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JUDUL: **AMYLASE IMMOBILIZATION ON CALCINED-KAOLIN AND SODIUM ALGINATE BEADS.**

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Saya : **NOR SA'ADAH BINTI MOHAMED**
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Alamat Tetap:
N0. 354 Felda Chini 01,
26690 Chini,
Pahang Darul Makmur

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Supervisor : Rozaimi bin Abu Samah

Date : May 2009

AMYLASE IMMOBILIZATION ON CALCINED-KAOLIN AND SODIUM ALGINATE BEADS

NOR SA'ADAH BT MOHAMED

A thesis submitted in fulfillment of the
requirements for the award of the degree of
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MAY 2009

DECLARATION

I declare that this thesis entitled "Amylase Immobilization on Calcined-Kaolin and Sodium Alginate Beads" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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Date : May, 2009

Special Dedication of This Grateful Feeling to My….

Beloved parents
Mr. Mohamed bin Idris
Mrs. Rosmawati binti Hamzah

Supportive Lecturer
Mr. Rozaimi bin Abu Samah

My sisters and brothers and all my friends

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ABSTRACT

The objective of this research was to study the techniques for α-amylase immobilization on two different support materials. α-Amylase from *Bacillus* sp was immobilized by two different techniques which were physical adsorption onto calcined-kaolin and entrapment in sodium alginate beads. The advantages of attaching enzymes to a solid support and a few of the major reasons are single batch of enzyme can be used either multiple or repetitive and at the same time it can save cost. The immobilized α-amylase exhibited better thermostability than the free one. It is believed that kaolin and alginates has tremendous potential to be utilized as solid support material. It was estimated that the percentage of protein loading was 59% for adsorption onto calcined-kaolin, whereas for entrapment in sodium alginate, 74% of protein was immobilized. The immobilized α-amylase was tested for its capacity of converting starch into glucose, as well as its optimum pH and temperature. The optimum pH and temperature for both immobilized α-amylase were found to be pH 8 and 70°C, respectively.

ABSTRAK

Matlamat kajian ini adalah untuk mengkaji teknik immobilisasi ke atas α-amylase menggunakan dua bahan bantu lekatan yang berbeza iaitu calcined-kaolin dan sodium alginate. α-Amylase daripada *Bacillus* sp telah dipegunkan menggunakan dua teknik yang berbeza, iaitu teknik lekatan fizikal dan teknik pemerangkapan. Teknik ini telah digunakan untuk membolehkan enzim digunakan semula berulang kali disamping menjimatkan kos. Disamping itu, α-amylase yang telah dipegunkan mempunyai kestabilan suhu yang lebih baik berbanding α-amylase bebas. Sebanyak 59 % protein yang telah dipegunkan untuk teknik lekatan fizikal keatas calcined-kaolin, manakala untuk teknik pemerangkapan keatas sodium alginate, 74% protein telah dipegunkan. Kebolehan α-amylase yang telah dipegunkan menukar kanji kepada glukosa telah diuji dengan optimum pH dan suhu. Suhu dan pH optimum ialah 70°C dan pH 8 bagi kedua-dua teknik yang telah digunakan

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%	-	percent
°C	-	degree Celcius
g	-	gram
g/l	-	gram per liter
h	-	hour
kg	-	kilogram
L	-	liter
L/h	-	liter per hour
min	-	minute
ml	-	milliliter
mm	-	millimeter
Rpm	-	rotation per minute
wt %	-	weight percent
w/v	-	weight per volume
µg/ml	-	microgram per milliliter
pI	-	isoelectric point

CHAPTER 1

INTRODUCTION

1.1 Introduction

Enzymes are attractive catalysts, highly effective and specific under ambient conditions. Research over the last four decades has focused on understanding the modes of enzymatic deactivation, as well as developing methods to overcome this shortcoming of the biocatalytic approach. Most of the enzymes are stable when stored at low temperatures and neutral pH in aqueous media. This condition is fragile and can be easily disturbed by external stresses such as high pressures and temperatures, extreme pH, organic solvents, freezing, drying, and by oxidative or denaturing agents. The resulting activity loss may be reversible or irreversible. The severity of these effects varies with the type of enzyme and the nature and the intensity of the stress (Drevon *et al.*, 1997).

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly and continuously. Enzyme will be attached to an inert and insoluble material. There is a variety of methods by which enzymes can be immobilized, ranging from covalent chemical bonding to physical entrapment (Zaborsky, 1973). Immobilization refers to the preparation of insoluble biocatalytic derivatives and involves the coupling of enzymes to solid supports that are either organic or inorganic.

The use of a relatively expensive catalyst as an enzyme requires, in many instances, it is recovery and reuse to make an economically feasible process. Moreover, the use of an immobilized enzyme permits to greatly simplify the design of the reactor and the control of the reaction (Mateo *et al.*, 2007). Usually biocatalyst industry required immobilization to the use of an enzyme, and the simplest solution to the solubility problem of these interesting biocatalysts. There are four main categories methods have been used for the immobilization of enzymes which are adsorption, entrapment, covalent binding and cross linking.

1.2 Research statement

Enzymes work by lowering the activation energy for a reaction, hence the rate of the reaction significantly increase. Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. Since with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions.

Despite the advantages above, enzymes not as an ideal industrial-scale catalyst because of several factors, mainly high cost of the enzymes, and enzymes instability. Enzymes are cost-effective only when it can be re-used many times. Enzymes need to be separated from the process liquid for recirculated. This is possible when the enzymes are attached to a carrier which can be filtrated and recovered. The cost to recover the enzyme after reactions is expensive and only limited to batch operation.

Immobilized enzymes are used in analytical chemistry and as catalysts for the production of chemicals, pharmaceuticals, and food. Immobilized enzymes will retain at it support as substrate and flow through of enzymes in reactor packing. Immobilization can eliminate problems with recovery of enzyme and purity of products which are important to economic operation.

1.3 Research Objective

The objective of this research is to study the techniques for α-amylase immobilization on two different support materials

1.4 Research Scope

The scopes of this research are:

a) Immobilization of α-amylase by adsorption onto calcined-kaolin.
b) Immobilization of α-amylase by entrapment in sodium alginate beads.
c) Determine the amount of immobilized α-amylase for both techniques
d) Determine the optimum pH and temperature for both immobilized α-amylase

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The industrialization of a continuous process for the optical resolution of DL-amino acids using immobilized aminoacylase was succeeded by Dr.Chibata *et al*.1969 at Tanabe Seiyaku Corporation. This is the first industrial application of immobilized enzymes in the world.

The term of immobilized enzyme was recommended at the First Enzyme Engineering Conference in 1971.before the conference, various terms such as water-insoluble enzyme, trapped enzyme, fixed enzyme, and matrix-supported enzyme had been used.

The major application of immobilized enzyme in dairy industry is in the preparation of lactose-hydrolyzed milk and whey, using ß-galactosidase. A large population of lactose intolerants can consume lactose-hydrolyzed milk. This is a great significance in a country like India where lactose intolerance is quite prevalent (Konechy, 1984). Pharmaceutical industry immobilized enzyme used in the production of 6-aminopenicillanic acid by the deacylation of the side chain of, using penicillin acylase (Pastore *et al.*, 1976).

Enzyme immobilization is preferred to enhance enzyme stability, multiple and repetitive use and the easy way of enzyme removal from the reaction mixture (Hasirci *et al.,* 2006). Immobilization can be achieved by two different ways, which are chemical and physical methods (Tumturk *et al.,* 2000).

