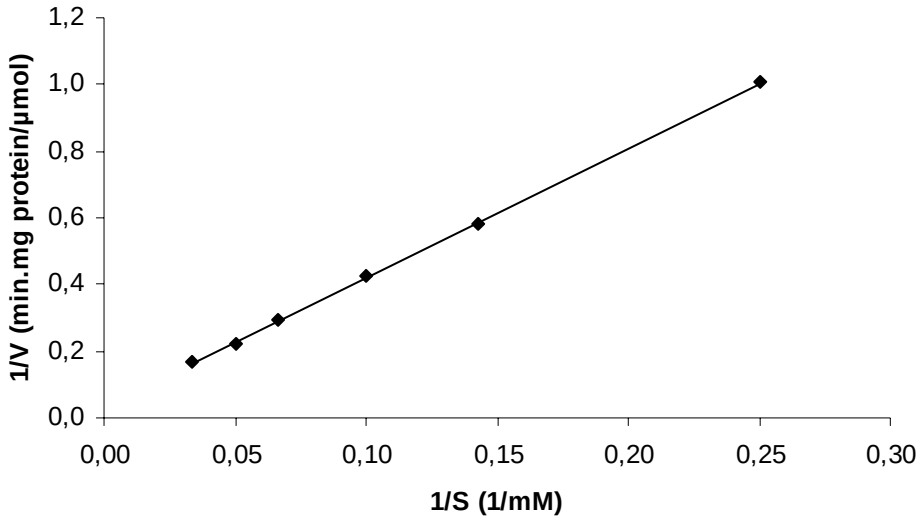


Figure 6.7 Lineweaver Burk plot for free catalase. Reactions were carried out in phosphate buffer (pH 7.0, 50 mM) at 35°C.

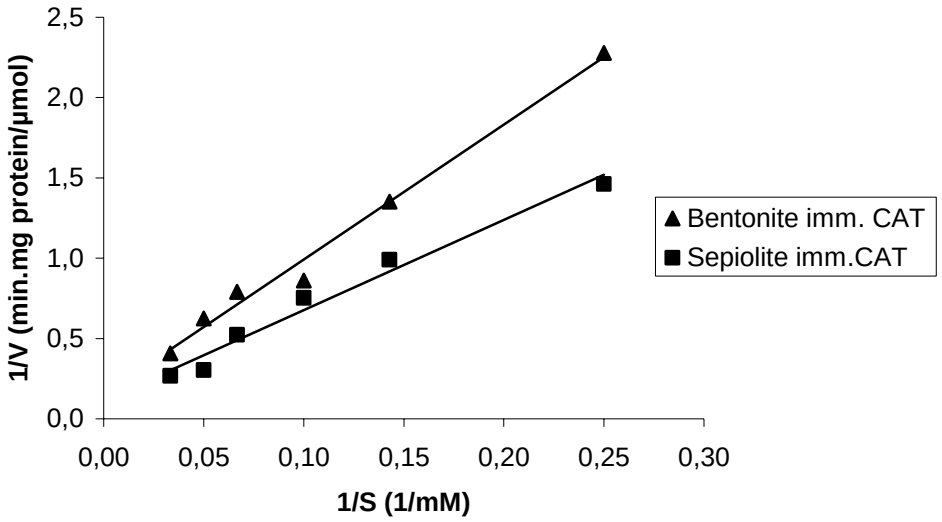


Figure 6.8 Lineweaver Burk plot for immobilized catalase. Reactions were carried out in phosphate buffer (pH 7.0, 50 mM) at 35°C.

6.4 Thermal Stability of Free and Immobilized Catalase

The enzyme thermal stability is one of the important criteria for its long-term and commercial application (Costa et al., 2001). It is often observed that immobilized enzyme has a higher thermal stability than the corresponding free enzyme because of the reduction of conformational flexibility in the immobilized enzyme (Çetinus & Öztop, 2003).

To determine the thermal stabilities of free and immobilized catalases, the samples in the phosphate buffer (pH 7.0) were incubated at various temperature values for different time intervals and then their activities were found at 25°C.

The thermal stability of free catalase at various temperatures for different preincubation times was shown in Figure 6.9.

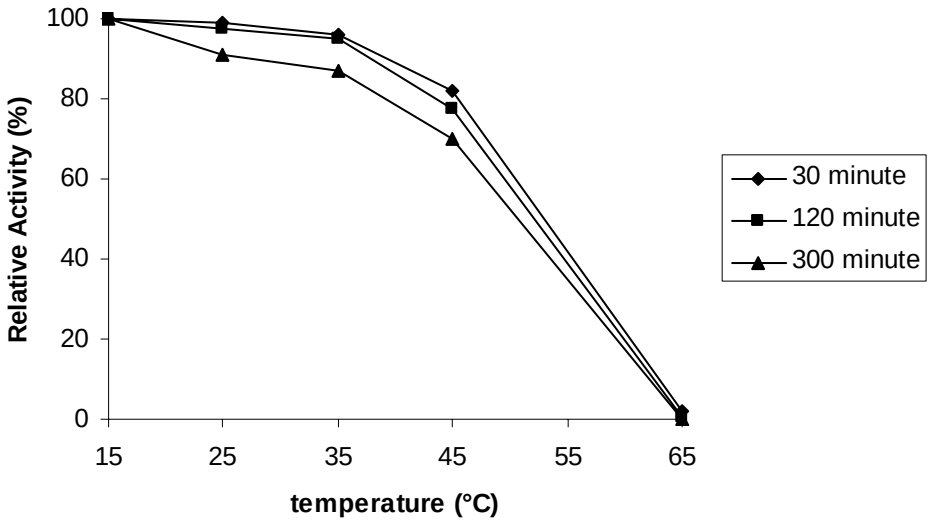


Figure 6.9 Thermal stability at various temperature of free catalase.Enzyme activity was determined at 25ºC in phosphate buffer pH 7.0 (50 mM).

As it is seen in Figure 6.9, the activity loss is increased when the preincubation time is increased, too. Free catalase lost all its activity at 65°C.

Figure 6.10 and 6.11 indicate the thermal stability of free and immobilized catalases at various temperature values as a function of different preincubation time.

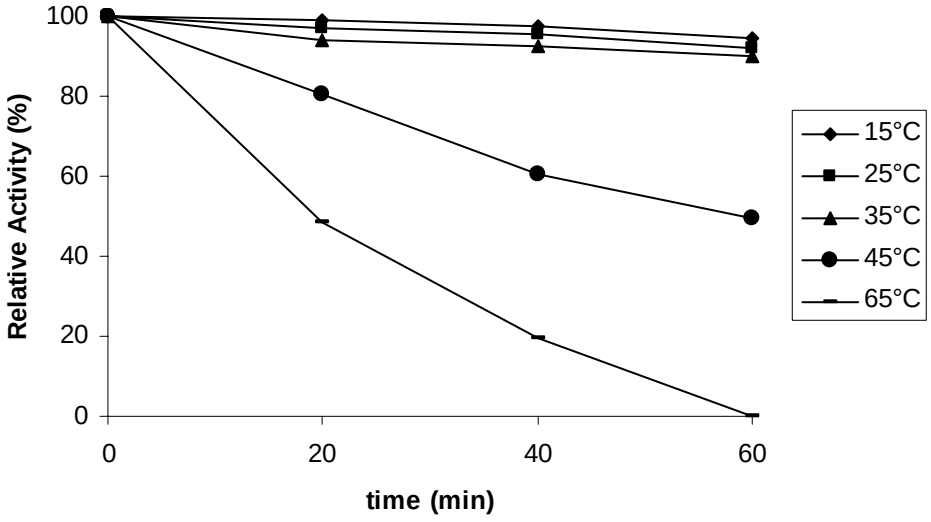
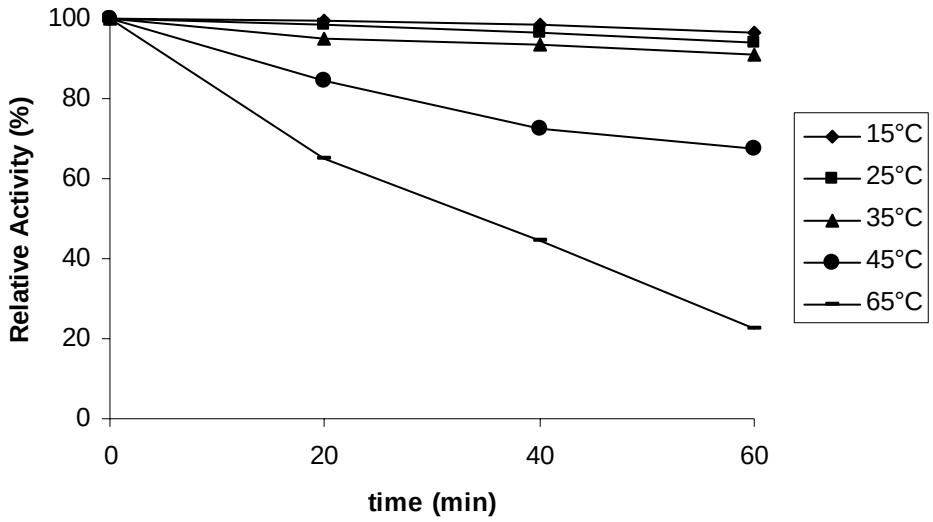
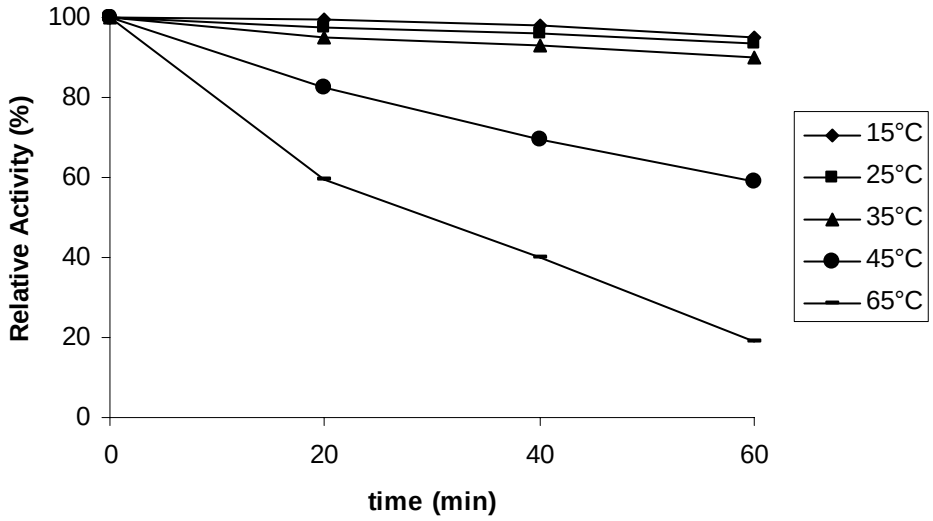


Figure 6.10 Thermal stability of free catalase at various temperature values. Enzyme activity was determined at 35°C in phosphate buffer pH7.0 (50mM).



a.



b.

Figure 6.11 Thermal stability of immobilized catalase at various temperature values. Enzyme activity was determined at 35°C in phosphate buffer pH7.0 (50mM). a. Bentonite immobilized catalase, b. Sepiolite immobilized catalase.

