

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

IMMOBILIZATION OF AN ALCOHOL DEHYDROGENASE
FROM BACILLUS THURINGIENSIS SEROVAR
ISRAELNESIS ON EUPERGIT C250 L

MOHAMED SHEHATA
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF CHEMISTRY

GEBZE
2017

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IMMOBILIZATION OF AN ALCOHOL
DEHYDROGENASE FROM *BACILLUS*
THURINGIENSIS SEROVAR *ISRAELNESIS* ON
EUPERGIT C 250 L**

MOHAMED SHEHATA
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF CHEMISTRY

THESIS SUPERVISOR
PROF. DR. AZIZ TANRISEVEN

GEBZE
2017

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

BACILLUS THURINGIENSIS SEROVAR
ISRAELNESIS`DEN ALKOL
DEHYDROGENAZIN EUPERGIT C 250 L
ÜZERİNE İMMOBİLİZASYONU

MOHAMED SHEHATA
YÜKSEK LİSANS TEZİ
KİMYA ANABİLİM DALI

DANIŞMANI
PROF. DR. AZİZ TANRISEVEN

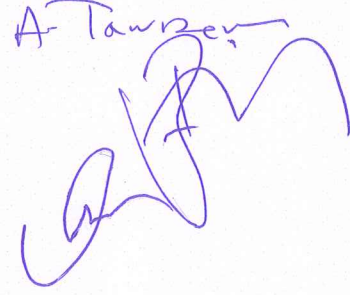
GEBZE
2017

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 12 / 04 / 2017 tarih ve 2017 / 21 sayılı kararıyla oluşturulan jüri tarafından 02 /05 / 2017 tarihinde tez savunma sınavı yapılan MOHAMED SHEHATA'nın tez çalışması Kimya Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE

(TEZ DANIŞMANI) : Prof. Dr. Aziz Tanriseven



ÜYE

: Yard. Doç. Dr. Barış Binay

ÜYE

: Yard. Doç. Dr. Fatih Aktaş

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

...../...../..... tarih ve/..... sayılı kararı.

İMZA/MÜHÜR

Doç. Dr. Arif Çağdaş AYDINOĞLU
Gebze Teknik Üniversitesi
Fen Bilimleri Enstitüsü Müdürü

SUMMARY

Over the past decade, there is an exponential increase in the application of enzymes in such industries as food, pharmaceuticals, and detergents. Global market of industrial enzymes reached about \$4.2 billion dollars in 2014 and expected to increase to \$6.2 billion dollars during the next five years. This is a reflection of the exceptional properties of enzymes.

Alcohol dehydrogenases ADHs are a group of enzymes that exist in many eukaryotic and prokaryotic organisms including humans, animals, yeast, and bacteria. ADHs catalyze the oxidation of primary and secondary alcohols to ketones and aldehydes, respectively with the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. ADHs have gained more interest in recent years because of their importance in the production of enantiometrically pure molecules such as; chiral alcohols, aldehydes, and ketones which are important in drug industries. Using enzymes in large scale production is still limited by their instability against harsh reaction and storage conditions. Moreover, enzymes in soluble form are not reusable which make it expensive for the large scale applications. On the other hand, usage of immobilized enzymes can overcome most of these drawbacks.

In this thesis work, the covalent immobilization of alcohol dehydrogenase from *Bacillus thuringiensis serovar israelensis* (BtADH) on Eupergit C 250 L was optimized, resulting in 100% immobilization and 85.02% activity yields. The immobilized enzyme was used in the biotransformation of 2-propanol to acetone. An inactivation of the immobilized enzyme due to the multiple exposures to 2-propanol and acetone was determined and avoided by optimizing the substrate concentration. The immobilized enzyme retained 88.77% of its initial activity in 12 subsequent uses. The effects of pH and temperature on both of the soluble and immobilized enzymes were also studied.

Keywords: Enzyme immobilization, alcohol dehydrogenase, biooxidation, bioreduction, *Bacillus thuringiensis serovar israelensis*.

ÖZET

Enzimlerin gıda, ilaç ve deterjan gibi endüstriyel alanlarda kullanımı son on yılda dikkate değer bir şekilde artmıştır. Endüstriyel enzimlerin küresel Pazar payı 2014 yılında yaklaşık 4.2 milyar dolar iken gelecek beş yıl içinde bunun 6.2 milyar dolara ulaşacağı tahmin edilmektedir. Bu artışın sebebi enzimlerin özelliklerinden dolayıdır.

Bir oksidoredüktaz grubu enzimi olan alkol dehidrogenaz insan, hayvan maya ve bakteri gibi okaryot ve prokaryot organizmalarda bulunur. Bu enzim nikotin adenin dinükleotit kofaktörünü indirgeyerek primer ve sekonder alkollerin aldehit ve ketona dönüşümünü sağlar. İlaç endüstrisine için oldukça önemi olan kiral alkoller, aldehit ve ketonlar gibi saf enantiyomerik maddelerin üretiminde kullanılan bu enzimlerin önemi son yıllarda giderek artmıştır. Enzimlerin büyük ölçekli üretimlerde kullanımı reaksiyon ve depolama şartlarında dayanıklılıklarıyla sınırlıdır. Ayrıca, serbest halde kullanılan enzimlerin tekrar kullanımı mümkün olmadığından büyük ölçekli üretimlerde kullanımları pahalıya mal olur. Diğer taraftan, immobilize enzimlerin kullanımı bu gibi sorunları giderebilir.

Bu tez çalışmasında, *Bacillus thuringiensis serovar israelensis* alkol dehidrogenaz'ın Eupergit C 250 L üzerine kovalent immobilizasyonu optimize edildi. Çalışmalar sonucunda, %100 immobilizasyon ve % 85.02 aktivite verimleri elde edildi. Immobilize enzim 2-propanolün asetona dönüşümünde kullanıldı. Immobilize enzim 2-propanol ve aseton tarafından inhibe ediliyor bulundu. Bu problemi gidermek için substrat optimizasyon çalışmaları gerçekleştirildi. Optimizasyon çalışmaları sonucunda, immobilize enzimin ard arda 12 defa kullanımı sonucunda % 88.77 seviyesinde aktivitesinin kaldığı gözlemlendi. Bu tez çalışmasında, immobilize ve serbest enzim için optimum sıcaklık ve pH değerleri de belirlendi.

Anahtar kelimeler: Enzim immobilizasyonu, *Bacillus thuringiensis serovar israelensis*, alkol dehidrogenazlar, biyoyükseltgenme, biyoindirgenme.

ACKNOWLEDGEMENTS

I would like first to express my sincere gratitude to my thesis supervisor Prof. Dr. Aziz Tanriseven whose office door was always open whenever I ran into a trouble spot or had a question about my research or writing. He consistently allowed this thesis to be my own work, but steered me in the right direction whenever he thought I needed it.