2.2 Immobilized enzyme

2.2.1 Overview

Enzymes are protein molecules which are used to increase the chemical reactions rate of living cells. Without enzymes, most biochemical reactions would be too slow to even carry out life processes. Enzymes present great specificity and not permanently modified by their participation in reactions. It is cost-effective to use them more than once, since they are not changed during the reactions. However, it is difficult to separate if the enzymes are in solution with the reactants or products.

Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. Immobilized enzyme means, enzyme is unable to move or stationary. Immobilized enzyme is an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product. For immobilization of enzyme, it is necessary that functional group in the active center should not be involved in the reaction leading to immobilization of the enzyme.

There are some advantages to attaching enzymes to a solid support and a few of the major reasons are single batch of enzyme can be used either multiple or repetitive. Other than that the reaction is able to stop rapidly by removing the enzyme from the reaction solution.

Beside that, enzymes are usually stabilized by bounding at high temperature and across a large pH range and immobilized enzymes are useful in the food and pharmaceutical industries because of the product are not contaminated with the enzyme. Further more, the use of immobilized enzyme eliminate the enzyme separation step from the main process thus simplifying and increasing the overall process yield.

On the other hand, there are some disadvantages of immobilized enzyme which is smaller surface area of enzyme. Immobilization also can affect the shape of active side so fewer enzyme substrate complexes form. At the same time immobilized enzyme have a little kinetic energy. Beside that, the substrate needs to diffuse through the gel or membrane and enzyme at lower concentration when immobilized.

2.2.2 Properties of immobilized enzyme

According to the definition of immobilized enzyme by katzir, 1960 three types of immobilized enzyme can be classified which is heterogenization of the soluble enzyme by coupling to an insoluble support by adsorption or covalent binding, by cross-linking of the enzyme or entrapment in a lattice or in microcapsules such as alginate beads. The other types of immobilized enzyme are retention of the enzyme by means of ultrafiltration membranes and use of whole cells for biotransformation using their enzyme apparatus.

Changes in physical and chemical properties of immobilized enzyme are important. Enzyme would be expected to undergo upon insolubilization if the best use is to be made of the various immobilization techniques available. The physical and chemical changes have been observed in the stability and kinetic properties of enzyme.

The stability of the enzymes might be expected to either increase or decrease on insolubilization, depends on the carrier provides a microenvironment which capable of protein denatured.

Changes in activity of enzymes due to the actual process of insolubilization usually decrease the specific activity of an enzyme upon insolubilization, and it can be attributed to protein denatured. The new microenvironment may be a result of the physical and chemical character of the support matrix alone, or it may result from interactions of the matrix with substrates or products involved in the enzymatic reaction.

2.2.3 Application of immobilized enzyme

Since early 1970, the used of enzymes in food as well as in pharmaceutical and chemical industries has been increased steadily.

Table 2.1 Industrial use of immobilized enzymes

Enzyme	**Substrate**	**Product**
Penicillin amidase	Penicillin G	6-Amino Penicillanic acid
Cephalosporin amidase	Glutaryl-7-ACA	7-ACA
Amino acylase	Acryl-D-L-amino acid	L-amino acid
Aspartase	Fumaric acid	L-malic acid
Fumarase	Fumaric acid	L-malic acid
Lactase (galactosidase)	Lactose GalGlc	Low lactose milk Gal+Glc
Aspartase β-decarboxylase	Aspartic acid	L-alanine
Nitrile hydratase	Acrylonitile	Acrylamide
Glucose isomerase	Glucose	High fructose corn syrup
Lipase	Rac-1-phenylethyl-amine	(*S*)-1-phenylrthyl-amine

By far, the most important application of immobilized enzymes is in dairy industry and pharmaceutical industry specifically for the conversion of glucose syrups to high fructose syrups as well as production of 6-aminopenicillanic acid.

2.2.3.1 Immobilized enzyme in industry

One of the major applications of immobilized enzyme in dairy industry is in the preparation of lactose-hydrolysed milk and whey, using β-galactosidase. This is of great significance in a country like India where lactose intolerance is quite prevalent (Konechy, 1984).

The sweetness and solubility of the sugar enhances because of the hydrolysis of lactose and future potentials in preparation of variety of dairy products are found. Lactose hydrolysed whey may be used as a component of whey-based baverages, leaving agents, feed stuffs, or may be fermented to produced ethanol and yeast, then converting an inexpensive byproduct into a highly nutritious, good quality food ingredient (Kubal *et al.,* 1990).

2.2.3.2 Immobilized enzyme in pharmaceutical

One of the major applications of immobilized enzyme in pharmaceutical industry is the production of 6-aminopenicillanic acid by the deacylacation of the side chain in either penicillin G or V, by using penicillin acylase (Pastore, 1976).

More than 50% of 6-aminopenicillanic acid produced today is enzymatically using the immobilized route. Squibb (USA), Astra (Sweden) and Riga Biochemical Plant (USSR) is the first industries was setting up the process for the production of 6-aminopenicillanic acid in 1970s. Currently, most of the pharmaceutical make used of this technology.

A number of immobilized systems have been commercially produced for penicillin acylase which make use of a variety of techniques either using the isolated enzyme or the whole cells (Wiseman, 1985). This is also one of the major applications of the immobilized enzyme technology in India.

2.2.4 Immobilized techniques

Methods for immobilized enzyme can be classified into three basic categories, which are carrier-binding method, cross-linking method and entrapping method.

1. Carrier-binding method: the binding of enzymes to water-insoluble carrier such as polysaccharide derivatives, synthetic polymers, and porous glass.
2. Cross-linking method: intermolecular cross-linking of enzymes by means of bifunctional or multifunctional reagents such as glutaraldehyde, bisdiazobenzidine and hexamethylene diisocyanate.
3. Entrapping method: incorporating enzymes into the lattice of a semipermeable gel on enclosing the enzymes in a semipermeable polymer membrane, such as collagen, gelatin cellulose, triacetate polyacrylamide, and *k*-carrageenan.

Table 2.2 Preparation and characteristics of immobilized enzyme

Characteristic	Carrier-binding method		Cross-linking	Entrapment
	Adsorption	Covalent binding		
Preparation	Easy	Difficult	Difficult	Difficult
Enzyme activity	Low	High	Moderate	High
Substrate specificity	Unchangeable	Changeable	Changeable	Unchangeable
Binding force	Weak	Strong	Strong	Strong
Regeneration	Possible	Impossible	Impossible	Impossible
General applicability	Low	Moderate	Low	High
Cost of immobilization	Low	High	Moderate	Low

The carrier-binding method is the oldest enzyme immobilization method. This method can be divided into three according to the binding mode of the enzyme, that is, physical adsorption, ionic binding, and covalent binding.

2.2.4.1 Adsorption

Among immobilization techniques, adsorption may have a higher commercial potential than other techniques because it is the simplest of all the techniques, less expensive and high catalytic activity may be retained and does not grossly change the activity of the bound enzyme.

In case of enzymes immobilized through ionic interactions, adsorption and desorption of the enzyme depends on the basicity of the ion exchanger. Moreover, a dynamic equilibrium is normally observed between the adsorbed enzyme and the support which is often affected by pH as well as the ionic strength of the surrounding medium. This property of reversibility of binding has often been used for the economic recovery of the support.

This technique has been successfully adapted in industry for the resolution of racemis mixtures of amino acids, using amino acid acylase (Decleire *et al*., 1987). This technique also offers the reusability of expensive supports after inactivation of immobilized enzyme.

Kolot (1981) have been investigated a variety of commercially available ion exchangers for this purpose. One of the techniques, which have gained importance more recently, is the use of polyethylenimine for imparting polycationic characteristics to many of the neutral supports based on cellulose or inorganic materials. Enzymes with low pI have been bound through adsorption followed by cross-linking on polyethylenimine-coated supports.