As seen in these figures there is no significant activity loss at 15, 25 and 35°C for free and immobilized catalases. But at 45°C; although free catalase lost about 50 % of its initial activity for 1 hour preincubation time, sepiolite immobilized catalase lost 41.1 % and bentonite immobilized catalase lost only 32.6 % of their initial activity

values for 1 hour preincubation time. And at 65°C free catalase lost all its initial activity. But sepiolite immobilized catalase and bentonite immobilized catalase remained about 19.1 % and 22.3 % of their initial activities for 1 hour preincubation time, respectively.

These results show that immobilized catalases have a higher thermal stability than free catalase for 1 hour preincubation time.

6.5 Operational Stability of Immobilized Catalase

The stability of immobilized enzymes provide a lot of advantageous in various biotechnological applications unlike its free counterparts (Akgöl et al, 2001). Unlike free enzymes, immobilized enzymes can be easily separated from product solutions and reused (Katchalski-Katzir, 1993).

Operational stability of the immobilized catalases were determined for 10 successive batch reactions at 25°C for 60 min. The results were presented in Figure 6.12 and Figure 6.13 for bentonite immobilized catalase and sepiolite immobilized catalase, respectively.

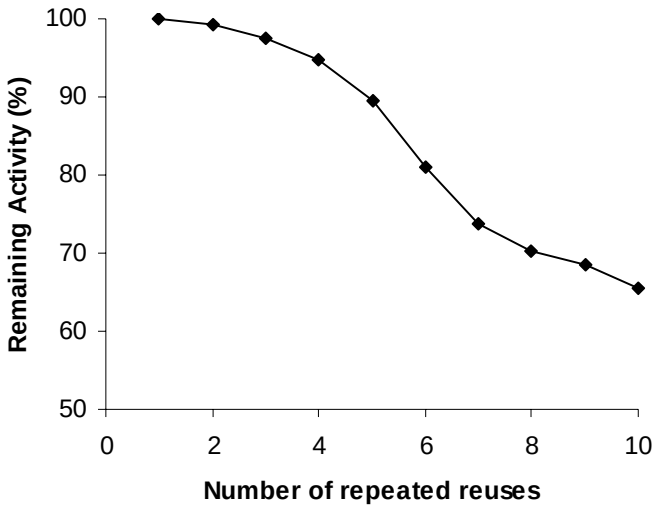


Figure 6.12 Operational stability of bentonite immobilized catalase.

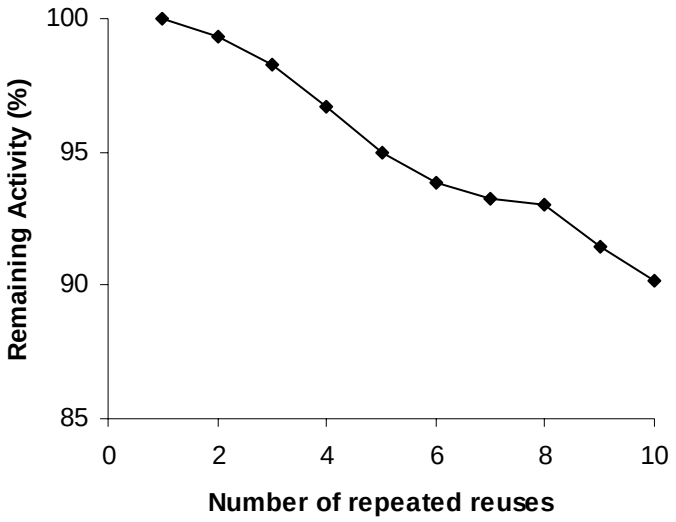


Figure 6.13 Operational stability of sepiolite immobilized catalase.

These figures indicate that bentonite immobilized catalase lost about 35 % of its initial activity after 10 cycles of batch experiments and sepiolite immobilized catalase lost about 10 % of its initial activity after 10 cycles of batch experiments, respectively.

This activity loss might have been formed because of the poisoning effect of substrate (Akgöl et al., 2001).

6.6 Storage Stability of Free and Immobilized Catalase

To find the storage stability of free and immobilized catalases, the samples were stored in a phosphate buffer (0.05 M, pH 7.0) at -15, 4 and 25°C and the activity measurements were carried out for a period of 95 days. Figure 6.14, Figure 6.15 and Figure 6.16 indicate the results for -15, 4 and 25°C, respectively.

Both free and immobilized catalases which were stored at -15°C were more stable than others stored at 4°C and 25°C.

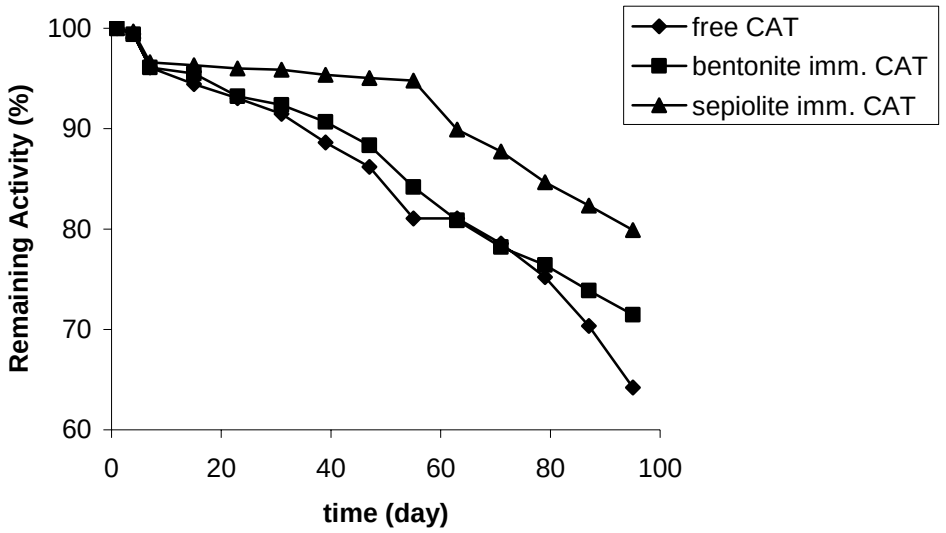


Figure 6.14 The change of storage stability of free and immobilized catalases at -15°C.

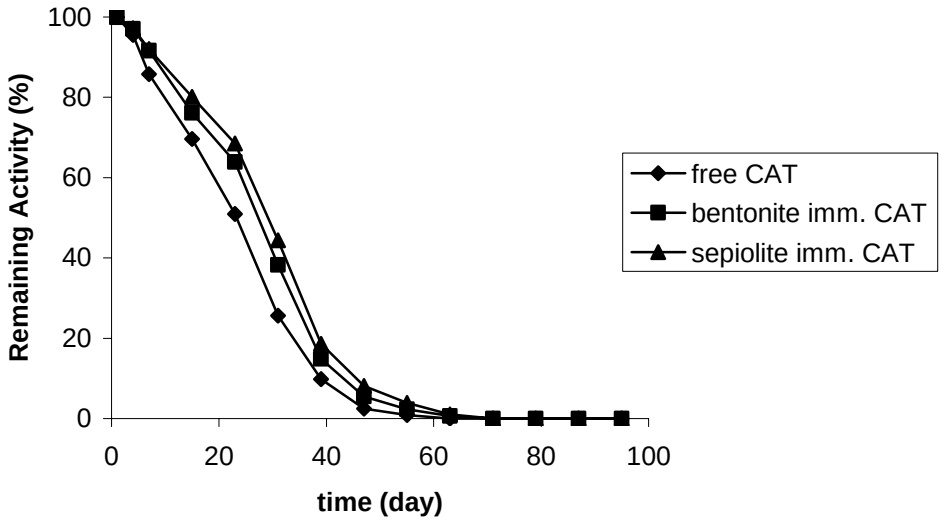


Figure 6.15 The change of storage stability of free and immobilized catalases at 4°C.

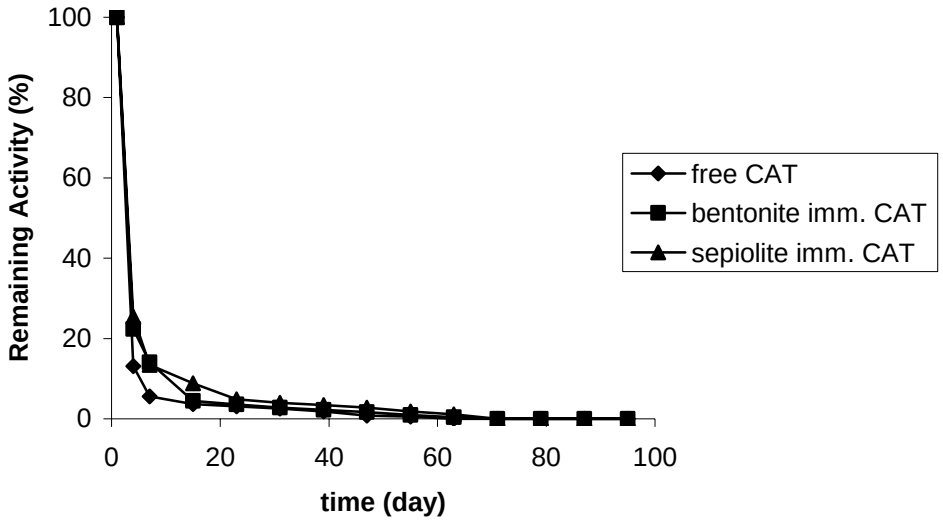


Figure 6.16 The change of storage stability of free and immobilized catalases at 25°C.

At -15°C, free enzyme lost about 35.78 % of its activity after 95 days. In the same interval, bentonite immobilized catalase and sepiolite immobilized catalase lost 28.52 % and 20.11 % of their activities, respectively. The enzyme residual activity decreased with the increase of the temperature. At 4°C and 25°C, all samples lost their activities within 71 days.

6.7 The Effect of Contact Time on The Immobilization of Catalase onto Bentonite and Sepiolite

To determine the optimum contact time for immobilization of catalase onto bentonite and sepiolite, the equilibrium concentrations were measured at certain times by spectrophotometrically and the amounts of catalase immobilized onto clays were calculated by the equilibrium concentrations. The curves that indicated the effect of contact time on adsorption was plotted the amount of catalase immobilized (mg/g) measured in the certain time intervals versus time.

The effect of contact time on the immobilization of catalase onto bentonite and sepiolite for various initial catalase concentrations (from 30 μg/mL to 200 μg/mL) were shown in Figure 6.17 and Figure 6.18, respectively. As seen in Figure 6.17 and

Figure 6.18, the immobilization reached equilibrium within the first hour for all initial catalase concentrations studied. And it was observed that increasing catalase concentrations were well in line with the increased amount of immobilized catalase.

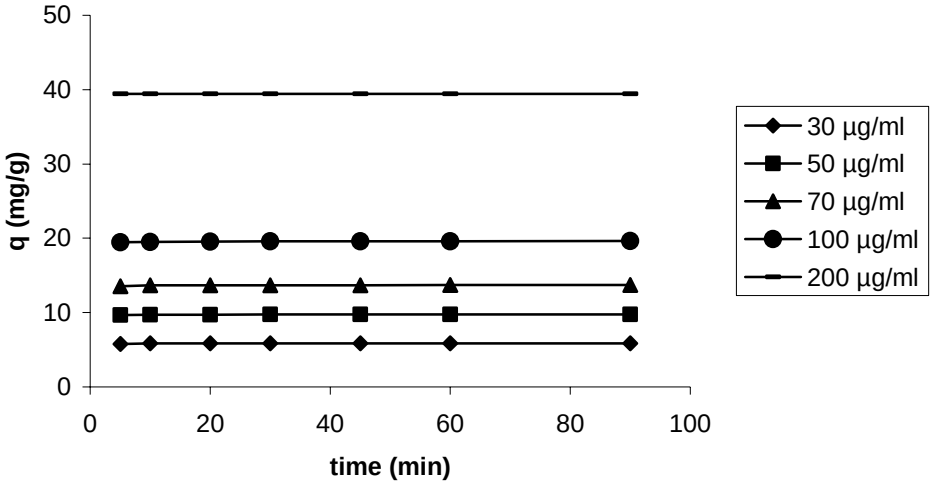


Figure 6.17 Adsorption kinetics of catalase onto bentonite at different initial catalase concentrations.