I would also like to thank my former thesis advisor Prof. Dr. Ayhan Çelik for allowing me to undertake this project and also for his motivation, vision, unfailing support, and commitment since my first day in his lab.

My sincere thanks also go to Asst. Prof. Dr. Fatih Aktaş for his efforts during cloning and characterization of the enzyme used in this thesis work and also for his insightful comments and encouragement. I would like to thank my fellow labmates Fatma Ertan, Dr. Aişe ünlü, Dr. Gülden Yetiş, Ünzile Güven, Uğur pala, and Meltem Çorbacıoğlu for their unlimited support and for the good times we shared during the past three years.

A special thanks to my family. Words cannot express how grateful I am to my parents for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far.

Finally, a very special gratitude goes to my fiancée and my soulmate Nour Alnajjarine for her love, patience, and support. Thank you for everything we have lived together during the past years. I shall always love you and stay with you till infinity and beyond, as we used to say.

TABLE OF CONTENTS

	<u>Sayfa</u>
SUMMARY	v
ÖZET	vii
ACKNOWLEDGEMENTS	vii
TABLE OF CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	X
LIST of FIGURES	xii
LIST of TABLES	xiv
1. INTRODUCTION	1
1.1. Biocatalysis	1
1.2. Enzyme immobilization	1
1.3. Objective of the study	2
2. LITERATURE REVIEW	4
2.1. Enzymes	4
2.2. Classification of enzymes	4
2.3. Oxidoreductases	5
2.4. Alcohol Dehydrogenases (ADHs)	5
2.5. <i>Bacillus thuringiensis</i> serovar <i>israelensis</i> Alcohol Dehydrogenase	7
2.6. Enzyme immobilization	8
2.7. Non covalent immobilization techniques	8
2.8. Covalent immobilization	9
2.9. Eupergit C and Eupergit C 250 L	11
2.10. Immobilization of Alcohol Dehydrogenases	12
3. MATERIALS AND METHODS	14
3.1. Materials	14
3.1.1. Chemicals	14
3.1.2. Kits	14
3.1.3. Bacterial strains	14
3.1.4. Data bases and software	14
3.1.5. Instruments	15

3.2. Methods	15
3.2.1. Investigating the structural features of BtADH	15
3.2.2. Prepration of competent cells	15
3.2.3. Isolation and transformation of the plasmid DNA	16
3.2.4. Overexpression of recombinant BtADH	16
3.2.5. Purification of BtADH	16
3.2.6. Protein analysis	17
3.2.7. Activity assay of soluble and immobilized enzymes	17
3.2.8. Immobilization of BtADH on Eupergit C 250 L	18
3.2.9. Optimization of BtADH immobilization	18
3.2.10. Determination of the substrate-product inhibition	19
3.2.11. Optimization of the substrate concentration	19
3.2.12. Determiation of optimal pH and temperature	20
3.2.13. Reusability of the immobilized enzyme	20
4. RESULTS AND DISCUSSION	21
4.1. Investigating the structural features of BtADH	21
4.2. Overexpression and Purification of BtADH	24
4.3. Immobilization of BtADH of BtADH on Eupergit C 250 L	24
4.4. Optimization of BtADH immobilization on Eupergit C 250 L	25
4.5. Optimization of the substrate concentration	27
4.6. Determination of the optimum pH and Temperature	33
4.7. Reusability of the immobilized enzyme	34
5. Future work	35
REFERENCES	37
BIOGRAPHY	41
APPENDICES	42

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u> <u>and Acronyms</u>	<u>Explanations</u>
μ	: Micro
μL	: Microliter
μM	: Micro molar
mL	: Milliliter
mM	: Millimolar
$^{\circ}\text{C}$	: Degree Celsius
Mg^{2+}	: Magnesium cation
MgCl_2	: Magnesium chloride
NaOH	: Sodium Hydroxide
NaCl	: Sodium Chloride
Zn^{2+}	: Zinc cation
ADHs	: Alcohol dehydrogenases
Amp	: Ampicillin
APS	: Ammonium per sulfate
BSA	: Bovine serum albumin
BtADH	: <i>Bacillus thuringiensis serovar israelensis</i> Alcohol Dehydrogenase
DMSO	: Dimethyl sulfoxide
EDTA	: Ethylenediaminetetraacetic acid
<i>E. coli</i>	: <i>Escherichia coli</i>
G	: Gram
HCl	: Hydrogen chloride
kDa	: Kilo Dalton
LB	: Luria Broth
M	: Molar
NAD^{+}	: Nicotinamide adenine dinucleotide
PAGE	: Polyacrylamide Gel Electrophoresis
pH	: The negative of the base 10 logarithm of the molar concentration, measured in units of moles per liter, of hydrogen ions in a solution
PMSF	: Phenylmethane sulfonyl fluoride

RPM	: Revolution per minute
SDS	: Sodium dodecyl sulfate
SOC	: Super Optimal broth with catabolite repression
TEMED	: Tetramethylethylenediamine
U	: Unit

LIST of FIGURES

<u>Figure No:</u>	<u>Page</u>
1.1:	Schematic presentation of immobilization techniques. 2
2.1:	Alcohol dehydrogenase catalyzed reactions. 5
2.2:	Horse liver alcohol dehydrogenase complexed with NAD ⁺ and trifluoroethanol. 6
2.3:	Yeast alcohol dehydrogenase complexed with bound coenzyme. 7
2.4:	Non-covalent immobilization techniques. 9
2.5:	Covalent immobilization using epoxy resins . 10
2.6:	Covalent immobilization on amino activated resins. 10
2.7:	Crosslinking of enzymes using poly (glutaraldehyde). 11
2.8:	Immobilization of enzymes on Eupergit C 12
4.1:	Sequence alignment of BtADH and 4eex. 21
4.2:	BtADH homology model. 22
4.3:	BtADH surface lysine and glutamic acid residues. 23
4.4:	Electrostatic potential surfaces of BtADH. 23
4.5:	SDS-PAGE analysis of BtADH purification. 24
4.6:	Effect of the reusability on immobilized BtADH activity using 500 mM 2-propanol. 28
4.7:	Effects of acetone and 2-propanol on the activity of immobilized BtADH. 29
4.8:	Effect of the reusability on immobilized BtADH activity using 1 mM 2-propanol. 30
4.9:	Effect of the reusability on immobilized BtADH activity using 0.8 mM 2-propanol. 30
4.10:	Effect of the reusability on immobilized BtADH activity using 0.6 mM 2-propanol. 31
4.11:	Effect of the reusability on immobilized BtADH activity using 0.4 mM 2-propanol. 32
4.12:	Effect of the reusability on immobilized BtADH activity using 0.1 mM 2-propanol. 32