However, adsorption is generally not very strong and some of the adsorbed protein will desorbed during washing and other operation steps. Thus, immobilization via adsorption requires an electrostatic interaction between the enzyme and support.

2.2.4.2 Entrapment

The entrapping technique is based on confining enzymes in the lattice of a polymer matrix or enclosing enzyme in semipermeable membranes, and it can be classified into the lattice and microcapsules type. These techniques are different from the covalent binding and cross-linking method which the enzyme itself does not bind to the gel matrix or membrane.

However, if a chemical polymerization reaction is used for entrapping, relativity severe conditions are required and lose of enzyme activity occurs in some cases. Therefore, it is necessary to select the most suitable conditions for the immobilized of various enzyme.

Entrapment has been extensively used for the immobilization of cells, but not for enzymes. The main restriction of this technique for the immobilization of enzymes is the possibility of slow leakage during continuous use in view of the small molecular size compared to the cells. In this technique, enzymes have been entrapped in natural polymers like agar, agarose and gelatine through thermoreversal polymerization, but in alginate and carrageenan by ionotropic gelation (Tampion *et al.*, 1987).

Meanwhile, a number of synthetic polymers have also been investigated. Notable among them are the photo-crosslinkable resins, polyurethane prepolymers (Mosbach, 1987) and acrylic polymers like polyacrylamide (Deshpande *et al.,* 1987). Polyacrylamide may not be a useful support for food industry because of the toxicity, but it can have potentials in the waste treatment and fabrication of analytical devices containing biocatalysts. One of the major limitations of entrapment technique is the diffusion limitation as well as the steric hindrance, especially when used the starch and protein as a macro molecular substrates.

2.2.4.3 Covalent binding

The covalent binding technique is based on the binding of enzymes and water-insoluble carriers by covalent bonds. Immobilization of enzyme by covalent binding is carried out under relatively severe conditions in comparison with those of physical adsorption or ionic binding.

The condition for immobilization by covalent binding is much more complicated and less mild than in the cases of physical adsorption and ionic binding. Therefore, covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength.

Covalent binding is not a good technique for the immobilization of cells. The several functional groups extensively investigated are the amino, carboxyl, and the phenol group of tyrosine (Hartmier, 1988). Enzymes are covalently linked to the support through the functional groups in the enzymes, which are not essential for the catalytic activity. It is often desirable to carry out the immobilization in the presence of its substrate or a competitive inhibitor so as to protect the active site. The covalent binding also should be optimized so as not to alter its conformational flexibility. A number of industrially useful enzymes are glycoprotein wherein the carbohydrate moiety may not be essential for its activity.

2.2.4.4 Cross-linking

The cross-linking method is based on the formation of chemical bond, but water-insoluble carriers are not used in this method. The immobilized enzyme is performed by the formation of intermolecular cross-linkages between the enzyme molecules by means of bifunctional or multifunctional reagents.

Biocatalysts can also be immobilized through chemical cross-linking using homo- as well as heterobifunctional cross-linking agents. Among these, glutaraldehyde which interacts with the amino groups through a base reaction has been extensively used cause of the low cost, high efficiency, and stability. The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen (Hartmier *et al*., 1988).

Adsorption followed by cross-linking has also been used for the immobilization of enzymes. The technique of cross-linking in the presence of an inert protein also can be applied to either enzymes or cells. The technique can also be used for the immobilization of enzymes by cross-linking the cell homogenates

2.3 Supports material for immobilization

Several types of supports material are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support depends on the nature of the enzyme, nature of the substrate and its ultimate application. Reagents used should be considered for selection of proper support materials.

Support materials are classified as inorganic supports, synthetic polymers and natural macromolecules (Ye *et al*., 2005). Generally, the main characteristics of a support are to elicit high chemical and mechanical stability, a hydrophilic nature and also non-toxic behavior. Polymeric materials are suitable candidates due to their reactive functional groups, good mechanical properties, easy to prepare and applicability to introduce bio-friendly components for improving biocompatibility (Rebros *et al*., 2007 and Sankalia *et al.,* 2007).

The most important requirements for support materials are must be insoluble in water, high capacity to bind enzyme, chemically inert and mechanically stable. The enzyme binding capacity is determined by the available surface area, both internal (pore size) and external (bead size), the ease with which the support can be activated and the resultant density of enzyme binding sites. The inertness refers to the degree of non-specific adsorption and the pH, pressure and temperature stability. In addition, the surface charge and hydrophilicity must be considered. The activity of the immobilized enzyme will also depend upon the bulk mass transfer and local diffusion properties of the system.

There are several type of support material are widely used in immobilization of enzyme such as Calcined Kaolin and Sodium Alginate.

2.3.1 Calcined kaolin

Kaolin is a clay mineral with the chemical composition $Al_2Si_2O_5(OH)_4$. It is a layered silicate mineral, with one tetrahedral sheet linked through oxygen atoms to one octahedral sheet of alumina octahedral**.** Kaolin is a flexible mineral with a variety of uses and benefits for the modern day industry (Deer *et al*., 1992).

Kaolin is used in ceramics, medicine, coated paper, food additive, in toothpaste, as a light diffusing material in white incandescent light bulbs, and in cosmetics. It is also used in paint to extend titanium dioxide (TiO_2) and modify gloss levels, in rubber for semi-reinforcing properties to improve extrusion and in adhesives to improve adhesion. The largest use is in the production of paper, including ensuring the gloss improved on some grades of paper.

Commercial grades of kaolin are supplied and transported as dry powder, semi-dry noodle or as liquid slurry. A more recent, and more limited, use is as a specially formulated spray applied to fruits, vegetables, and other vegetation to repel or deter insect damage.

2.3.2 Sodium alginate

Term of alginate usually used for the salts of alginic acid and it can also refer to all the derivatives of alginic acid and alginic acid itself. Alginate**s** are presented in the cell walls of brown algae as the calcium, magnesium and sodium salts of alginic acid. The uses of alginates based on three main properties.

1. The ability to increase the viscosity of aqueous solutions.
2. The ability to form gels. Gel form when a calcium salt is added to a solution of sodium alginate in water. The gel forms by chemical reaction, the calcium displaces the sodium from the alginate, holds the long alginate molecules together and produced a gel. No heat is required and the gels do not melt when heated. This is in contrast to the agar gels where the water must be heated to about 80°C to dissolve the agar and the gel forms when cooled below about 40°C.
3. The ability to form films of sodium or calcium alginate and fibres of calcium alginates.

Alginates also used in textile printing and food. In textile printing, alginates are used as thickeners for the paste containing the dye. These pastes are applied to the fabric either by screen or roller printing equipment. The thickening property of alginate is useful in sauce, syrups and toppings for ice cream. Addition of alginate can make icings non-sticky and allow the baked goods to be covered with plastic wrap.

2.4 Amylase

2.4.1 Overview

Amylase is an enzyme that breaks down starch or glycogen. Amylase is produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi produce amylases to the outside of their cells to carry out extra-cellular digestion. When they have broken down the insoluble starch, the soluble end products such as glucose or maltose are absorbed into their cells. Pancreas also makes amylase (α- amylase) to break down dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy.

Amylases are classified based on how they break down starch molecules, which are α-amylase reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose, and β-amylase breaks down the glucose-glucose bonds by removing two glucose units at a time, thereby producing maltose, and γ-Amylase breaks successive bonds from the non-reducing end of the straight chain, producing glucose.