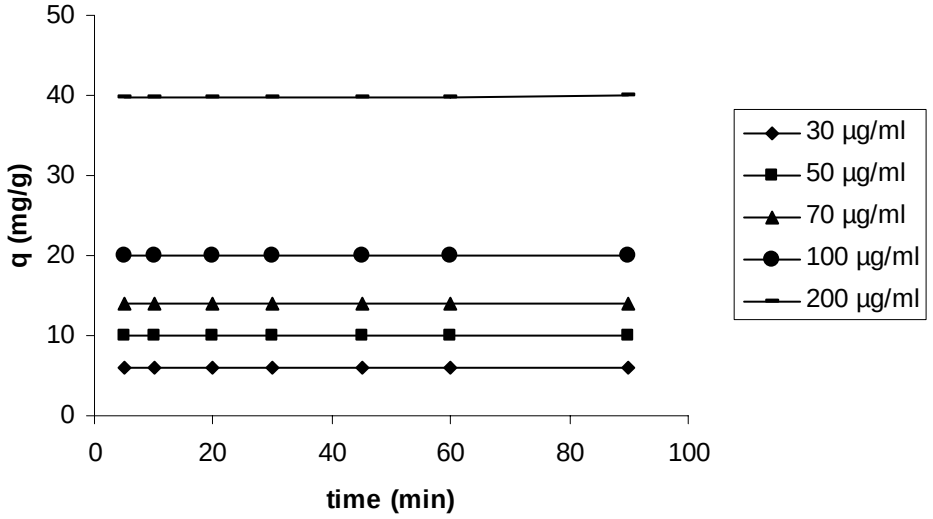


Figure 6.18 Adsorption kinetics of catalase onto sepiolite at different initial catalase concentrations.

6.8 Immobilization of Catalase onto Bentonite and Sepiolite

A set of samples for the catalase – bentonite and catalase – sepiolite suspensions were prepared with different concentrations. These samples were agitated in a temperature controlled shaker (Memmert) with shaking speed 105 rpm for 1 hour. Than the samples were centrifuged (5000 rpm, 5 min, 4°C) and the amount of free catalase was determined spectrophotometrically at 240 nm with Aebi method. The amount of immobilized catalases were calculated by measuring the initial and equilibrium concentrations of catalase in the reaction medium. The data obtained was used to form adsorption isotherms of catalase onto bentonite and sepiolite.

The adsorption isotherms of catalase on bentonite and sepiolite were determined by plotting the amount of catalase adsorbed by clays (q mg/g) versus equilibrium concentration of catalase (C_e mg/L). Figure 6.19 and Figure 6.20 show the catalase sorption isotherms at 15, 25, 35 and 45°C. In terms of the slope of initial portion of the curves, the shapes of these isotherms correspond to S type according to the Giles classification (Giles, McEwan, Nakhwa, & Smith, 1960).

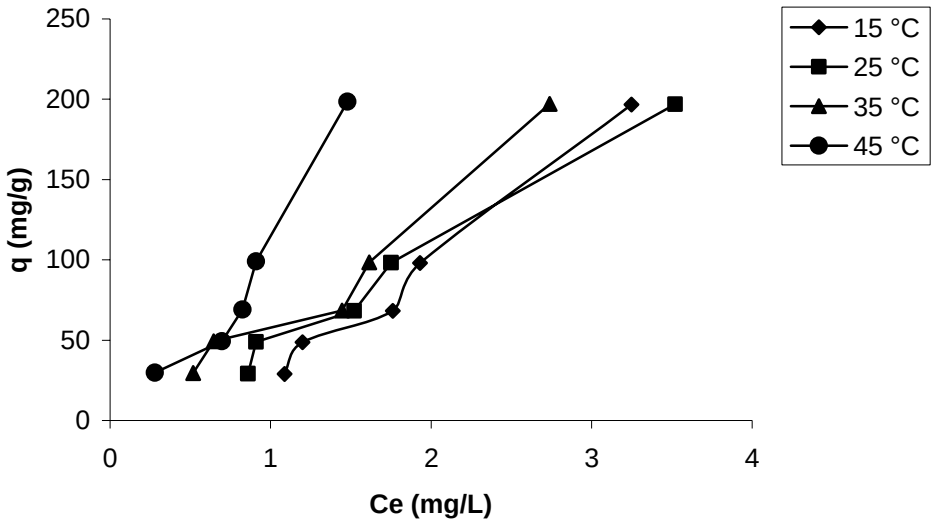


Figure 6.19 Adsorption isotherms of catalase onto bentonite at different temperatures

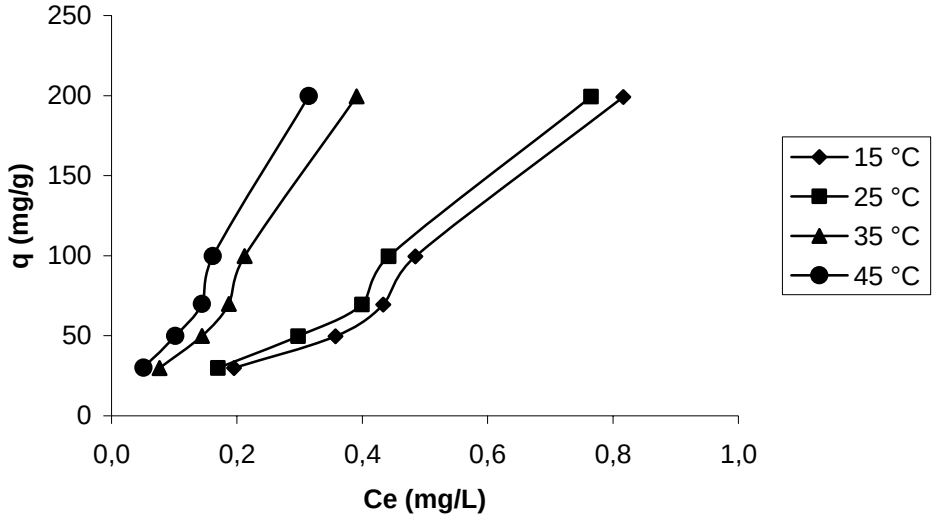


Figure 6.20 Adsorption isotherms of catalase onto sepiolite at different temperatures

As can be seen that adsorption ishoterms are found as S type which reflects weak interaction between adsorbate and adsorbent or van der Waals type of interaction. Physical adsorption (Multilayer adsorption) may be occured.

The fit of adsorption data to the Langmuir and Freundlich equations were investigated to find sorption capacities of the bentonite and sepiolite by using linear forms as below

$$1/q = 1/q_m + (1/bq_m).(1/C_e) \quad (1)$$

$$\log q = \log K_f + (1/n).\log C_e \quad (2)$$

where

C_e = the liquid phase concentration of adsorbate at equilibrium (mg/L),

q = the solid phase concentration of adsorbate at equilibrium (mg/g),

q_m = the maximum adsorption capacity (maximum amount that can be adsorbed by adsorbent as a monolayer (mg/g),

b = Langmuir constant regarding the adsorption energy,

K_f = adsorption capacity

$1/n = n_f$ = heterogeneity factor (adsorption intensity) (a measurement of linearity).

q_m and b parameters are obtained by plotting 1/q versus $1/C_e$ for the adsorption process of catalase onto bentonite and sepiolite at different temperatures (Figure 6.21 and Figure 6.22).

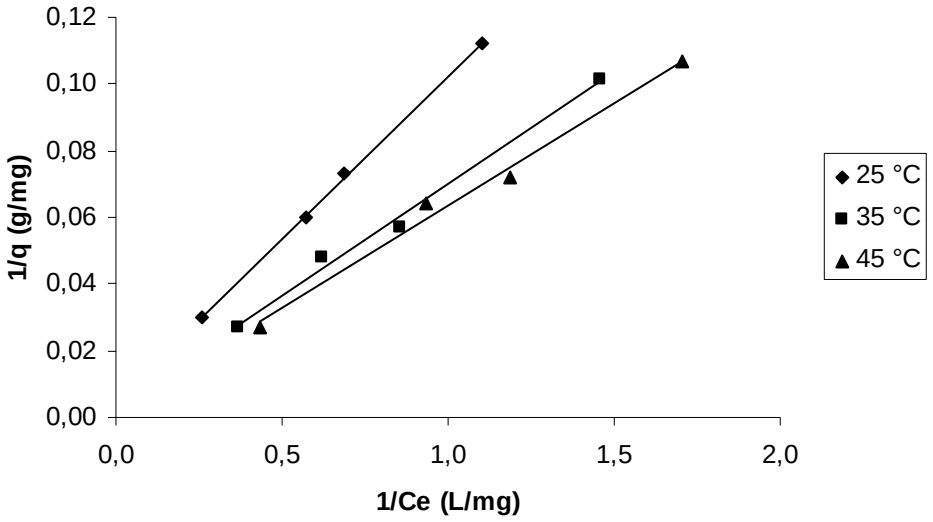


Figure 6.21 Langmuir isotherm plots for adsorption of catalase by bentonite.

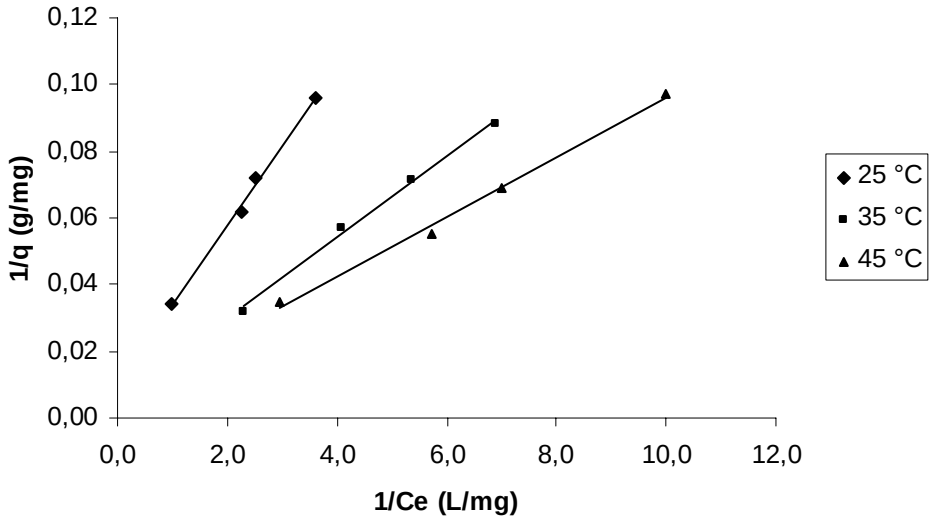


Figure 6.22 Langmuir isotherm plots for adsorption of catalase by sepiolite.