4.13:	Effect of pH on the activities of soluble and immobilized enzymes.	33
4.14:	Effect of temperature on the activities of soluble and immobilized enzymes.	34
4.15:	Effect of the reusability on the immobilized activity	35

LIST of TABLES

<u>Table No:</u>	<u>Page</u>
2.1: Classification of enzymes.	4
4.1: Effect of matrix amount on BtADH immobilization.	25
4.2: Effect of pH on BtADH immobilization.	26
4.3: Effect of buffer concentration on BtADH immobilization.	26
4.4: Effect of coupling time on BtADH immobilization.	27

1. INTRODUCTION

1.1. Biocatalysis

Over the past decade, there is an exponential increase in the application of biocatalysis to industry [1]. The conventional synthetic catalysts have been employed in industry for a long time but, having different disadvantages such as; hazard chemical conditions, complicated processes, high energy consumption, and generation of non-desired by-products paved the way for the employment of biocatalysts [2]. The replacement of synthetic catalysts with biocatalysts results in simple processes and cleaner products which are basic requirements for pharmaceutical and chemical industries. Enzymes are biocatalysts found in all organisms. Enzymes accelerate chemical reactions by lowering the reaction activation energy. Global market of industrial enzymes reached about \$4.2 billion dollars in 2014 and expected to increase to \$6.2 billion dollars during the next five years [3]. This is a reflection of the exceptional properties of enzymes e.g. high selectivity and specificity, non-toxicity, mild reaction conditions, low energy requirements, and less by-products [4]. Enzymes are classified into six main classes; oxidoreductases, transferases, lyases, isomerases, ligases, and hydrolases.

Enzymes can be used in the large scale production of valuable, optically active molecules which cannot be produced using synthetic catalysts [5]. However, using enzymes in large scale production is limited by instability of enzyme against harsh reaction conditions, high susceptibility to presence of other chemicals such as organic solvents, difficult separation from the reaction mixture and lack of the long-term operational stability. Moreover, enzymes in soluble form are not reusable which make it expensive for the large scale applications. On the other hand, usage of immobilized enzymes can overcome most of these drawbacks.

1.2. Enzyme immobilization

Enzyme immobilization can be defined as confining enzymes to inert, solid, and insoluble supports over which a substrate is converted to product. Immobilization of enzymes on different supports imparts improved properties to

enzymes such as improved stability, ease of separation from the reaction mixture, protection against inactivation and denaturation, and stability against organic solvents [6]. Immobilized enzymes are cost effective in industrial productions compared to soluble ones since they can be reused in productions. There are several immobilization techniques including covalent, adsorption, entrapment, cross-linking, and encapsulation (Figure1.1).

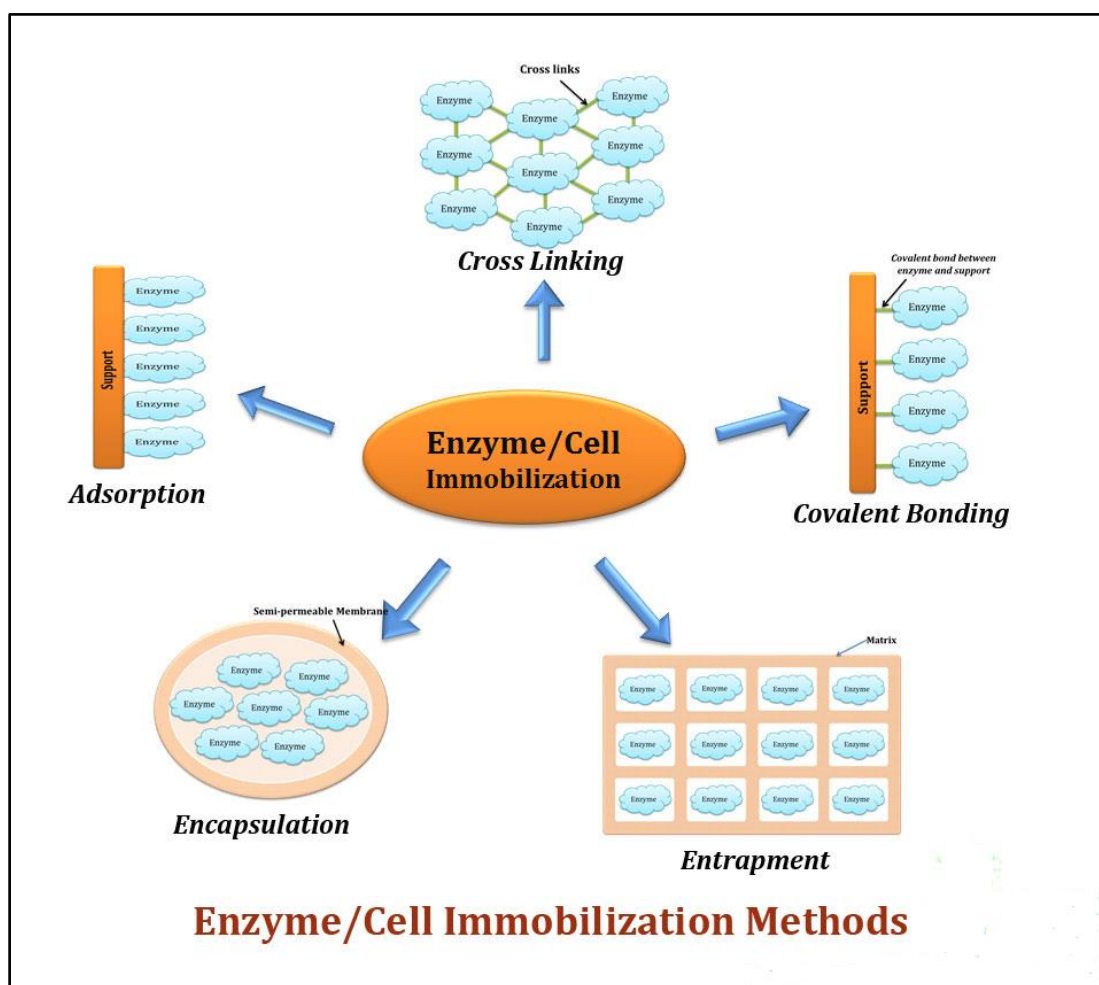


Figure 1.1: Schematic presentation of immobilization techniques.