Microbial amylases was exploited by humans for several purposes, such as for the preparation of high fructose corn syrup, as an additive to detergents for removing stains, as a saccharification of starch for production of alcohol and as a brewing. Although many microorganisms produce this enzyme, the most commonly used in industrial production are *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquifaciens* and *Aspergillus niger (*Abe *et al*., 1988)

2.4.2 Amylase immobilization

The research on α-amylase immobilization has done by Kahraman *et al*., (2007). α-Amylase was covalently immobilized onto phthaloyl chloride-containing amino group functionalized glass beads. The activity of immobilized α-amylase was assayed at various temperatures.

The results showed that the maximum catalytic activity of immobilized α-amylase was obtained at 50°C compared to free α-amylase whereas the maximum catalytic activity is 30°C. e

An enzyme in solution can have a different optimal pH from the same enzyme immobilized depends on the nature of the enzyme-bound and pH value in the immediate area of the enzyme environment. Changes in the optimum pH normally results in insolubilization of enzymes, depending upon the polymer used as support.

The maximum pH of the immobilized α-amylase was shifted 1.0 pH unit to the acidic region. The shift depends on the enzyme reaction as well as on the structure and the charge of the matrix. In this research, the shift to acidic region could be result of some secondary interactions between the enzyme and the polymeric matrix.

2.4.3 Application of Amylase

The studies of using amylase have done by Prasanna, 2005 based on their research entitle of amylases and their applications. Amylases are widely distributed and one of the most studied enzymes. This enzyme has widely used ranging from textile to effluent treatment.

Amylases are starch degrading enzymes, which is distributed in microbial, plant and animal kingdoms. Starch and related polymers was degrading to yield products characteristic of individual amylolytic enzymes. Term amylase was initially used originally to designate enzymes capable of hydrolyzing α-1, 4- glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Bernfeld *et al*., 1955). Amylase act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involve.

The history of the industrial production of enzymes dates back to the time in 1894 when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat bran koji culture of *Aspergillus oryzae.* Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase began in 1959.

Since then, α-amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major part of the starch processing industry (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch to products containing glucose, maltose, and others, is brought by controlled degradation (Norman *et al.*, 1978). Some of the applications of amylase are:

1. Liquefaction.

   Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis using thermostable amylase.

2. Manufacture of high fructose containing syrups.

   High fructose containing syrups produced by enzymic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzymic liquefaction and saccharification.

3. Manufacturing of maltose.

   Maltose is naturally occurring disaccharide and it is widely used as sweetener and also as intravenous sugar supplement. Because of the low tendency to be crystallized and is relatively nonhygroscopic it is suitable used in food industries.

4. Other applications.

   Amylases, especially alkaline amylase are used in detergents. To some extent amylases are also used as digestive aids to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients (Beazell, 1942).

CHAPTER 3

MATERIALS & METHOS

3.1 Introduction

This chapter will discuss about preparation processes of immobilization α-amylase by two different methods which are adsorption, and entrapment based on several parameters. Free α-amylase and immobilized α-amylase was reacted at different temperature and different pH to determine the optimum pH and temperature. Amount of protein content was determined via Modified Lowry method and glucose concentration by Dinitrosalicylic Colorimetric method.

3.2 Research procedures

3.2.1 Immobilized α-amylase

α-Amylase from *Bacillus* sp was immobilized by two different techniques. The first techniques, α-amylase was immobilized onto calcined-kaolin by physical adsorption and the second techniques are entrapment of α-amylase in sodium alginate beads.

Using the equation for each method, percent of immobilized α-amylase was determined based on the protein content for each sample.

3.2.1.1 Adsorption

1.50 gram of α-amylase from *Bacillus* sp was dispersed into 30 ml of distilled water. The mixture was stirred for an hour then the immobilization of α-amylase was carried out by continuous shaking at 100 rpm of 4 gram of calcined-kaolin with partially purified 15 ml of α-amylase solution for 1 hour at room temperature. The immobilized α-amylase was then separated by filtration and washed with distilled water to remove the unabsorbed soluble enzyme. The immobilized α-amylase was then lyophilized in freeze drier for further analysis (Shahrul Nazmi, 2008)

% Immobilization = [(B-A)/B] x 100

Where: B = Total amount of protein in supernatant before immobilization
A = Total amount of protein in supernatant after immobilization

3.2.1.2 Entrapment

An equal volume of α-amylase solution and sodium alginate solution was mixed to give a 4 % (w/v) final concentration of sodium alginate solution in the mixture. The mixture obtained was extruded dropwise through a pastuer pipette (1mm diameter) into a gently stirred 2 % (w/v) $CaCl_2.2H_2O$ solution for 2 h to give bead size of 3 mm. The sodium alginate beads containing the α-amylase are thoroughly washed with distilled water and used for further analysis (Dey *et al*., 2003)

% Immobilization yield = (I/A-B) x 100

Where: A = added enzyme
B = unbound enzyme
I = immobilized enzyme

3.3 Procedure analysis

3.3.1 Protein assay

The amount of protein concentration in a solution before and after immobilization was determined by using Modified Lowry Method. The Lowry method is best used with protein concentrations of 0.01-1.0 mg/mL. This method based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin's reagent in the Folin-Ciocalteau reaction. It was the accurate method for protein determination of cell fraction, chromatography fractions, enzyme preparations, and so on. The modified Lowry is done entirely at room temperature.

3.3.1.1 Modified Lowry Method procedure

A reaction mixture that contains 1.0 ml modified Lowry reagent and 0.2 samples was incubated at room temperature for 10 minutes. Distilled water is used as a blank. At the end of the incubation period, 0.1 ml 1 N Folin-Ciocalteu reagent is added into the reaction mixture and left at room temperature for 30 minutes. The absorbance of the sample was measured at 750 nm. Based on the BSA standard calibration curve plotted, protein concentration was determined for each sample (Rozaimi, 2006).

3.3.2 Glucose assay

Amount of reducing sugar in immobilized and free α-amylase was determined by Dinitrosalicylic Colorimetric method.

3.3.2.1 Dinitrosalicylic Colorimetric method (DNS)

The reaction mixture containing 3 ml Dinitrosalicylic Colorimetric reagent and 3 ml sample was incubated at 90°C for 5-15 minutes and red-brown color was appeared, then, the reaction mixture was cooled at room temperature before. The absorbance of the sample was measured at 540 nm. Based on the Glucose standard calibration curve plotted, glucose concentration was determined for each sample

3.4 Characterization of immobilized α-amylase

3.4.1 Effect of temperature

The digested product was assayed for α-amylase activity using DNS in order to determine amount of glucose concentration at different temperature, and the optimum temperature for α-amylase compared to free α-amylase.

3.4.1.1 Adsorption

The reaction mixture containing 1gram of immobilized α-amylase and 3ml of 2% (w/v) starch solution were incubated at different temperature (50, 60, 70, 80 and 90°C) in oven until the sample was dripped. The digested product was assayed for α-amylase activity using DNS according to Bernfeld method (1955).

3.4.1.2 Entrapment

The reaction mixture, containing 20 ml of 2% (w/v) starch solution in acetate buffers (0.1 M, pH 4.5) and 2g of sodium alginate beads were incubated at different temperature (50, 60, 70, 80 and 90°C) in a waterbath shaker. After the enzymatic reaction had proceeded for 10 min, 0.5 ml of the digested products was assayed for α-amylase activity using DNS according to Bernfeld method (1955). One unit was defined as the amount of amylase that produced 1 mmole of reducing sugar under assay condition per gram of bead.

3.4.2 Effect of pH

Immobilized α-amylase was reacted at different pH to determine the activity of α-amylase, percent of relative activity and optimum pH of α-amylase using Modified Lowry method.