The sorption parameters of catalase on bentonite and sepiolite corresponding to Langmuir equation were summarized in Table 6.2 and Table 6.3. q_m (maximum adsorption capacity) values for bentonite are 212.77, 294.12 and 526.32 at 25°C,

35°C and 45°C, respectively. In addition, b values are 0.047 at 25°C; 0.051 at 35°C and 0.054 at 45°C. q_m values for sepiolite are 95.24, 188.68 and 147.06 at 25°C, 35°C and 45°C, respectively. In addition, b values are 0.44 at 25°C; 0.55 at 35°C and 0.76 at 45°C.

Table 6.2 The sorption parameters of Langmuir equation for catalase onto bentonite at different temperatures

Temperature (°C)	**R^2**	**q_m (mg/g)**	**b (L/mg)**
25°C	0.9995	212.77	0.047
35°C	0.9920	294.12	0.051
45°C	0.9904	526.32	0.054

Table 6.3 The sorption parameters of Langmuir equation for catalase onto sepiolite at different temperatures

Temperature (°C)	**R^2**	**q_m (mg/g)**	**b (L/mg)**
25°C	0.9956	95.24	0.44
35°C	0.9953	188.68	0.55
45°C	0.9938	147.06	0.76

The data are well in line with Langmuir model for bentonite and sepiolite. As seen from Table 6.2 and Table 6.3 bentonite's relative sorption capacity are higher than sepiolite's.

One should keep in mind that the isotherm shapes themselves can never prove the adsorption mechanism for adsorption; they can only provide a reasonable mechanism for adsorption. It can be added that adsorption mechanism must be proved by molecular spectroscopy, the other isotherm equation such as Dubinin Radushkevich (DR) equation and thermodynamic parameters. (http://www.dekker.com /servlet/product/productid/E-ESCS/toc).

To determine the adsorption process is favorable or unfavorable, the dimensionless constant separation factor was determined by Weber & Chakkravorti (1974) as below:

$$R_L = 1/ (1+bC_0) \quad (3)$$

where
b = Langmuir isotherm constant (L/mg),
C_0 = initial catalase concentration (mg/L).

The parameter R_L indicates the shape of the isotherm. If the R_L values are higher than 1, R_L indicates unfavourable adsorption. If the R_L values are 1, R_L indicates linear adsorption. R_L values between 0 and 1 indicate favourable adsorption. If the R_L values are zero, R_L indicates irreversible adsorption (Kadirvelu & Namasivayam, 2003).

Table 6.4 The sorption parameters of Langmuir equation for catalase onto bentonite at different temperatures

Temperature (°C)	**b (L/mg)**	**C_0 (mg/L)**	**R_L**
25°C	0.047	100	0.175
35°C	0.051	100	0.164
45°C	0.054	100	0.156

Table 6.5 The sorption parameters of Langmuir equation for catalase onto sepiolite at different temperatures

Temperature (°C)	**b (L/mg)**	**C_0 (mg/L)**	**R_L**
25°C	0.44	100	0.022
35°C	0.55	100	0.018
45°C	0.76	100	0.013

The values of R_L at different temperatures were reported in Table 6.4 and Table 6.5. As seen in these tables, dimensionless constant separation factor, R_L, were between 0 and 1. So the values show that adsorption of catalase onto bentonite and sepiolite is favourable.

In order to determine the constants K_f (relative sorption capacity) and n_f, (measure of the linearity of the sorption) the linear form of the equation shown below may be used to produce a graph of lnq against lnC_e.

$$lnq = \ln K_f + n_f \ln C_e \quad (4)$$

The graphs indicating adsorption of catalase onto bentonite and sepiolite which are formed by plotting lnq versus lnC_e are shown in the Figure 6.23 and 6.24, respectively.

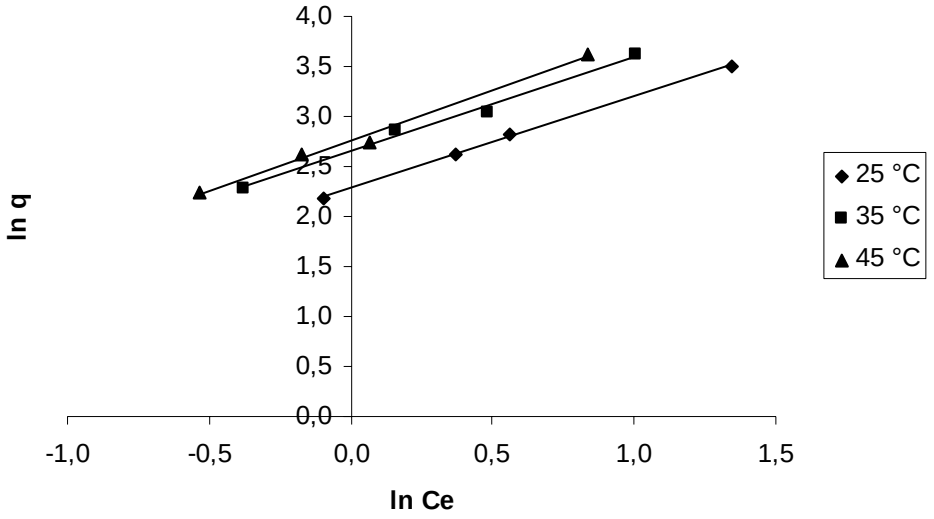


Figure 6.23 Freundlich isotherm plots for adsorption of catalase by bentonite.

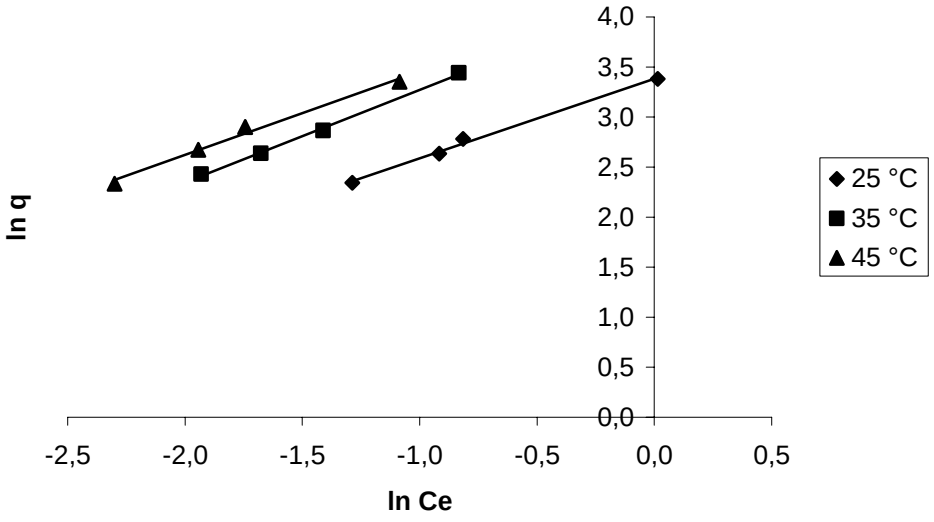


Figure 6.24 Freundlich isotherm plots for adsorption of catalase by sepiolite.

If the Langmuir and Freundlich equation is compared, it is said that the Langmuir isotherm yielded best fits to the experimental data.

Table 6.6 The sorption parameters of Freundlich equation for adsorption of catalase onto bentonite at different temperatures.

Temperature (°C)	**R^2**	**K_f (mg/g)**	**n_f**
25°C	0.9995	9.795	0.916
35°C	0.9902	14.218	0.934
45°C	0.9920	15.719	0.995

Table 6.7 The sorption parameters of Freundlich equation for adsorption of catalase onto sepiolite at different temperatures.

Temperature (°C)	**R^2**	**K_f (mg/g)**	**n_f**
25°C	0.9947	29.421	0.7946
35°C	0.9974	66.733	0.9277
45°C	0.9877	72.937	0.8338

K_f (relative sorption capacity) and n_f (measure of the linearity of the sorption) were found by using the linear regression method applied to the straight lines in Figure 6.23 and Figure 6.24. The K_f and n_f values were shown in Table 6.6 and 6.7 for bentonite and sepiolite. As can be seen from Table 6.6 and 6.7, K_f values increase by increasing temperature. This result indicates that adsorption capacities of bentonite and sepiolite increase when temperature increases. It can be easily understood that the values of n_f are smaller than 1, reflecting the favorable adsorption (Ghiaci, Abbaspur, Kra, & Seyedeyn, 2004). Although n_f values decrease from bentonite to sepiolite, K_f values increase from bentonite to sepiolite. This observation indicates that the adsorption capacities increase from bentonite to sepiolite.

Both these theories suffer from the disadvantage that equilibrium data over a wide concentration range can not be fitted with a single set of constants (Mc Kay, El Geundi & Nassar, 1987). The n_f values are less than 1 in the form of linear Freundlich equation. n_f is indicator of heterogeneity factor. If the value of n_f is equal

to unity, the adsorption is linear. It indicates that adsorption sites are not homogeneous in energy.

6.9 Adsorption Kinetics

In order to characterize the kinetic behavior of a reaction it is desirable to determine how the rate of reaction varies as the reaction progresses. Lagergren's first order kinetic equation has been most widely used to describe the solute adsorption on various adsorbents (Ho, 2003). The first-order Lagergren expression based on the solid capacity is (Lagergren, 1898)

$$\log (q_e-q) = \log q_e - (k_1/2.303)t \quad (5)$$

where q_e and q are the amount of adsorbate adsorbed at equilibrium and at time t, respectively (mg/g), and k_1 is the rate constant (min^{-1}).

Another widely used kinetic expression is the pseudo second-order rate expression derived by Ho and McKay (Ho & McKay, 2003) where sorption capacity was assumed to be proportional to the number of active sites occupied on the sorbent. The pseudo second-kinetic rate law can be expressed as:

$$t/q = 1/k_2q_2^2 + (1/q_2)t \quad (6)$$

where k_2 is the pseudo second-order rate constant with a unit of g/mg.min.

And also the intraparticle diffusion model was used to find out adsorption mechanism.

$$q_t = k_p\, t^{0,5} + C \quad (7)$$

where q_t is the amount of adsorbate adsorbed on adsorbent at various time t (mgg^{-1}), k_p is the intraparticle diffusion rate constant (mg g^{-1} $min^{-0,5}$) and C is the intercept for the intraparticle diffusion model.

In this thesis both of these models were tested for the adsorption of catalase onto bentonite and sepiolite. The straight-line plots of $1/q_t$ versus 1/t for the pseudo-first-order reaction and t/q_t vs (versus) t for the pseudo-second order reaction for the adsorption of catalase onto bentonite and sepiolite have also been tested to obtain the rate parameters (Özcan & Özcan, 2004). The best-fit model was determined based on the linear regression correlation coefficient values. The results were presented in Figure 6.25 and Figure 6.26. The pseudo second order model is well in line with our experimental results. (Table 6.8 and Table 6.9).

Table 6.8 The pseudo-second-order parameters for adsorption of catalase onto bentonite at different initial catalase concentrations.