1.3. Objectives of the study

Enzymatic oxidation of alcohols is one of the most important biotechnological processes in fine chemical and pharmaceutical industries. In a previous study in our biochemistry lab, a novel alcohol dehydrogenase from *Bacillus thuringiensis serovar israelensis* (BtADH) was cloned, overexpressed

in *Escherichia coli*, and utilized in soluble form for the biotransformation of various alcohols, ketones and aldehydes [7]. Considering the results obtained, the objective of this present work was to develop a reusable immobilized alcohol dehydrogenase to be more convenient for biotransformations. The experimental work of the thesis was focused on;

- Construction of the enzyme homology model and studying the nature of enzyme surface and the active site residues.
- Optimization of the covalent immobilization of the enzyme on an epoxy (oxirane)-activated supports (Eupergit C 250 L).
- Characterization of the immobilized enzyme.
- Usage of the immobilized enzyme in the biotransformation of 2-propanol to acetone.
- Optimization the reaction conditions leading to minimizing substrate-product inhibition effect on the immobilized enzyme activity.

2. LITERATURE REVIEW

2.1. Enzymes

Enzymes are proteins that catalyze the chemical reactions within the cell. Enzymes have always been a hot topic of research and were extensively investigated. Following the production of the first recombinant protein in the early 1970s, there was a revolution in the industrial uses of enzymes. The development in biotechnology during the last four decades has been reflected in a significant increase in enzyme application to numerous such industries as food, beverage, textile, detergents, and pharmaceuticals [8]. Enzymes are superior to chemical catalysts because of high regio- and chemo- selectivity, mild reaction conditions, lower energy consumption, less by-products formation, and eco-friendly processes [9]. However, enzymes have some disadvantages including high cost, denaturation and inactivation, difficult separation from the reaction media, and weak operational/storage stabilities [10].

2.2. Classification of enzymes

Enzymes are classified into six main classes; oxidoreductases, transferases, lyases, isomerases, ligases, and hydrolases (Table 2.1).

Table 2.1: Classification of enzymes.

Class of enzyme	Catalyzed reaction	Example
Oxidoreductases	Oxidation-reduction reactions where electron or atom is transferred from substrate to another.	Dehydrogenase, peroxidases, oxygenases
Transferases	Transfer of specific group from a substrate to another	Methyltransferase, transaminase,
Lyases	Nonhydrolytic bond cleavage	Dehydratases, decarboxylases,
Isomerases	Isomeric reactions	Racemases, mutases
Ligases	Covalent bond formation	Synthetase, RNA polymerases
Hydrolases	Hydrolysis reactions	Proteases, lipase, helicases

2.3. Oxidoreductases

Oxidoreductases are cofactor dependent enzyme group that catalyze the transfer of electrons from one molecule to another during the oxidation-reduction reactions. The cofactors used in these reactions are NAD^+ , NADP^+ , and FAD . Most of metabolic pathways involve oxidoreductase-catalyzed reactions. Oxidoreductases consist of 22 different enzyme subclasses including; alcohol dehydrogenases, oxygenases, and peroxidases. Oxidoreductases have been utilized in different industrial applications such as; synthesis of chiral molecules for pharmaceuticals, production and functionalizing of polymers, biosensors, and organic pollutants degradation [11]. The commercializing of oxidoreductases is limited because of their expensive cofactors. Different strategies have been developed to overcome this problem by regeneration of cofactors.

2.4. Alcohol Dehydrogenases (ADHs)

Alcohol dehydrogenases (EC 1.1.1.1) are NAD^+ dependent enzymes that catalyze the inter-conversions of ketones, alcohols, and aldehydes (Figure 2.1). Alcohol dehydrogenases exist in many eukaryotic and prokaryotic organisms such as; humans, animals, yeast, and bacteria [12]. Alcohol dehydrogenases play a vital role in many important biological processes including biosynthesis of different metabolites in mammals and fermentation reactions in yeast and bacteria.

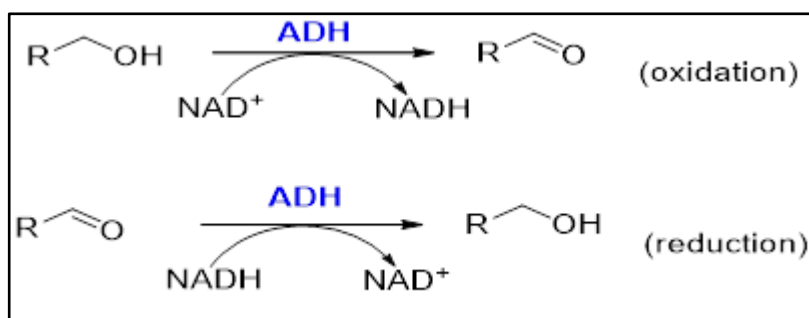


Figure 2.1: Alcohol dehydrogenase catalyzed reactions.

The first alcohol dehydrogenase was isolated from *Saccharomyces cerevisiae* (brewer's yeast) in 1937 by Erwin Negelein and his coworkers. Many alcohol dehydrogenases have been isolated from different organisms. In 1948, Bonnichsen and Wassen isolated and crystallized the first ever horse liver alcohol dehydrogenase. It was found that the properties of these enzymes are different from the alcohol dehydrogenase isolated from yeast [13]. The yeast alcohol dehydrogenases have two times molecular weight and are 100 times more active in comparison to horse liver alcohol dehydrogenases [14].

Alcohol dehydrogenases are classified according to their primary sequence length into three main dehydrogenase families; (i) short chain (SDR), (ii) medium chain (MDR), and long chain (LDR) dehydrogenases [15]. ADHs were among the first enzymes, the three-dimensional structures and amino acid sequences of which were determined [16]. Horse liver alcohol dehydrogenases have two subunits each of which contains two zinc atoms. The active site is located in the space between the cofactor binding and the catalytic domains. (Figure 2.3) [17].

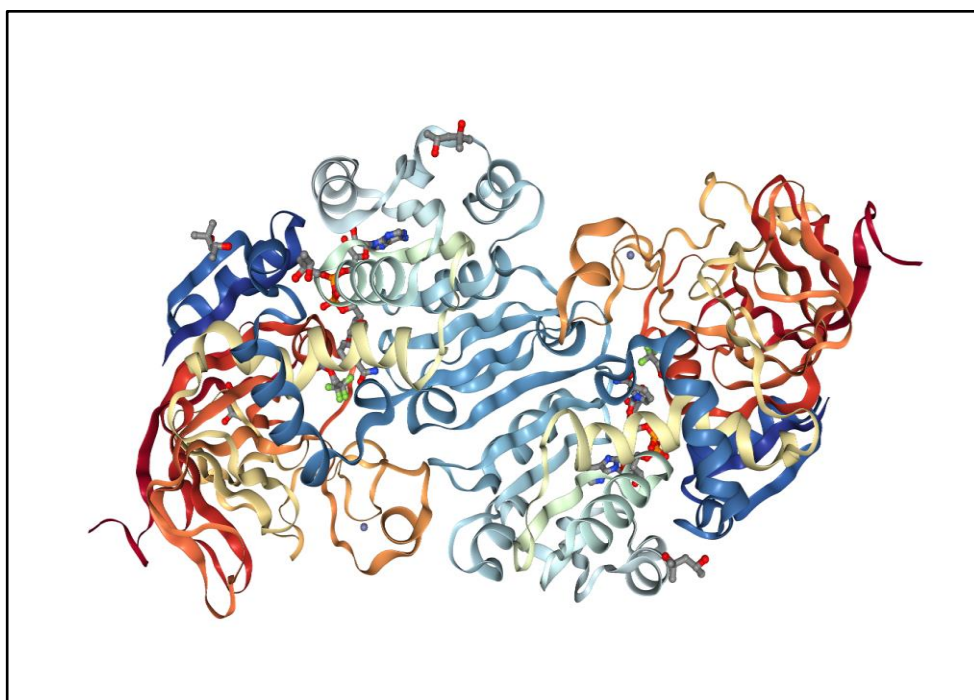


Figure 2.2: Horse liver alcohol dehydrogenase complexed with NAD⁺ and trifluoroethanol.