The mixtures were reacted at different pH (5. 6, 7, 8, and 9) for 15 minutes at 50 rpm in a waterbath shaker. The relative activities are determined as percentage yield of activities at optimum temperature compared to the activity of reaction at optimum pH.

$$\textit{Relative activity (\%)} = \frac{\textit{Yield at different pH}}{\textit{Maximum \% activity (optimum pH)}} \times 100$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

α-Amylase from *Bacillus* sp was immobilized via two different methods and different support material. It was immobilized onto calcined-kaolin by physical adsorption and entrapment in sodium alginate beads. Amount of protein loading for both techniques was estimated based on Modified Lowry method. Beside that, the Immobilized and free enzyme was analyzed to determine the concentration of glucose via Dinitrosalicylic Colorimetric method (DNS). The optimum temperature and pH was obtained based on the concentration of glucose and enzyme activity observed.

4.2 Calibration curve

4.2.1 BSA standard calibration curve

The amount of protein content was determined by using Modified Lowry Method. Figure 4.1 shows the BSA standard calibration curve. The samples were analyzed in triplicates as shown in table 4.1.

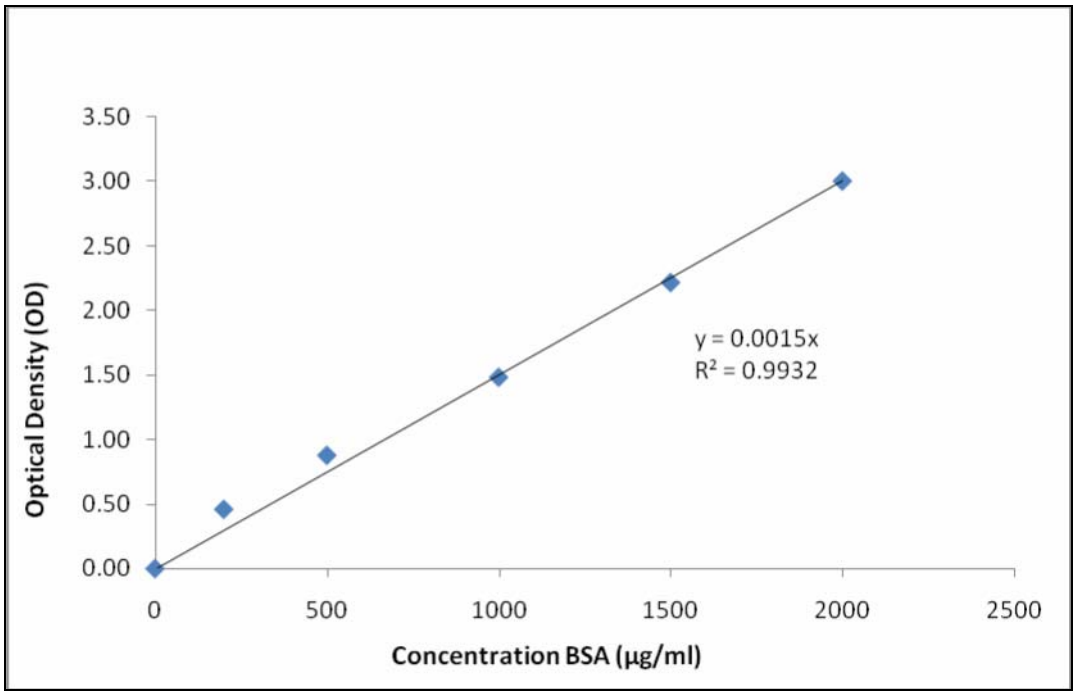

Figure 4.1 BSA standard calibration curve

Table 4.1 Optical density at different Concentration of BSA

BSA concentration (µg/ml)	Optical Density			
	1	2	3	Average
0	0.000	0.000	0.000	0.000
200	0.518	0.427	0.432	0.459
500	0.867	0.879	0.885	0.877
1000	1.445	1.486	1.513	1.481
1500	1.967	2.310	2.366	2.214
2000	3.000	3.000	3.000	3.000

4.2.1 Glucose standard calibration curve

Amount of glucose content was determined using Dinitrosalicylic Colorimetric method (DNS). Figure 4.2 shows the glucose standard calibration curve. The samples were analyzed in triplicates as shown in table 4.2.

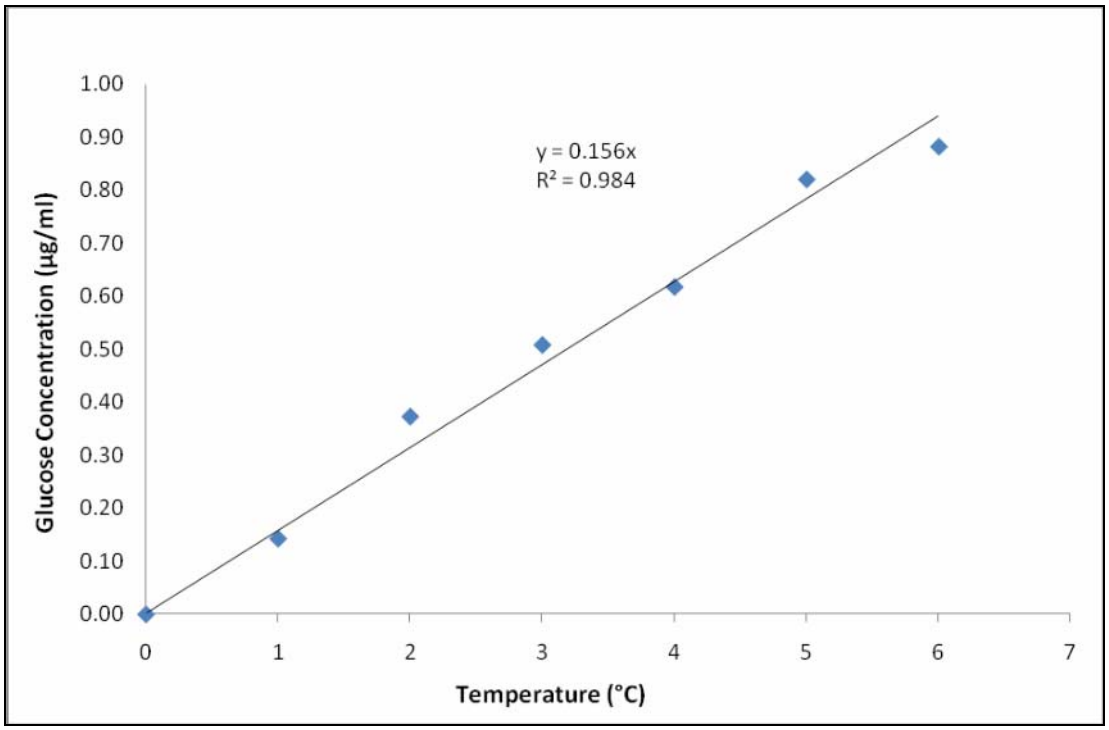

Figure 4.2 Glucose standard calibration curve

Table 4.2 Optical density at different concentration of glucose

Glucose concentration (µg/mL)	Optical Density			
	1	2	3	Average
0	0	0	0	0.000
1	0.144	0.142	0.142	0.143
2	0.372	0.373	0.373	0.373
3	0.504	0.505	0.514	0.508
4	0.617	0.617	0.617	0.617
5	0.820	0.819	0.820	0.820
6	0.881	0.882	0.881	0.881

4.3 Immobilization of α-amylase

After immobilization of α-amylase, the protein content in supernatant before and after immobilization of two different techniques was found. Percent of immobilization determined using the equation at previous chapter. Based on the optical density of α-amylase, it was estimated that amount of protein in supernatant have been immobilized onto the support for both techniques were found to be 59% and 74% respectively.