Concentration (µg/mL)	**R^2**	**k_2 (g/mg.min)**	**$q_{2(exp)}$(mg/g)**	**$q_{2(cal)}$(mg/g)**
30	1.0000	14.54	5.86	5.87
50	1.0000	3.29	9.74	9.75
70	1.0000	1.56	13.70	13.72
100	1.0000	1.37	19.80	19.61
200	1.0000	1.28	39.46	39.53

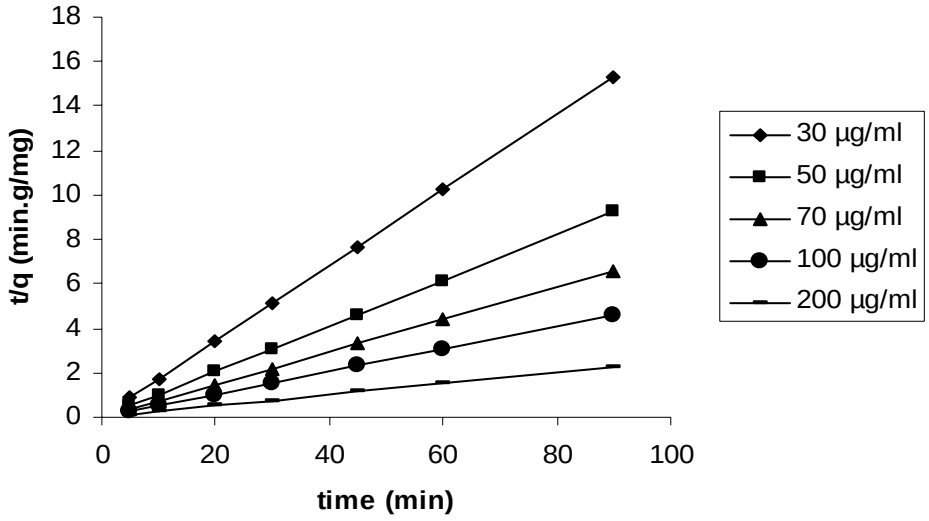


Figure 6.25 Pseudo-second-order plots for adsorption of catalase onto bentonite at different initial catalase concentrations.

Table 6.9 The pseudo-second-order parameters for adsorption of catalase onto sepiolite at different initial catalase concentrations.

Concentration (µg/mL)	**R^2**	**k_2(g/mg.min)**	**$q_{2(exp)}$(mg/g)**	**$q_{2(cal)}$(mg/g)**
30	1.0000	11.43	5.99	5.99
50	1.0000	10.04	9.98	9.98
70	1.0000	6.39	13.99	13.99
100	1.0000	1.67	19.98	20.00
200	1.0000	0.70	39.85	39.84

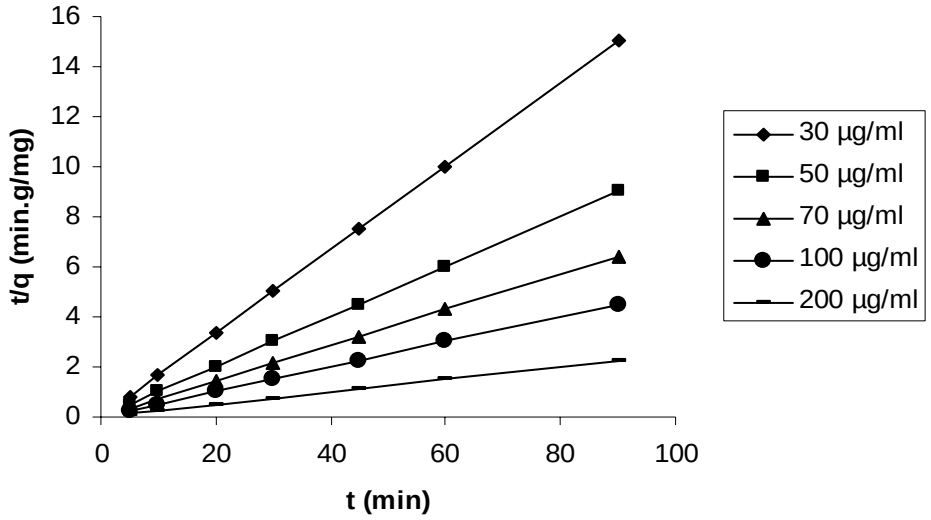


Figure 6.26 Pseudo-second-order plots for adsorption of catalase onto sepiolite at different initial catalase concentrations.

Table 6.10 The pseudo-second-order parameters for adsorption of catalase onto bentonite at different temperatures.

Temperature (°C)	**R^2**	**k_2(g/mg.min)**	**q_{cal}(mg/g)**
15°C	1.0000	5.202	19.608
25°C	1.0000	6.477	19.646
35°C	1.0000	8.602	19.685
45°C	1.0000	12.852	19.724

Table 6.11 The pseudo-second-order parameters for adsorption of catalase onto sepiolite at different temperatures.

Temperature (°C)	R^2	k_2(g/mg.min)	q_{cal}(mg/g)
15°C	1.0000	5.040	19.920
25°C	1.0000	6.300	19.920
35°C	1.0000	6.275	19.960
45°C	1.0000	8.367	19.960

6.10 Thermodynamic Parameters of Adsorption

In order to understand the effect of temperature better, it is important to know the change of thermodynamic parameters such as standard Gibbs free energy change, ΔG, standard enthalpy, ΔH, and standard entropy, ΔS. The Gibb's free energy change of adsorption is calculated from the following equation (Özcan & Özcan, 2004):

$$\Delta G^\circ = -RT \ln K_d \quad (8)$$

where

K_d = The equilibrium constant at temperature T,

R = gas constant (8.314 J mol^{-1} K^{-1}),

T = absolute temperature (K).

For each temperature, the same t value was selected (60 min.) and from this value, firstly q_t then C_s and C_e values were calculated by using Pseudo-second-order equation. K_d was estimated using the following equation;

$$K_d = C_s / C_e \quad (9)$$

where

C_e = equilibrium concentration

C_s = the amount of adsorbed

Table 6.12 The equilibrium constant (K_d) for adsorption of catalase onto bentonite at different temperatures.

Temperature (K)	**C_s**	**C_e**	**K_d**	**ln K_d**	**$1/Tx10^{+3}$**
298	98.149	1.851	53.025	3.971	3.36
308	98.564	1.436	68.638	4.229	3.25
318	99.029	0.971	101.987	4.625	3.14

Table 6.13 The equilibrium constant (K_d) for adsorption of catalase onto sepiolite at different temperatures.

Temperature (K)	**C_s**	**C_e**	**K_d**	**ln K_d**	**$1/Tx10^{+3}$**
298	99.510	0.490	203.082	5.314	3.36
308	99.695	0.305	326.869	5.790	3.25
318	99.790	0.210	475.190	6.164	3.14

Standard enthalpy, ΔH°, and standard entropy, ΔS°, were calculated by using van't Hoff equation:

$$\ln K_d = (\Delta S°/R) - (\Delta H°/RT) \quad (10)$$

The curve was plotted ln K_d versus (1/T) (Figure 6.27). The slope of van't Hoff plot is equal to ΔH°/R and the intercept is equal to ΔS°/R. These thermodynamic parameters were given in Table 6.14 and in Table 6.15.

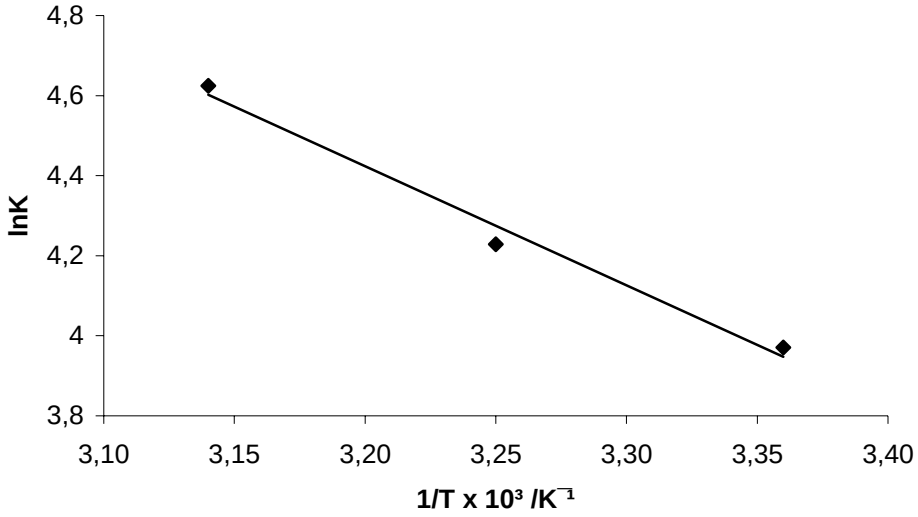


Figure 6.27 van't Hoff of $\ln K_d$ vs 1/T for adsorption of catalase onto bentonite.

Table 6.14 Thermodynamic parameters for adsorption of catalase onto bentonite

Temperature/K	**ΔH°/kJ mol^{-1}**	**ΔS°/J mol^{-1}K^{-1}**	**−ΔG°/kJ mol^{-1}**
298	24.72	115.9	9.84
308			10.83
318			12.23

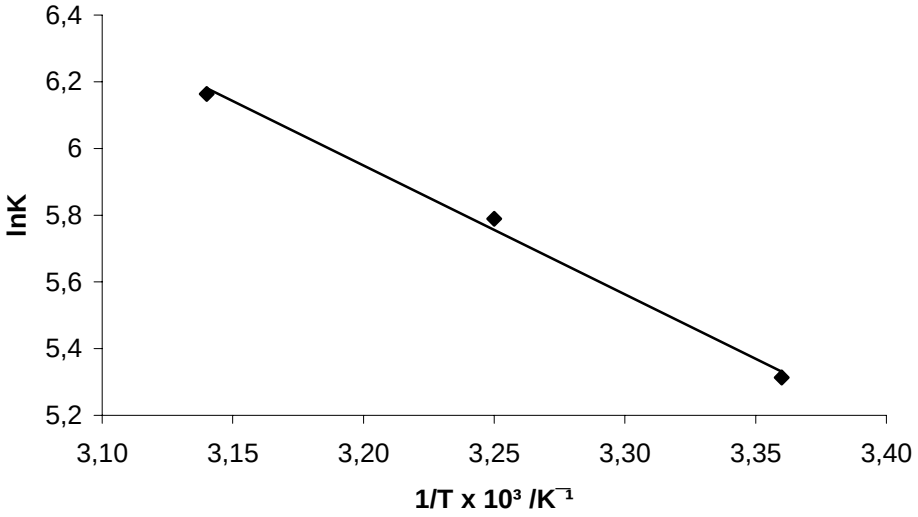


Figure 6.28 van't Hoff of $\ln K_d$ vs 1/T for adsorption of catalase onto sepiolite.

Table 6.15 Thermodynamic parameters for adsorption of catalase onto sepiolite

Temperature/K	ΔH°/kJ mol^{-1}	ΔS°/J mol^{-1}K^{-1}	−ΔG°/kJ mol^{-1}
298	32,13	152.3	13.17
308			14.82
318			16.30

The negative values of ΔG^0 indicate that the adsorption of catalase onto bentonite and sepiolite are spontaneous. The Gibbs free energy value at 318K is the highest negative value than the values of other temperatures. For that reason more energetically favourable adsorption is occurred at 318K for both of the adsorbent. ΔH° were found to be 24.72 kJ/mol for bentonite and 32.13 kJ/mol sepiolite. These values confirm endothermic nature of adsorption. ΔS° was calculated as 0.116 kJ/mol for bentonite and 0.152 kJ/mol for sepiolite. The positive ΔS° values indicate the increased randomness at the solid/solution interface during the adsorption of catalase on bentonite and sepiolite.