On the other hand, the fungal, bacterial, and yeast alcohol dehydrogenases have tetrameric structures in which, two dimers are interacted and the active sites are exposed in opposite directions [18].

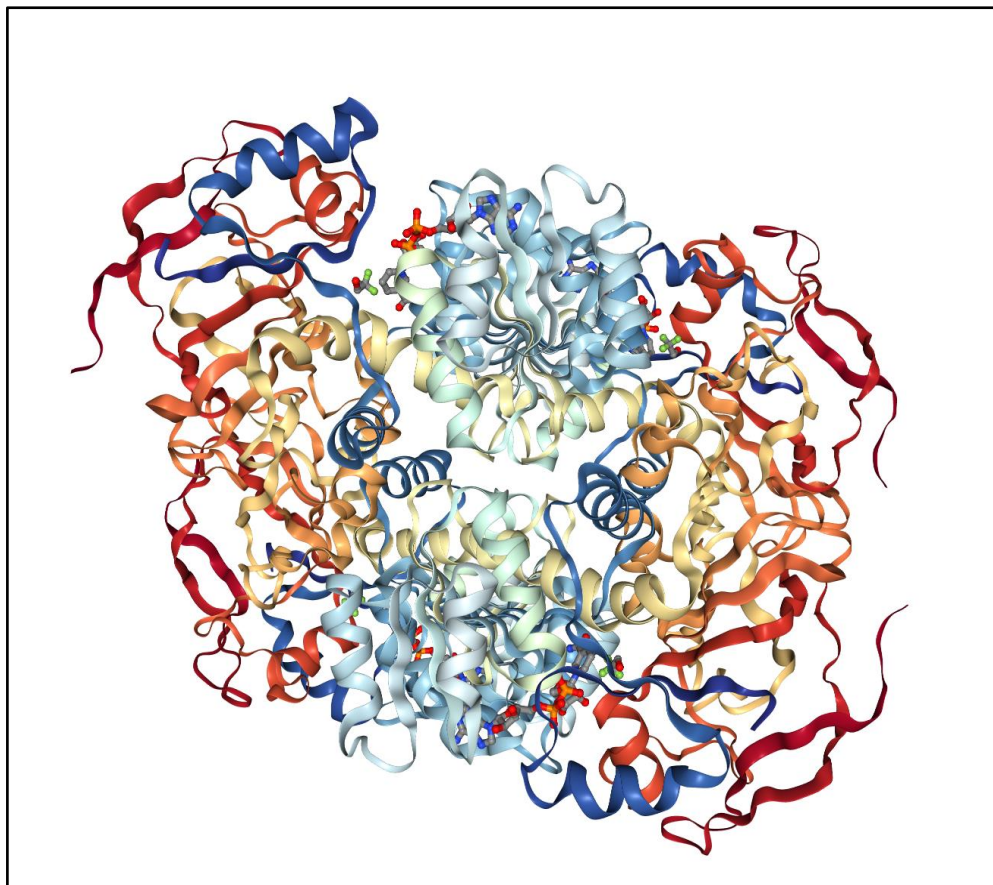


Figure 2.3: Yeast alcohol dehydrogenase complexed with bound coenzyme.

ADHs have gained more interest in recent years because of their importance in the production of enantiometrically pure molecules such as; chiral alcohols, aldehydes, and ketones which are important in many industries including pharmaceuticals and fine chemicals. Several metal catalysts could be used in place of ADHs in the enantioselective synthesis of chiral compounds however, usage of these catalysts leads to many difficulties such as; lower optical activity, purification problems, hazardous byproducts, and requirements of sophisticated procedures[19], [20]. On the other hand, alcohol dehydrogenases can be used in production of optically pure molecules through environmentally benign processes.

2.5. *Bacillus thuringiensis* serovar *israelensis* Alcohol Dehydrogenase (BtADH)

Bacillus thuringiensis serovar *israelensis* alcohol dehydrogenase (BtADH) is a novel NADH dependent zinc containing enzyme [7]. BtADH is a medium chain dehydrogenase consists of 345 amino acids. This enzyme has high affinity towards short and medium chain primary substrates. However, it has lower affinities towards long chain and cyclic substrates. BtADH can be utilized in large scale production of different alcohols, aldehydes, and ketones but, it shares the common disadvantages of enzymes including high cost and low operational and storage stabilities. Different such strategies as protein engineering and enzyme immobilization methods have been developed to overcome these obstacles.

2.6. Enzyme Immobilization

Enzyme immobilization can be defined as confining enzymes to inert, solid, and insoluble supports over which substrates are converted to products. Immobilization of enzymes is the best and the simplest solution for most of the problems associated with the application of enzyme at industrial scale[21]. Different advantages can be achieved by immobilizing enzymes including;

- Increased operational and storage stabilities
- Pure products
- Better control of the reactions
- Reusability
- Reduced inhibition and denaturation

2.7. Non-covalent Immobilization Techniques

Non-covalent immobilization is a simple, fast and cheap technique (Figure 2.5). It's based on non-covalent interactions such as; Van der Waals force and ionic interactions. Several non-covalent immobilization procedures have been developed such as;

- Entrapment of enzymes in polymeric materials such as; sol-gel.
- Adsorption of enzymes to the surface of different carriers such as; organic, mineral, and modified resins.
- Encapsulation of enzymes in semi-permeable capsules such as; nylon and nitro capsules.