Compared to usage of lipase by adsorption technique, the percent of immobilized is higher (68%). Calcined-kaolin provides good distribution of lipase on mass transfer and preventing lipase particles from aggregation thus helps dispersion of lipase in the reaction media (S.Nazmi, 2007). Beside that, the lipase molecules may generally be immobilized on the surfaces and inside the support that possess pores due to the shrinking effects as it was heated at high temperatures. (Sabir *et al*., 2001).

Immobilization by entrapment has been done by Dey *et al*., 2003. The immobilization efficiency was found to be highest (75%) for a final concentration of 4% (w/v) sodium alginate solution. It has been reported that the porosity of the calcium alginate beads depend upon the alginate type and the concentration (Longo *et al*., 1992). Higher immobilization efficiency could not be attained due to some leakage of the enzyme into the solution. Although, in practice reducing the size of the pores can reduce leakage, some initial leakage of the enzyme molecule is certain to occur (Zaborsky, 1973). The lower immobilization efficiency in case of lower percentage sodium alginate solution might be due to larger pore size and consequently greater leakage of the enzyme from the matrix.

4.4 Optimum pH for immobilized α-amylase

The immobilized α-amylase was used to convert soluble starch into glucose at pH 5, 6, 7, 8, and 9 for 15 minutes at 70°C and 50rpm in waterbath shaker. The relative activities of immobilized α-amylase are determined using previous equation at chapter 3.

Immobilized α-amylase exhibited maximum activities at pH 8. Figure 4.3 and 4.4 shows a dependence of enzyme activity on pH at different techniques of immobilization. Maximum activity was shown at pH 8 for both techniques. It is estimated, the optimum pH of immobilized α-amylase is pH 8. It is being active at optimum pH and eventually inactive due to denaturation. It was observed that the higher activity of enzyme was obtained at the higher of pH

Tien *et al.,* 1999 has been optimized the pH of immobilized α-amylase by entrapment using response RSM. They found the optimum pH is 5.5 and the highest specific activity was 21.85 U/mg. While the optimum pH for immobilized α-amylase on zirconia by adsorption was found in the range 6 to 8. The stability of the enzyme against pH was significantly improved upon immobilization. This expansion is possible due to the stabilization of enzyme molecules resulting from multipoint attachment on the surface of support material used and due to the charge effects of the support.

The pH at which the immobilization takes place is very important in maintaining the enzyme function as well as maximizing the amount of enzyme immobilized. It was shown that adsorption of protein on to zirconia requires that the surface charges of support and the protein be compatible (Reshmi *et al*., 2007). Immobilization improved the pH stability of the enzyme.

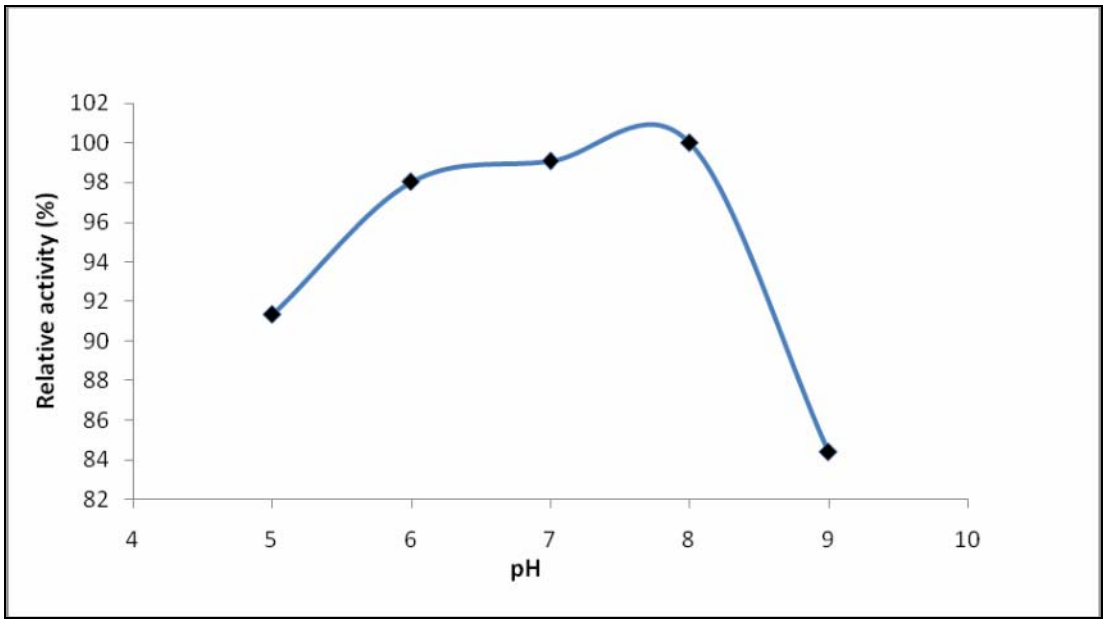

Figure 4.3 Immobilized α-amylase activity at different pH by adsorption onto calcined-kaolin.

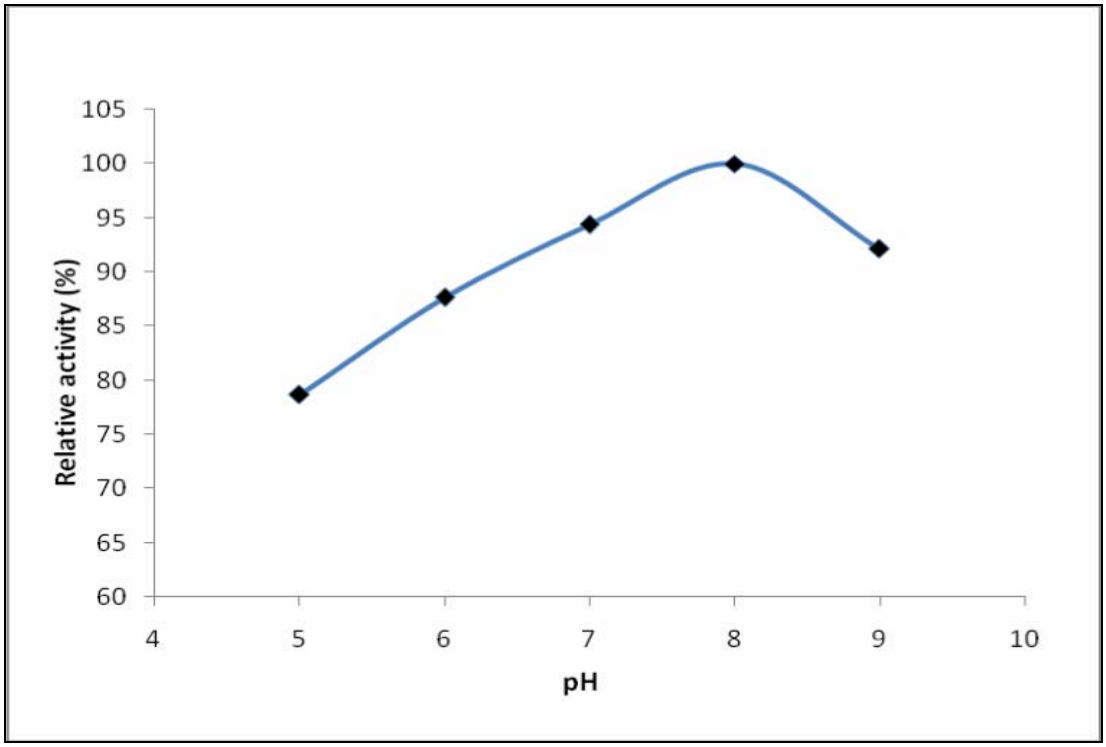

Figure 4.4 Immobilized α-amylase activity at different pH by entrapment in sodium alginate beads.