6.11 SEM Analysis

The surface morphologies of clay and catalase loaded clay which examined scanning electron microscopy (SEM) were presented in Figure 6.31-Figure 6.32 and Figure 6.33-Figure 6.34 for bentonite and sepiolite, respectively.

In these figures, silicate layers with different sizes can be seen and disperse in various direction. It can be added that the distribution of silicate particles are heterogeneous.

After loading, in the photographs of catalase loaded clay, small white grains can be seen. This can be due to fact that catalae particles were accumulated on the surface of clay sample.

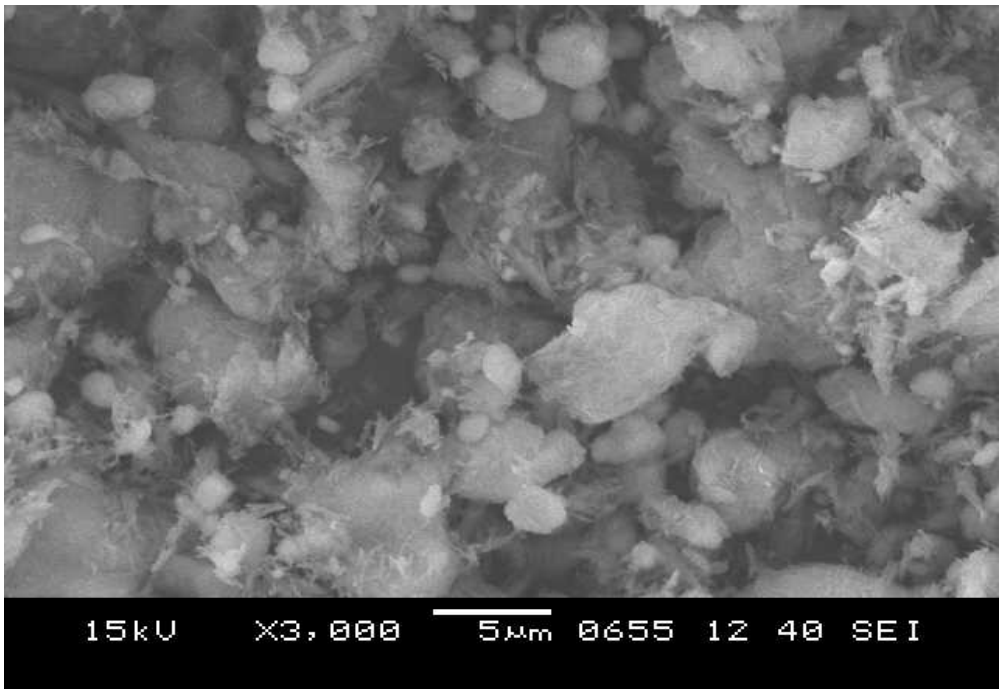


Figure 6.31 SEM Micrographs of bentonite (x3000)

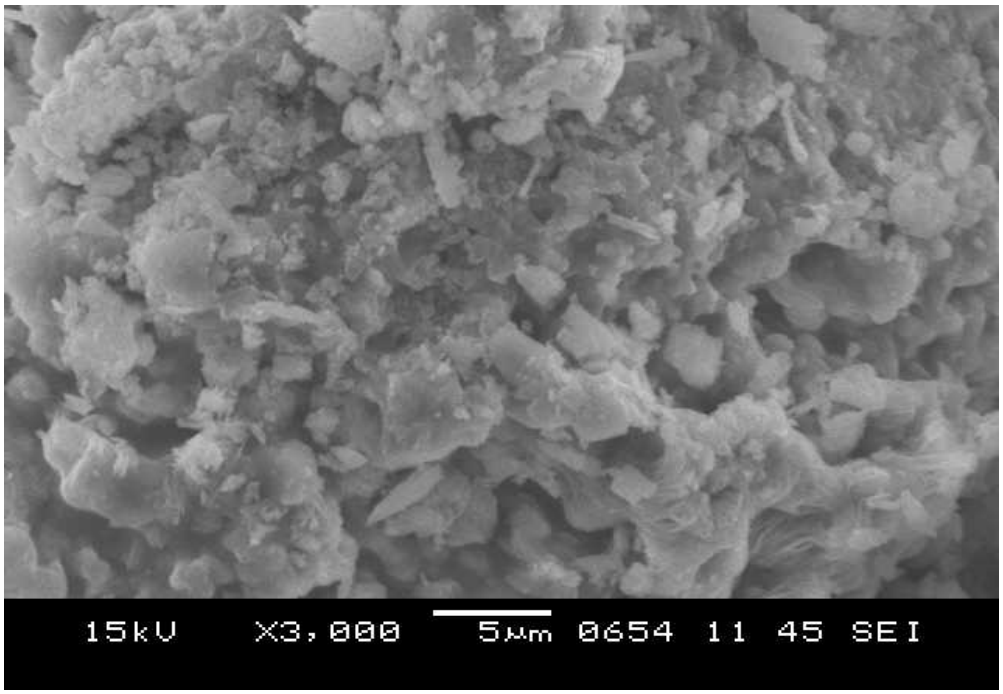


Figure 6.32 SEM Micrographs of catalase immobilized bentonite (x3000)

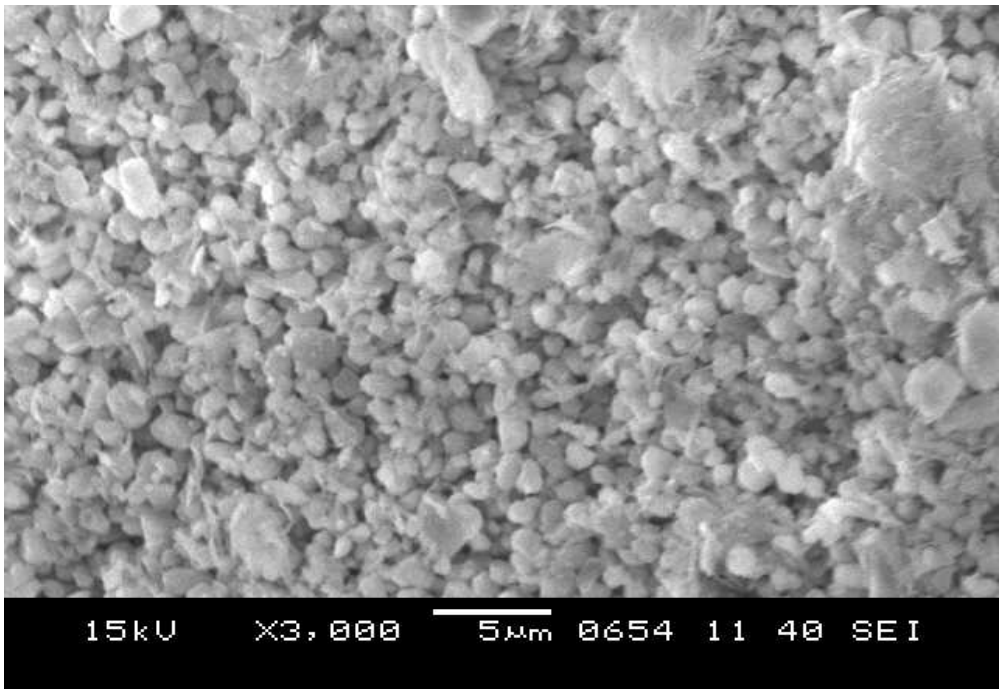


Figure 6.33 SEM Micrographs of sepiolite (x3000)

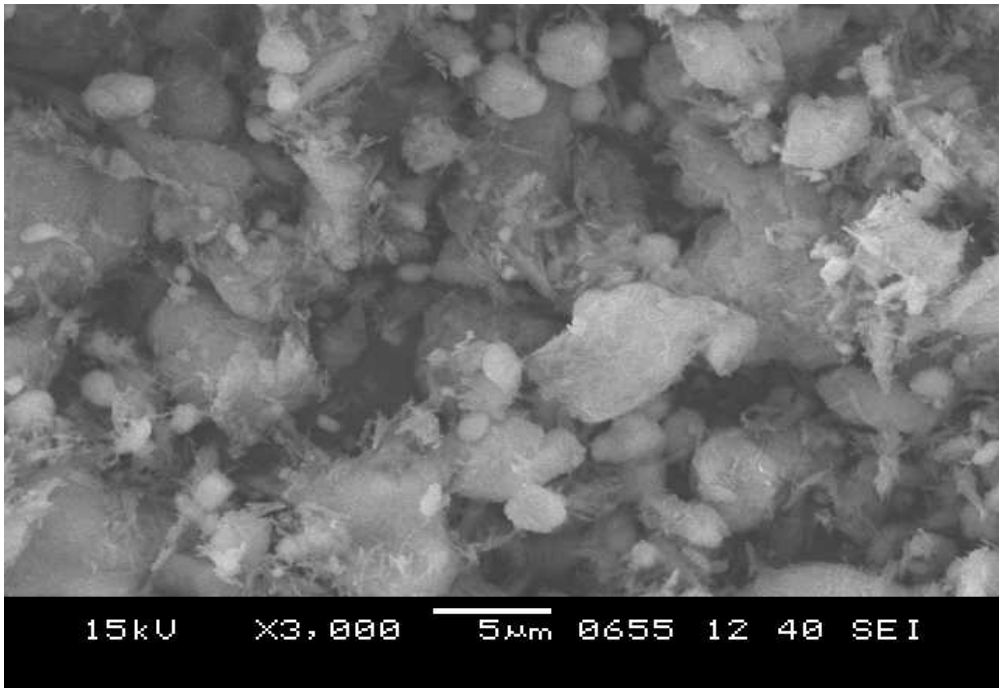


Figure 6.34 SEM Micrographs of catalase immobilized sepiolite (x3000)

CHAPTER SEVEN
CONCLUSION

7. Conclusion

The effects of the temperature on activity of free and immobilized catalases were studied within the temperature range of 15 to 65°C (Figure 6.1). As can be seen from results, the maximum activities were found at 35°C for free and immobilized catalases. Immobilized catalases showed higher activity than free catalase at high temperatures. The results may be caused by changing physical and chemical properties of the enzyme by immobilization. Li et al (2004) proposes that the covalent bond may reduce the conformational flexibility of the immobilized enzymes, which makes the immobilized enzymes require a higher temperature to form the proper conformation to recognize and bind the substrate molecules and thereby increase the optimum temperature. Accordingly, it also results in a higher temperature resistance than free enzyme. The increased resistance of the immobilized enzyme in the various temperature values indicates good temperature stability of immobilized enzyme. Our results were agree with the results of Li et al (2004). In addition, energy from heat may positively effect the rate of enzymatic catalysis.

The pH of the environment has a considerable influence on the performance of any enzyme (Costa et al., 2002). In this study, it is found that the activities of immobilized catalases at alkaline pHs was higher than the activity of free catalase. On the other hand, formation of covalent bonding between carrier and enzyme provide stability via optimizing pH of reaction. Ye et al (2005) also concluded that strong interactions between carrier and immobilized enzyme may be associated with intra-molecular forces which affect conformational stability of enzyme. Shentu, Wu & Song (2005) also indicated that pH of the experimental conditions are important for immobilization of catalase. Catalase and clays are different ionisation state. Inasmuch as catalase has no net charge at its isoelectrical point, maximum immobilization from aqueous solution is generally observed at this point. In the present study the optimum immobilization pH for catalase is found as 7. However,

catalase is negatively charged in this pH likewise support material. Therefore, it can be said that hydrophobic interactions between catalase and support material can be more effective than electrostatic repulsion.