This technique has been utilized in the immobilization of different enzymes but, despite of its simplicity and low cost it suffers from the following problems;

- Mechanically unstable at the industrial harsh conditions
- Enzyme leakage from the matrix
- Pore size limitations that result in substrates/products diffusion problems

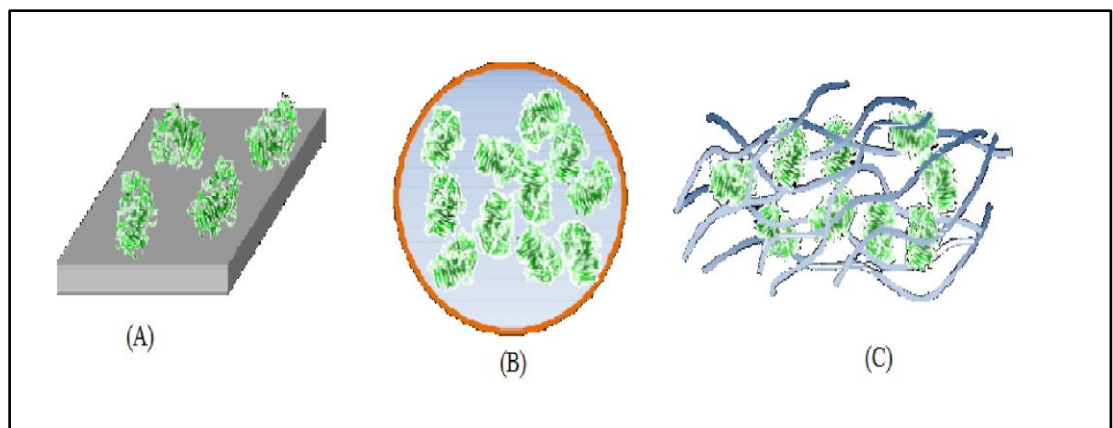


Figure 2.4: Non-covalent immobilization techniques, (A) adsorption, (B) encapsulation, (C) entrapment.

2.8. Covalent Immobilization

Covalent immobilization involves binding of enzymes to solid supports. It can be achieved by the reaction between the support functional groups and the reactive groups found on the enzyme surface such as; amino, carboxyl, hydroxyl, and sulfhydryl groups. Covalent immobilization is always the method of choice because of the followings;

- High mechanical stability

- No desorption or diffusion problems
- Availability of several supports with various functional groups
- Large amount of enzyme loading

Covalent immobilization can be achieved by using different resins and reagents as shown in (Figure 2.6) and (Figure 2.7).

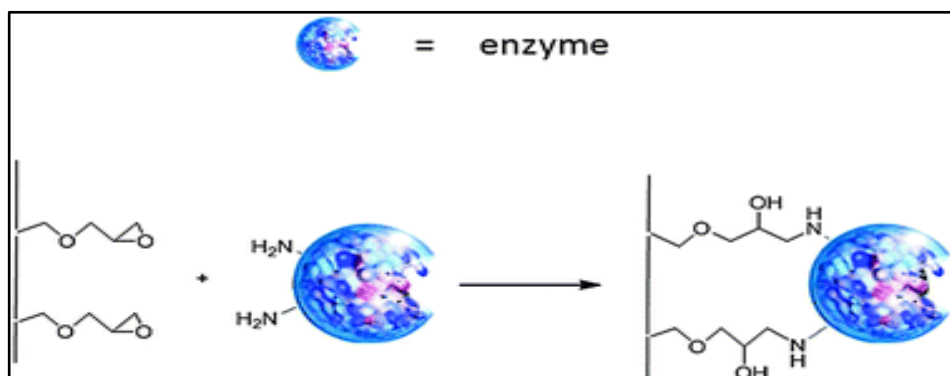


Figure 2.5: Covalent immobilization using epoxy resins.

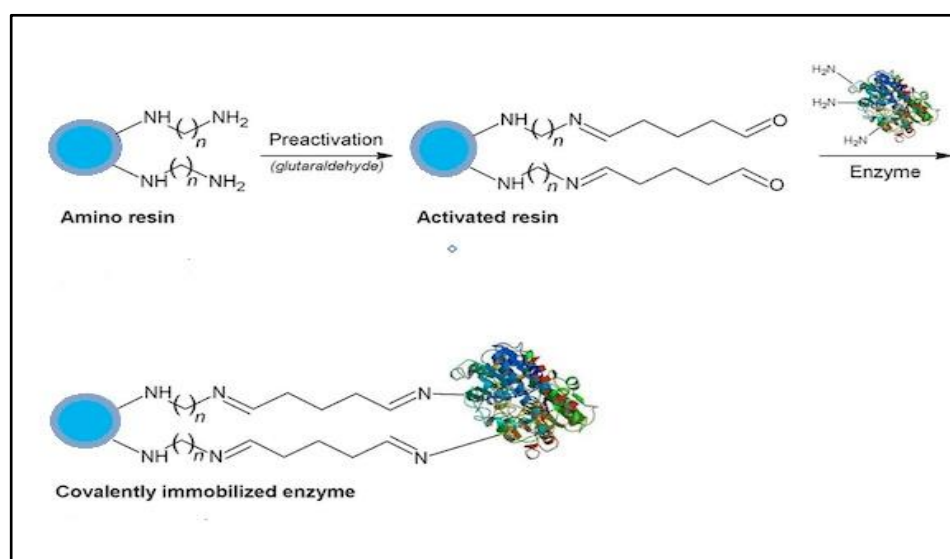


Figure 2.6: Covalent immobilization on activated amino resins.

Enzymes also could be crosslinked using multi-functional reagents leading to an insoluble 3 dimensional enzyme aggregate which could be used immobilized biocatalysts [22]. Different chemical reagents can be used in this methods such as; hexamethylene diisocyanate, glutaraldehyde, and bisdiazobenzidine (Figure 2.7).