4.5 Optimum temperature for immobilized α-amylase

For adsorption technique, immobilized α-amylase was incubated at different temperature (50, 60, 70, 80 and 90°C) in oven until the sample was dripped. While, for entrapment technique immobilized α-amylase were incubated for 10 minutes at different temperature in waterbath shaker. After the enzymatic reaction, the digested product was assayed for α-amylase activity using DNS according to Bernfeld method (1955).

Concentration of glucose was found to be higher at the range 40°C to 70°C and become lowest in order to the denaturation of enzyme occur While, the optimum temperature for both adsorption and entrapment techniques of immobilized and free amylase is 70°C respectively. The heat energy from the reaction temperature may affect enzymatic rate and functional group of substrate involved in the reaction. The excess heat energy provided to the enzymatic system has inhibited the conversion of product

Compared to the immobilization of lipase, it was expected to be active at optimum temperature of 37°C and eventually being inactive due to denaturation towards 60°C (A.Rahman *et al*., 2005). Enzymatic rate might be affected due to heat energy applied from the reaction. Lipase may become inactivated due to denaturation because of the excess heat energy provided to the enzymatic system has inhibited the conversion of product.

Beside that, Tien *et al.,* 1999 has been done the immobilization of α-amylase in sodium alginate by entrapment. Based on the research, the optimum temperature was found to be 41°C. The temperature profile observed in case of amylase from *Bacillus circulan*s may be because of some conformational effects due to entrapment, which protects the enzyme against heat denaturation.

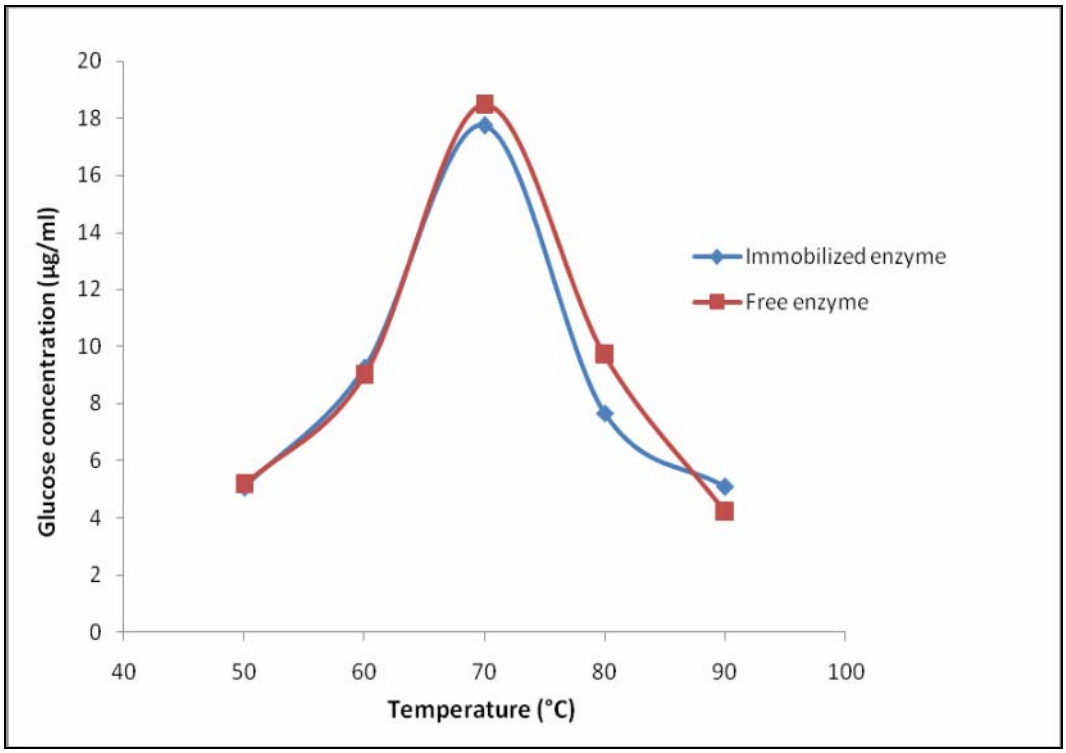

Figure 4.5 Glucose concentrations of Immobilized and free α-amylase at different temperature by adsorption onto calcined-kaolin

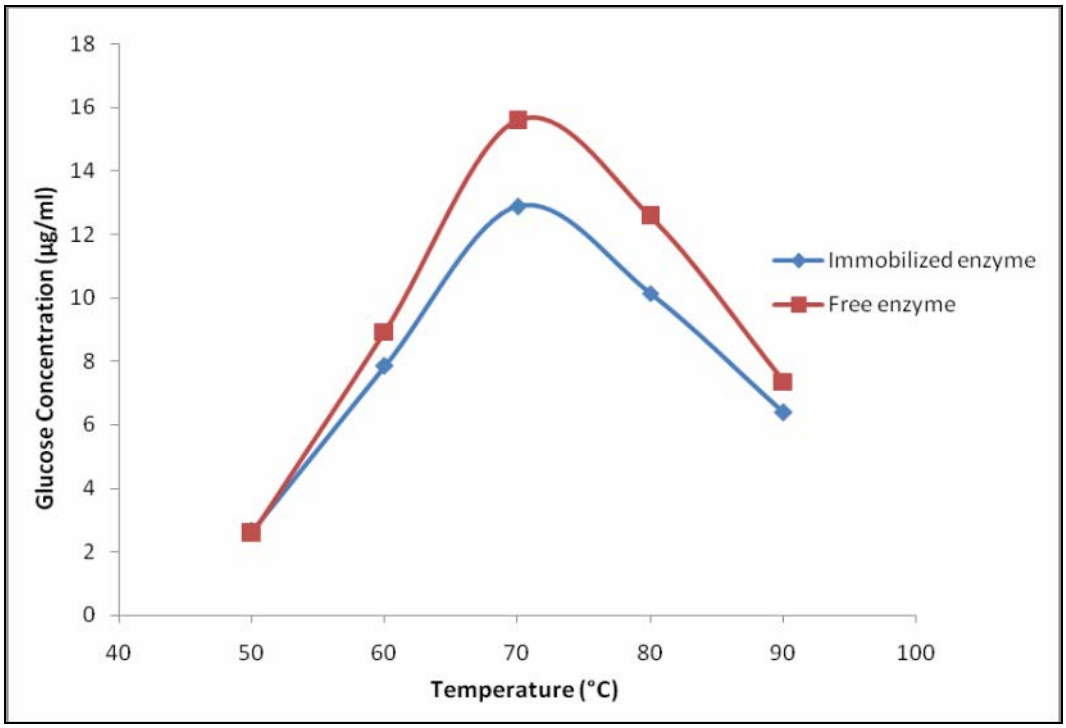

Figure 4.5 Glucose concentrations of Immobilized and free α-amylase at different temperature by entrapment in sodium alginate beads.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Immobilization of α-amylase from *Bacillus* sp using two different techniques which adsorption and entrapment was done successfully. Calcined-kaolin was used as support material in adsorption whereas, sodium alginates used in entrapment of α-amylase. It is estimated that 59% of free α-amylase was immobilized onto calcined-kaolin, although 74% of free α-amylase was immobilized into 4% (w/v) of sodium alginates solution.