The activities of free and immobilized catalases were decreased with increasing ionic strength as it was expected. Since ionic strength increases the electrostatic interactions, generally enzymes loss their activities at higher ionic conditions.

Optimum conditions for immobilization were determined as 45ºC, pH 7 and 50 mM. When the temperature is increased, the adsorption capacities of clays are decreased.

The initial rates of H_2O_2 decomposition were obtained for various levels of H_2O_2 solutions. These data were plotted according to the method of Lineweaver–Burk and are shown in Fig.6.7 for free catalase and in Fig.6.8 for immobilized catalases. V_{max} and K_m values are found to be 30.1 μmol/min mg protein and 116.8 mM, respectively, for free catalase; 6.6 μmol/min mg protein and 55.6 mM, respectively, for bentonite immobilized catalase and 8.7 μmol/min mg protein and 49.2 mM, respectively, for sepiolite immobilized catalase.

V_{max} defines the highest possible velocity when all the enzyme is saturated with substrate, therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme, but may be affected by diffusion constrains. K_m is defined as the substrate concentration that gives a reaction velocity of 1/2 V_{max}. This parameter reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects. Comparison of the K_m for a given enzyme in both the immobilized and free states provides information about the interaction between the enzyme and its support. An increase in K_m once an enzyme has been immobilized, indicates that the immobilized enzyme has an apparent lower affinity for its substrate than that of the free enzyme does, which may be caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate

binding, or diffusional resistance to solute transport near the particles of the support. (Ye et al, 2005).

Figure 6.9 shows the thermal stability of free catalase at various temperature values. It indicates that the activity loss of free enzyme is well in line with increasing preincubation time. And also it indicates that the activity loss of free enzyme is increased when the temperature is increased, too.

The operational stabilities of bentonite immobilized catalase and sepiolite immobilized catalase were shown in Figure 6.12 and 6.13. The results showed that bentonite and sepiolite immobilized catalases remained 65 % and 90 % of their initial activities after 10 cycles of batch experiments, respectively.

Figure 6.14, 6.15 and 6.16 indicate the storage stabilities of free and immobilized catalases at various temperature values as a function of different time intervals. The samples which were stored at -15°C were more stable than others stored at 4°C and 25°C. Although free and immobilized enzymes showed similar trend in terms of activity losses dependent on time, immobilized CAT might be used industrially according to this result.

The immobilization of catalase onto bentonite and sepiolite from aqueous solution were studied in a batch system as a function of temperature, ionic strength, pH conditions and contact time. The optimum contact time for immobilization of catalase onto bentonite and sepiolite were found as 1 hour by plotting the amount of catalase immobilized (q, mg/g) measured in the certain time intervals versus time (see Fig. 6.17 and 6.18). Since the lower contact time such 1 hour, these materials can have advantages in immobilization of catalase to other support materials.

The shapes of the adsorption isotherms of catalase on bentonite and sepiolite correspond to S type according to the Giles classification as shown in Fig.6.19 and 6.20.

Linear form of Langmuir and Freundlich adsorption isotherms are applied to evaluate adsorption data. As can be seen Table 6.2 - 6.3 and Table 6.6 – 6.7 Langmuir adsorption isotherm gave a better fit than the Freundlich model based on the correlation coefficients (R^2) values. By using Langmuir equation, maximum adsorption capacities (q_m) of bentonite and sepiolite were obtained as 212.77 mg g^{-1} at 25°C, 294.12 mg g^{-1} at 35°C and 526.32 mg g^{-1} at 45°C (for bentonite) and 95.24 mg g^{-1} at 25°C, 188.68 mg g^{-1} at 35°C and 147.06 mg g^{-1} at 45°C (for sepiolite), respectively. Favourable adsorption for catalase was occurred since the values of R_L (dimensionless separation factor) were between 0-1 and Freundlich n_f (heterogeneity factor) values were smaller than 1. The values of R_L (Dimensionless separation factor) and n_f values at different temperatures are reported in Table 6.4 – 6.5 and Table 6.6 – 6.7 for bentonite and sepiolite immobilized catalases, respectively.

Two kinetic models including the pseudo-first-order equation and pseudo-second-order equation were employed to determine the kinetic nature of adsorption system. The kinetic data corresponded well with the pseudo-second-order kinetic model where the rate constants decreased as initial dye concentrations increased. It is clear that in Table 6.8 and 6.9 the sorption capacities at equilibrium, q_2, are increased with increasing initial catalase concentrations. As can be seen from Table 6.10 and 6.11, the values of the sorption capacity at equilibrium, q_2, are increased from 19.608 mg g^{-1} to 19.724 mg g^{-1} for bentonite and from 19.920 mg g^{-1} to 19.960 mg g^{-1} for sepiolite when the temperature is increased from 15ºC to 45ºC. This suggests that higher temperature values favour catalase immobilization.

The values of $\Delta H_.$ and ΔS were pozitive for the adsorption of catalase onto bentonite and sepiolite. The pozitive value of $\Delta H_.$ indicates that the adsorption process is endothermic in nature and the pozitive value of ΔS showed the increase in degree of freedom. The negative value of ΔG demonstrates a spontaneous and favourable adsorption process.

Consequently, since lower contact time, stability in wider temperature, pH and ionic strength conditions, bentonite and sepiolite can be used for immobilization of

catalase from aqueous solution. These materials can be easily found and modified for different purposes in our country. Therefore, the present study proposes an alternative use of clay materials.

REFERENCES

Adamczyk, Z. (2002). Adsorption of particles: Theory. *Encyclopedia of Surface And Colloid Science*. 499–516. Retrieved April 23, 2003, from http://dekker.com/servlet/product/DOI/101081EESCS12001 2922.

Aebi, H. E. (1974). Catalase In: Bergmeyer HU, editor. *Methos of enzymatic analysis* (3rd ed.) (672-84) Weinheim, Germany: Verlag Chemie.

Akgöl, S., & Dinçkaya, E. (1999). A novel biosensor for specific determination of hydrogen peroxide: catalase enzyme electrode based on dissolved oxygen probe. *Talanta*, 48, 363-367.

Akgöl, S., Kaçar, Y., Özkara, S., Yavuz, H., Denizli, A., & Arica, M. Y. (2001). Immobilization of catalase via adsorption onto L-histidine grafted functional pHEMA based membrane. *Journal of Molecular Catalysis B: Enzymatic*,15, 197-206.

Aksu, Z., & Kabasakal, E. (2004). Batch adsorption of 2,4-dichlorophenoxy-acetic acid (2,4-D) from aqueous solution by granular activated carbon. *Separation and Purification Technology*, 35, 223-240.

Alkan, S., Ceylan, H., & Arslan, O. (2005). Bentonite-supported catalase. *Journal of the Serbian Chemical Society*, 70, 721-726.

Allen,S. J., Gan, Q., Matthews, R., & Johnson, P. A. (2003). Comporison of optimized isotherm models for basic dye adsorption by kudzu. *Bioresource Technology*, 88, 143-152. Retrieved June 22, 2004, from Elsevier database.

Alvarez A., (1984) Sepiolite: Properties and Uses, in: E. Galán (Ed.), In Palygorskite–Sepiolite, Occurrences, Genesis and Uses, Dev. Sedimentol., vol. 37, Elsevier, Amsterdam.

Arslanoğlu, F. N., Kar, F., & Arslan, N. (2005). Adsorption of dark coloured compounds from peach pulp by using powdered- activated carbon. *Journal of Food Engineering*, 68, (4), 409-417.

Atkins, P., & Paula, J., (2006). *Physical Chemistry for the Life Sciences*. (2nd ed.) Oxford: New York.

Bajpai, A. K., & Sachdeva, R.. (2002). Immobilization of diastase onto acid-treated bentonite clay surfaces. *Colloid & Polymer Science*, 280, 892-899.

Bhattacharyya, K. G., & Gupta, S. S. (2006). Pb(II) uptake by kaolinite and montmorillonite in aqueous medium : Influence of acid activation of the clays. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, 277, 191-200.

Bilgiç C., (2005) Investigation of the factors affecting organic cation adsorption on some silicate minerals, *J. Colloid Int. Sci*., 281, 33-38.

Blanco, R. M., Terreros, P., Fernandez-Perez, M., Cristina, O., & Diaz-Gonzalez, G. (2004). Functionalization of mesoporous silica for lipase immobilization characterization of the support and the catalysts. *Journal of Molecular Catalysis B : Enzymatic*, 30, 83-93.

Blatt, H., Middleton, G., & Murray, R. (1980). Origin of sedimentary rocks (2d ed.): Englewood Cliffs, N.J., (766) Prentice-Hall.

Bouberka, Z., Kacha, S., Kameche, M., Elmaleh, S., & Derriche, Z. (2005). Sorption study of an acid dye from an aqueous solutions using modified clays. *Journal of Hazardous Materials B*, 119, 117-124.

Brauner, K. & Preisinger, A. (1956) Structure of Sepiolite, Miner. *Petrogr. Mitt*., 6, 120-140.

Burns, R. G. (1986). Interaction of enzymes with soil minerals and organic colloids. In: Huang, P. M., Schnitzer, M. (Eds.), Interaction of Soil Minerals with Natural Organics and Microbes. Soil Science Society of America, Madison, WI, pp. 439-452.

Catalase. (n.d.). Retived August 10, 2006, from http://www.all-science-fair-projects.com/science_fair_projects/105/901/69adbb37c02f0fb4f8b674fbae189d9f.html

Choi, M. M. F., & Yiu, T. P. (2004). Immobilization of beef liver catalase on eggshell membrane for fabrication of hydrogen peroxide biosensor. *Enzyme and Microbial Technology*, 34, 41-47.

Classifications of Enzymes. (n.d.). Retrieved November 3, 2006, from http://www.web-books.com/Mobio/Free/Ch2E1.htm

Costa S. A., Tzanov, T., Paar, A., Gudelj, M., Gübitz, G. M., & Cavaco-Paulo, A. (2001). Immobilization of catalases from Bacillus SF on alumina for the treatment of textile bleaching effluents. *Enzyme and Microbial Technology*, 28, 815-819.

Costa, S. A., Tzanov, T., Carneiro, A. F., Paar, A., Gübitz, G. M., & Cavaco-Paulo, A. (2002). Studies of stabilization of native catalase using additives. *Enzyme and Microbial Technology*, 30, 387-391.

Çetinus, Ş. A., & Öztop, H. N. (2000). Immobilization of catalase on chitosan film. *Enzyme and Microbial Technology*, 26, 497-501.

Çetinus, Ş. A., & Öztop, H. N. (2003). Immobilization of catalase into chemically crosslinked chitosan beads. *Enzyme and Microbial Technology*, 32, 889-894.

Eberhardt, A. M., Pedroni, V., Volpe, M., & Ferreira, M. L. (2004). Immobilization of catalase from Aspergillus niger on inorganic and biopolymeric supports for H_2O_2 decomposition. *Applied Catalysis B: Environmental*, 47, 153-163.

Freundlich, H., 1906. Adsorption in Solution. *Phys. Chemie*., 57, 384-410.