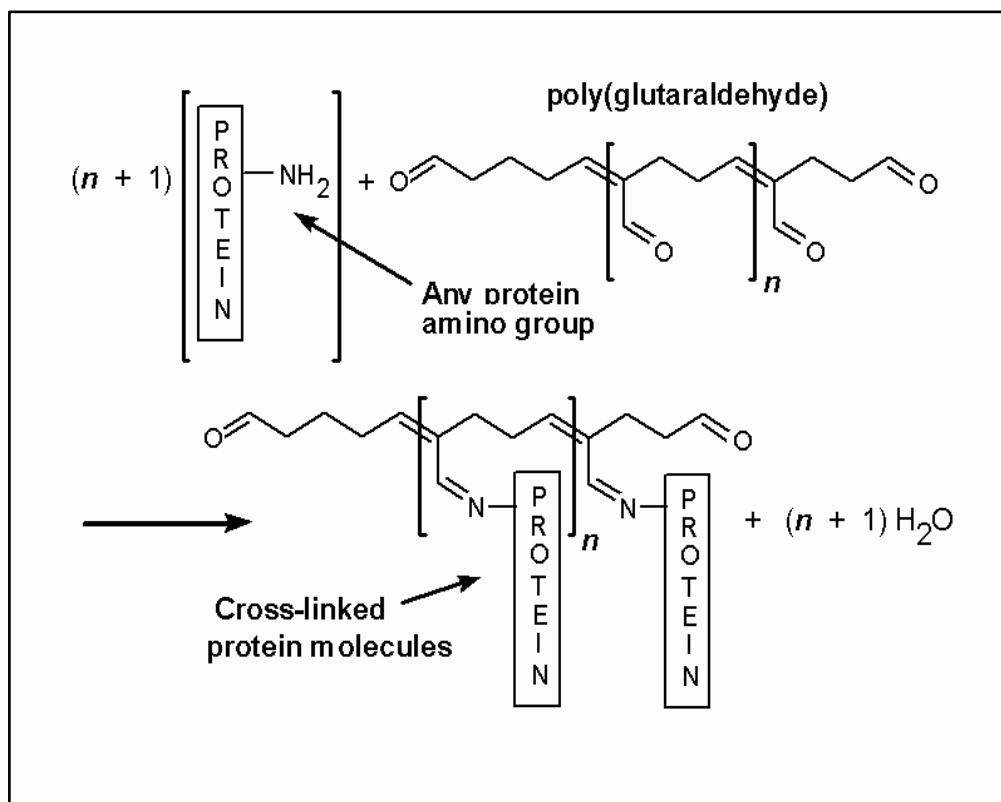


Figure 2.7: Crosslinking of enzyme using poly (glutaraldehyde).

Different types of polymers can be used as carriers in covalent immobilization depending on the nature of the enzyme [23]. These polymers can be classified into three main categories;

- Synthetic organic polymers such as; Eupergit C, Eupergit C 250 L, and Immobead.
- Inorganic polymers such as; silica and zeolite.
- Natural polymers such as; chitosan, alginate, starch, agarose, and DEAE-Sephadex.

2.9. Eupergit C and Eupergit C 250 L

Eupergit C and Eupergit C 250 L are epoxy (oxarane)-activated beads that are used widely in the covalent immobilization. These matrices have been commercially produced Rohm& Haas (Germany). They are synthetic copolymers made of different monomers including; glycidylmethacrylate, N,N'-methylene-bismethacrylamide, allyl ether, and methacrylamide [24]. Both of the two matrices

have a diameter between of 100-250 μm but, they differ in the pore size and the epoxy content. The pore sizes are 10 nm and 100 nm while the epoxy contents are 600 $\mu\text{mol/g}$ and 300 $\mu\text{mol/g}$ for Eupergit C and Eupergit C 250 L, respectively [25]. Immobilizing enzymes using these matrices is simple, fast, and doesn't involve any preactivation. Furthermore, they could be used in a wide range of pH from 0 to 14. Generally, the epoxy groups on the supports react with different nucleophilic groups on the enzyme surface such as; amino, phenolic, carboxylic and thiol forming multipoint covalent attachments (Figure 2.8) [26]. These kind of attachments impart higher conformational stability to the enzymes [27].

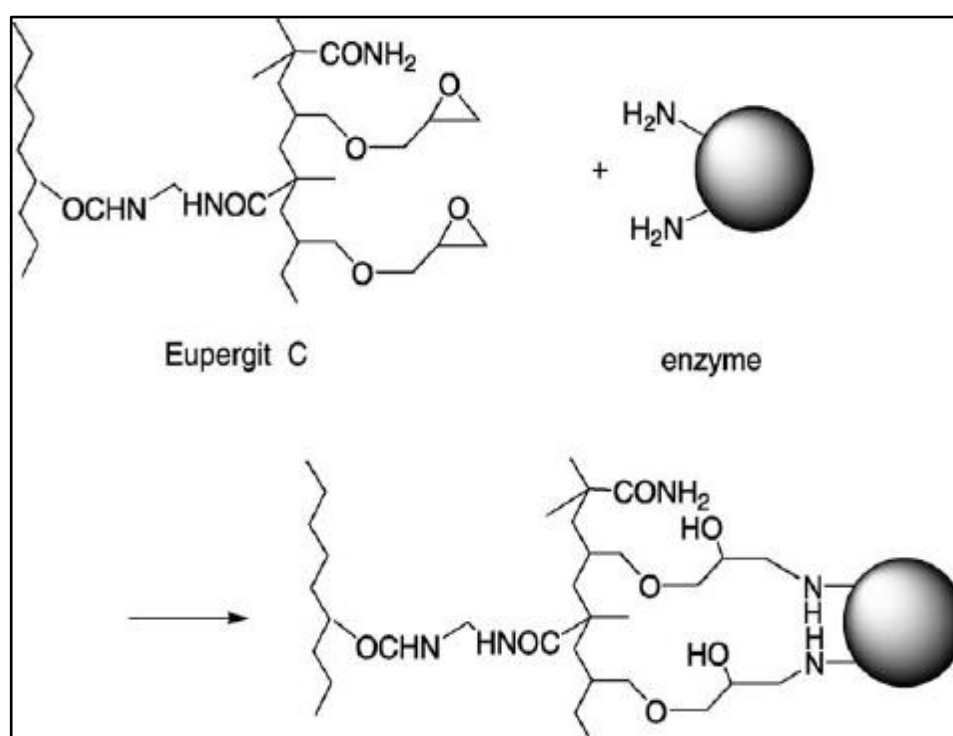


Figure 2.8: Immobilization of enzymes on Eupergit C.

2.10. Immobilization of Alcohol Dehydrogenases

Several studies have been reported on the immobilization of alcohol dehydrogenases on various supports using different techniques including covalent binding, entrapment, and adsorption.

A study was reported on the covalent immobilization of alcohol dehydrogenase from *Thermoanaerobium Brockii* (TBADH) on Eupergit C and cyanogen bromide-activated CL-Sepharose-4 [28]. The activity yields were 15%

and 30% for Eupergit C and cyanogen bromide-activated CL-Sepharose-4, respectively. However, the usage of Eupergit C was better CL-Sepharose-4 because of its higher mechanical stability. This immobilized enzyme was used in enantioselective reduction of 5-chloropentan-2-one yielding optically pure product.

Another study was reported on the covalent immobilization of an alcohol dehydrogenase from baker's yeast on Fe_3O_4 magnetic nanoparticles [29]. The activity yield was 62% and the immobilized enzyme exhibited improved stability in comparison to the free enzyme. Paradisi et al. reported the covalent immobilization of an alcohol dehydrogenase from *halophilic archaeon Haloferax volcanii* on a metal activated support (Ni-epoxy Sepabeads supports) [30]. The immobilization and activity yields were 100% and 61%, respectively. The immobilized enzyme exhibited higher thermal and conformational stabilities and organic solvents tolerance than the free enzyme.

Boliver et al. reported the covalent immobilization a horse liver alcohol dehydrogenase on glyoxyl-agarose supports [31]. The immobilized enzyme exhibited higher stability against different organic solvents, pH, and temperature. This improved stability is a reflection of the multipoint covalent attachments between the enzyme and the support. The immobilization and activity yields were 100% and 91%, respectively.