Kaolin is clay mineral and widely used in industry. It is a flexible mineral with a variety of uses and benefits for the modern day industry. Kaolin also exhibit good properties as support material in immobilized. Sodium alginate is one of the most frequently used polymers due to their mild gelling properties and non toxicity.

At the end of this research, the objective of my research has been achieved. In order to estimate the effectiveness techniques of immobilization, percentage of immobilization was determined based on the amount of protein loading onto solid support material. It was estimated the effectiveness techniques was entrapment with 74% amount of protein loading onto solid support material. Based on this estimation, believed that the possibility of α-amylase to immobilized into sodium alginates bead higher compare to kaolin.

Although entrapment was the effective techniques, it was used mainly for immobilization of cells, in case of immobilized cells, it causes diffusional and permeability problems which might impair the uptake and transport of substrate as well as excretion of products.

Based on the data analysis it was determined the optimum pH of immobilized α-amylase is at pH 8. It was observed that the activities of immobilized α-amylase are highest at this pH due to the activation of α-amylase and being eventually inactive due to denaturation.

Amount of reducing sugar in immobilized and free α-amylase was determined using Dinitrosalicylic Colorimetric method (DNS). Based on the data obtained, amount of reducing sugar higher at temperature 70°C. It was estimated the optimum temperature of immobilized and free α-amylase is 70°C. The highest concentration of glucose was obtained at that temperature and become lowest due to denaturation of enzyme.

5.2 Recommendation

Among the many matrices available, one of the most frequently used is entrapment within porous matrices, such as alginate often in the form of beads. This sort of system is reasonably safe, simple and cheap offering good mechanical strength. The conditions of entrapment like concentration of sodium alginate and bead size were optimized for highest apparent activity.

As for future it is recommended that, the process parameters, pH and temperature, affecting the performance of the immobilization were optimized using response surface methodology (RSM) (Box and Wilson, 1951). Response surface methodology can be defined as a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivarient equations. RSM was used to optimize the reaction conditions with reference to immobilized amylase activity.

It has been known that the net pores of sodium alginates gel beads are so large that enzymes can sometimes leak out from the gel. In order to prevent enzyme from leaking out of the gel beads, it is recommended that, beads were coated with chitosan and silicate. The catalytic activity of α-amylase entrapped beads retained being compared between non coated and coated beads

On the other hand, α-amylase can be replaced to other types of enzyme in order to determine the best enzyme can be used for that effective technique of immobilization. Type of solid support material used also can affect the rate of enzyme to immobilize. Sequentially to determine the support material that can give the higher immobilization, variety of support material must be used.

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APPENDIX

1.0 % of immobilization

1.1 Adsorption

% Immobilization = [(B-A)/B] x 100

Where: B = Total amount of protein in supernatant before immobilization
A = Total amount of protein in supernatant after immobilization

$$= \frac{(2000\text{mg} - 825.333\text{mg})}{2000\text{mg}} \times 100$$

= 59%

1.2 Entrapment

% Immobilization yield = (I/A-B) x 100

Where: A = added enzyme

B = unbound enzyme

I = immobilized enzyme

$$= \frac{590 \text{ u/g of bead}}{(2000 \text{ u/g of bead} - 1203 \text{ u/g of bead})} \times 100$$

= 74%

concentration of Sodium Alginate (%)	Optical Density		
	Added enzyme, A	Unbound enzyme, B	Immobilized enzyme, I
1	2.531	1.331	0.356
2	3	1.485	0.576
3	3	1.605	0.628
4	3	1.805	0.885

concentration of Sodium Alginate (%)	Added enzyme, A (u/g of bead)	Unbound enzyme, B (u/g of bead)	Immobilized enzyme, I (u/g of bead)	immobilized efficienci (%)
1	1687.333	887.333	237.333	30
2	2000	990	384	38
3	2000	1070	419	45
4	2000	1203	590	74

Immobilized efficiency at 4% concentration of sodium alginate

2.0 Relative activity

2.1 Adsorption

$$\% \text{ Relative activity} = \frac{\text{Yield at different pH}}{\text{Maximum \% activity}} \times 100$$

pH	Optical density	Concentration	enzyme activity	Relative activity (%)
5	1.295	863.333	57.556	91
6	1.390	926.667	61.778	98
7	1.405	936.667	62.444	99
8	1.418	945.333	63.022	100
9	1.197	798.000	53.200	84

Effect of pH

OD at pH 8 = 1.418
Base on BSA standard calibration curve, concentration = 798 µg/ml

$$\text{So, enzyme activity} = \frac{798\ \mu g/ml}{15\ \text{min}}$$

$$= 53.2\ \mu g/ml/min$$

$$\% \text{ relative activity} = \frac{53.2\ \mu g/ml/min}{63.022\ \mu g/ml/min} \times 100$$

$$= 84\%$$

2.2 Entrapment

$$\textit{Relative activity (\%)} = \frac{\textit{Yield at different pH}}{\textit{Maximum \% activity (optimum pH)}} \times 100$$

pH	Optical Density	Concentration	Enzyme activity	Relative activity (%)
5	0.070	46.667	3.111	79
6	0.078	52.000	3.467	88
7	0.084	56.000	3.733	94
8	0.089	59.333	3.956	100
9	0.082	54.667	3.644	92

Effect of pH

OD at pH 8 = 0.089
Base on BSA standard calibration curve, concentration = 54.667 µg/ml

$$\text{So, enzyme activity} = \frac{54.667\ \mu g/ml}{15\ min}$$

$$= 3.664\ \mu g/ml/min$$

$$\text{\% relative activity} = \frac{3.664\ \mu g/ml/min}{3.956\ \mu g/ml/min} \times 100$$

$$= 92\%$$

3.0 Optimum temperature

3.1 Adsorption

Temperature (°C)	Optical Density		Glucose concentration (µg/mL)	
	Immobilized enzyme	Free Enzyme	Immobilized enzyme	Free Enzyme
50	0.79	0.806	5.064	5.167
60	1.442	1.402	9.244	8.987
70	2.769	2.881	17.750	18.468
80	1.196	1.514	7.667	9.705
90	0.793	0.654	5.083	4.192

Effect of temperature

Free enzyme

OD at 70^0C = 2.881

Based on Glucose standard calibration curve, concentration = 0.156 µg/mL

So, Glucose concentration $= \frac{2.881}{0.156\ \mu g/mL}$

$= 18.468\ \mu g/mL$

Immobilized enzyme

OD at 70^0C = 2.769

Based on Glucose standard calibration curve, concentration = 0.156 µg/mL

So, Glucose concentration $= \frac{2.769}{0.156\ \mu g/mL}$

$= 17.750\ \mu g/mL$

3.2 Entrapment

Temperature (°C)	Optical Density		Glucose concentration (µg/mL)	
	Immobilized Enzyme	Free Enzyme	Immobilized Enzyme	Free Enzyme
50	0.412	0.402	2.641	2.577
60	1.224	1.393	7.846	8.929
70	2.009	2.432	12.878	15.590
80	1.582	1.963	10.141	12.583
90	0.996	1.147	6.385	7.353
100	0.368	0.305	2.359	1.955

Effect of temperature

Free enzyme

OD at 70^0C = 2.432

Based on Glucose standard calibration curve, concentration = 0.156 µg/mL

So, Glucose concentration $= \frac{2.432}{0.156\ \mu g/mL}$

$= 15.590\ \mu g/mL$

Immobilized enzyme

OD at 70^0C = 2.009

Based on Glucose standard calibration curve, concentration = 0.156 µg/mL

So, Glucose concentration $= \frac{2.009}{0.156\ \mu g/mL}$

$= 12.878\ \mu g/mL$