Fuentes, I. E., Viseras, C. A., Ubiali, D., Terreni, M., & Alcántara, A. R. (2001). Differrent phyllosilicates as supports for lipase immobilisation. *Journal of Molecular Catalysis B : Enzymatic*, 11, 657-663.

Fusi, P., Ristori, G.G., Calamai, L. & Stotzky, G. (1989) Influence of bacterial-amoebal interactions on sulfur transformations in soil. *Soil Biology and Biochemistry*. 21 911–920.

Garwood, G.A., Mortland, M.M. & Pinnavaia, T.J. (1983) *J. Mol. Catal*., 22, 153-163.

Ghiaci, M., Abbaspur, A., Kra, R., & Seyedeyn, A. F. (2004). Equilibrium isotherm studies for the sorption of benzene, toluene, and phenol onto organo-zeolites and as synthesized MCM, 41,40, 217-229.

Giese, R. F., Oss, C. J. van. (2002). *Collaoid and Surface Properties of Clays and Related Minerals*. New York. NY, USA: Marcel Dekker Incorporated, 2002. Retrieved May 10, 2004, from http://site.ebraray.com/lib/deulibrary/Doc?id= 10050774&page=2.

Giles, H., McEwan, T. H., Nakhwa, S. N., & Smith, D., (1960). Studies in adsorption Part II. A system of classification of solution adsorption isotherms. *Journal Chemical Society*., 4, 3973-3993.

Grim, R. E. (1968). *Clay Mineralogy*. New York: Mc. Graw Hill Book Comp.

Grim R. E., & Güven, N. (1978). Bentonites: *Geology, Mineralogy, Properties and Uses, Developments in Sedimentology*, 24. New York. Retrieved February 1, 2003, from Elsevier database.

Gorjup, B., Lampic, N., Penca, R., Perdih, A. & Perdih, M. (1999). Solvent effects on ligninases. *Enzyme and Microbial Technology,* 25, 15.

Gupta, S. S., & Bhattacharyya, K. G. (2006). Adsorption of Ni(II) on clays. *Journal of Colloid and Interface Science*, 295, 21-32.

Hall, K. R., Eagleton, L. C., Acrivos, A. & Vermeulen, T. (1966). *Industrial and Engineering Chemistry Fundamentals*, 5, 212-223.

Halliwell, B., & Gutteridge, J.M.C., (2003). *Free Radicals in Biology and Medicine*. (3rd ed.) Oxford: New York.

Hamdaoui, O. (2006). Batch study of liquid-phase adsorption of methylene blue using cedar sawdust and crushed brick. *Journal of Hazardous Materials B*, 135, 264-273.

Harter, R.D. & Stotzky, G. (1973). *Soil Sci. Soc. Am. Proc.*, 37, 116–123

Hillier, S. (1995). Erosion, sedimentation and sedimentary origin of clays, in Velde, B., ed., *Origin and mineralogy of clays*: New York, Springer-Verlag, p. 162-219.

Ho, Y. S. (2003). Citation review of Lagergren kinetic rate equation on adsorption reactions. *Scientometrics*, 59 (1), 171-177.

Ho, Y. S. & McKay, G. (2003). Pseudo-second-order model for sorption processes. *Process Biochemistry*, 34 (5), 451-465.

Hoda, N., Bayram, E., & Ayrancı, E. (2006). Kinetic and equilibrium studies on the removal of acid dyes from aqueous solutions by adsorption onto activated carbon cloth. *Journal of Hazardous Materials B*, 137, 344-351.

İnel, O. & Tümsek, F. (1999). The measurement of surface areas of some silicates by solution adsorption. *Turkish Journal of Chemistry*, 24, 9-20

Juma, N. G. (1998). The Pedospherre and its Dynamics: Minerology, 6.3 *Clay Crystals*. Retrieved May 19, 2005 from http://wwwsoils.rr.ualberta. ca/pedosphere /content/sectionof/page03.cfm.

Kadirvelu, K. & Namasivayam, C. (2003). Activated carbon from coconut coirpith as metal adsorbent: adsorption of Cd(II) from aqueous solution. *Advances in Environmental Research*, 7, 471-478.

Katchalski-Katzir, E. (1993). İmmobilized enzymes-learning from past successes and failures. *Trends Biotechnol,* 11, 471-478.

Kennedy, J. F. & Cabral, J. M. S. (1983). Immobilized enzymes. In: Scouten, W.H. (Ed.), *Solid Phase Biochemistry: Analytical and Synthetic Aspects*. Wiley, New York, 253-391.

Kilara, A. (1981). Immobilized proteases and lipases. *Process*, 25-27.

Kim, J., Jia, H., Lee, C., Chunk, S., Kwak, J. H., Shin, Y., Dohnalkova, A., Kim, B. G., Wang, P., & Grate, J. W. (2006). Single enzyme nanoparticles in nanoporous silica: A hierachical approach to enzyme stabilization and immobilization. *Enzyme and Microbial Technology*, 39, 474-480.

Lagergren, S. (1898). Zur Theorie der sogenannten adsorption gelöster stoffe. *Kungliga Svenska Vetenskapsakademiens*. Handlingar, Band 24 (4).

Langmuir, I. (1918). Adsorption of gases on plane surfaces of glass, mica and platinum. *J. Am. Soc*., 40, 1361-1403.

Li, S., Hu, J., & Liu, B. (2004). Use of chemically modified PMMA microspheres for enzyme immobilization. *BioSystems*, 77, 25-32.

Liu, C., Bo, A., Cheng, G., Lin, X. & Dong, S. (1998). Characterization of the structural and functional changes of hemoglobin in dimethyl sulfoxide by spectroscopic techniques. *Biochim. Biophys*. Acta, 1385, 53.

Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill Jr., C. G., & Admundson, C. H. (1990). Immobilized lipase reactors for modification of fats and oils- a review. *J. Am. Chem. Soc*., 12 (67), 890-910.

Malik, U.R., Hasany, S.M., & Subhani, M.S. (2005). Sorptive potantial of sunflower sterm for Cr (III) ions from aqueous solution and its kinetic and thermodynamic profile. *Talanta,* 66, 166-173.

Manoj, K. M., Lakner, F. J. & Hager, L.P. (2000). *J. Mol. Catal B: Enzymatic* 9, 107.

Marczewska, A. D.& Marczewski, A. W. (2002). Effect of adsorbate structure on adsorption from solutions. *Applied Surface Science*, 196, 264-272.

Marczewski, A. W. (2002). *A practical guide to isotherms of adsorption on heterogeneous surfaces*. Retrieved February 1, 2004, from http://www.2002/ adsorption org/awm/ads/Ads.htm.

McKay, G., El Geundi, M. & Nassar, M. M. (1987). Equilibrium studies during the removal of dyestuffs from aqueous solutions using bagasse pith. *Water Research*, 21 (12), 1513-1520.

Moelans, D., Cool, P., Baeyens, J., & Vansant, E. F. (2005). Using mesoporous silica materials to immobilise biocatalysis-enzymes. *Catalysis Communications*, 6, 307-311.

Murray, H. H. (1991). Overview — clay mineral applications. *Appl. Clay Sci*., 5, 379-395.

Naidja, A., & Huang, P. M. (1996) Deamination of aspartic acid by aspartase—Ca-montmorillonite complex. *Journal of Molecular Catalysis A Chemical*., 106, 255–265.

Ophardt C.E., (2006) *Virtual Chembook*. Retrieved 25 December, 2006. from http://www.elmhurst.edu/~chm/vchembook/570enzymes.html.

Önal, M., Sarıkaya, Y., & Alemdaroğlu, T. (2001). Investigation of the Microporous and Mesoporous Structures of the Reşadiye (Tokat/Turkey) Bentonite and its Fractions. *Turkish Journal of Chemistry*, 25, 241-249.

Özcan, A. S., & Özcan, A. (2004). Adsorption of acid dyes from aqueos solutions onto acid-activated bentonite. *Journal of Colloid and Interface Science*, 276, 39-46.

Pinnavaia, T. J. (1983). Intercalated clay catalysts. *Science*, 220, 365-371.

Robertson R.H.S., (1973), *Silicates Ind*., 38, 33.

Sanjay, G. & Sugunan, S. (2006). Enhanced pH and thermal stabilities of invertase immobilized on montmorillonite K-10. *Food Chemistry*, 94, 573-579.

Santaren, J., Sanz, J. & Ruiz-Hitzky, E. (1990). Structural fluorine in sepiolite. *Clay Miner*., 38, 63.

Senthilkumaar, S., Kalaamani, P., Porkodi, K., Varadarajan, P. R., & Subburaam, C. V. (2006). Adsorption of dissolved Reactive red dye from aqueous phase onto activated carbon prepared fom agricultural waste. *Bioresource Technology*, 97, 1618-1625.

Shentu, J., Wu, J., Song, W., & Jia, Z. (2005). Chitosan microspheres as immobilized dye affinity support for catalase adsorption. *International Journal of Biological Macromolecules*, 37, 42-46.

Sinegani, A. A. S., Emtiazi, G., & Shariatmadari, H. (2005). Sorption and immobilization of celllulase on silicate clay minerals. *Journal of Colloid and Interface Science*, 290, 39-44.

Singh, T.S. & Pant, K.K. (2004). Equilibrium, Kinetics and Thermodynamic Studies For Adsorption of As (III) on activated alumina. *Separation and Purification Technology*, 36, 139-147.

Staunton, S. & Quiquampoix, H. (1994). Regular Article Adsorption and Conformation of Bovine Serum Albumin on Montmorillonite: Modification of the Balance between Hydrophobic and Electrostatic Interactions by Protein Methylation and pH Variation. *J. Colloid Interface Sci*., 166, 89–94..

Stephen, A.N. (2001). *Clay Minerals*. Retrieved June 22, 2004 from http://tulane.edu./~soneison/geol211/clayminerals.htm.

Toth, J. (2002). *Adsorption Isotherms*. Encyclopedia of Surface And Colloid Science (2002) pages 212-224.

Trasar-Cepeda, C., Camina, F., Leiros, M. C., & Gil-Sotres, F. (1999). An improved method to measure catalase activity in soils. *Soil Biology and Biochemistry*, 31, 483-485.

Tsai, W. T., Lai, C. W., & Hsien, K. J. (2003). Effect of particle size of activated clay on the adsorption of paraquat from aqueous solution. Journal *of Colloid and Interface Science*, 263, 29-34.

Tukel, S. S., & Alptekin, Ö. (2004). Immobilization and kinetics of catalase onto magnesium silicate. *Process Biochemistry*, 39, 2149-2155.

Weber, T. W., & Chakkravorti, R. K. (1974). Pore and solid diffusion models for fixed bedadsorbers. *American Institude of Chemical Engineers Journal*, 20,228.

Velde, B. (1995). Composition and mineralogy of clay minerals, in Velde, B., ed., *Origin and mineralogy of clays*: New York, Springer-Verlag, p. 8-42.

Wilson, M. J. (Ed.). (1987). *A Handbook of Determinative Methods in Clay Minerology*. Published in the USA by Chapman and Hall. New York..

Ye, P., Xu, Z. K., Che, A. F., Wu, J., & Seta, P. (2005). Chitosan-tethered poly (acrylonitrile-co-maleic acid) hollow fiber membrane for lipase immobilization. *Biomaterials*, 26, 6394-6403.

Yeşiloğlu, Y. (2005). Utilization of bentonite as a support material for immobilization of Candida rugosa lipase. *Process Biochemistry*, 40, 2155-2159.