Sunil et al. reported the immobilization of a yeast alcohol dehydrogenase (YADH) via two different techniques, entrapment in a copolymer network (acrylamide-co-hydroxyethyl methacrylate) and covalent binding to preactivated chitosan beads [32]. The activity yields were 24% and 90% for the covalent and entrapment immobilization methods, respectively. Both of immobilized enzymes exhibited improved operational and storage stabilities in comparison to the soluble enzyme.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

2-propanol, Acetone, β -Mercaptoethanol, Coomassie brilliant blue R-250, PMSF (Phenylmethylsulfonyl fluoride), Sodium Dodecyl Sulphate (SDS), DMSO, Sodium phosphate dibasic, Sodium phosphate monobasic, Bradford reagent, Lysozyme, and Trizma were obtained from Sigma-Aldrich (Germany). Yeast extract granulated, Peptone, Sodium Chloride, Agar, NAD⁺ (Nicotinamide adenine dinucleotide), Ampicillin, TEMED, Acrylamide, Imidazole, and glucose were purchased from Merck (Germany). Albumin Fraction V (BSA), Lactose and Glycine were purchased from Applichem (Germany). Eupergit C250 L was provided by Degussa (Germany). All other biochemical reagents were obtained from various sources at the highest purity.

3.1.2. Kits

Plasmid isolation kit (QIAprep® Spin Miniprep Kit) was purchased from Qiagen, (Germany).

3.1.3. Bacterial strains

E.coli Top 10 and BL21 (DE3) were obtained from Invitrogen, (USA).

3.1.4. Data bases and software

National Center for Biotechnology Information (NCBI), Universal Protein Resource (UNIPROT), I-TASSER server, SDSC Biology Workbench, and Rosetta Commons protein data base were used for the protein sequence alignments. Swiss-Model, Geneious, and Chimera were used for the construction of the protein homology model and for studying the structural features of the enzyme.

3.1.5. Instruments

-80°C deep freezer (Forma - 86 °C ULT Freezer, Table top centrifuge (Beckman Allegra X-15R), Ultracentrifuge (Beckman Coulter™ Avanti™ J-30I Centrifuge), Table top centrifuge (Eppendorf Centrifuge 5810 R), Uv-vis Spectrophotometre (Shimadzu UV 3600), Mini-PROTEAN® 2-D electrophoresis system (Bio-RAD), Autoclave (HIRAYAMA, HICLA HVE-50), pH Meter (Inolab), +4°C and -20°C fridge (Arçelik), Water Bath (TECHNE, TE-10A Tempette), Ice flakes machine (Izmak), Microplate reader (SpectraMax Plus 384, Molecular Devices), Microwave oven (Binder), Magnetic stirrer (Heidolph), Incubator shaker (Excella E-25), Soniprep 150 sonicator (Sanyo, Tokyo, Japan), HiTrap™ column (Amersham Biosciences, UK), PD-10 desalting column (Amersham Biosciences, UK).

3.2. Methods

3.2.1. Investigating the Structural features of BtADH

In order to investigate the structural features of the enzyme, a homology model of the enzyme was constructed. Simply, sequence alignments were carried out using different protein data bases including; National Center for Biotechnology Information (NCBI), Universal Protein Resource (UNIPROT), I-TASSER, SDSC Biology Workbench, and Rosetta Commons. After that, the best template was used in the construction of the homology model by using Swiss-Model server. Then, the protein 3-D structure was studied and visualized by using Chimera software. The active site of the enzyme was studied using Geneious software.

3.2.2. Preparation of Competent Cells

Competent *E. coli* Top10 and BL21 cells were prepared using a standard protocol [33]. Briefly, a single *E. coli* colony from the stock culture was inoculated overnight in 3 ml LB medium at 30°C with continuous shaking at 170 rpm. After the overnight growth, 1 ml of the cell cultures was transferred to 100

ml fresh LB media. Cell cultures were grown to an optical density up to 0.3 at 600 nm. Cells were then collected by centrifugation for 10 min at 10000 rpm, +4°C. After discarding the supernatant, 1/10 TSS solution was used to re-suspended pellets on ice. For long-term storage, prepared competent cells were frozen and stored at - 80°C until future use.

3.2.3. Isolation and Transformation of Plasmid DNA

Plasmid DNA (pET14b-BtADH) was isolated from competent *E.coli* Top10 stock using Plasmid DNA purification kit (QIAprep® Spin Miniprep Kit). The transformation of the isolated plasmid into *E. coli* BL21 competent cells was carried out by mixing 3 µL of the previously isolated BtADH plasmid with 50 µL of competent cells. The mixture was incubated on ice for 30 minutes then; cells were transformed by heat shock at 42°C for 50 seconds in a water bath. Cells were cooled immediately on ice for 60 seconds and then added to 250 µL of S.O.C medium. Cells were grown in shaking incubator at 37°C (220 rpm) for 60 min. The cells were then plated on LB agar plates containing 100µg/ml ampicillin and incubated overnight at 37°C.

3.2.4. Overexpression of Recombinant BtADH

For BtADH expression, a modified auto induction procedure was used [34] . Briefly, the previously transformed cell was grown overnight on LB Agar plates containing 100 µg/ml ampicillin at 37 °C. A single colony from LB-Agar was used to inoculate a 5 ml of LB starter culture containing 100µg/ml ampicillin. This culture was then utilized in the induction of 500 ml Studier media supplemented with 100 µg/ml ampicillin in a 2 L baffled flask. The pH of the medium was adjusted to 7.00 and it was autoclaved. The growth medium was incubated at 30 °C with vigorous shaking (165 rpm) for 18 hours. Bacterial cells were then harvested by centrifugation at 5800 rpm and +4°C for 20 min. Pellets were stored at -20°C until later use.

3.2.5. Purification of BtADH

Four grams from stored cell pellets were retrieved and suspended in 16 ml 20 mM Tris-Cl buffer pH 7.8 containing 5 mM imidazole and 0.5 M NaCl. This was followed by addition of 40 μ L freshly prepared 100 μ g/ml lysozyme and 400 μ L of 0.1 mM (Phenylmethylsulfonyl fluoride) PMSF. Then, the mixture was incubated in ice bath for 45 min with gentle shaking. The cells were disrupted further mechanically by sonication on ice for 6 min with the pulse being on for 15 secs and off for 45 sec. In order to remove the cell debris, the crude cell free extract was centrifuged at $10000 \times g$ for 45 min. at $+4^{\circ}\text{C}$. The clarified extract was loaded onto a 2-ml Nickel column HiTrap™ column (Amersham Biosciences, UK). A buffer containing 100-400 mM imidazole was used for elution of His-tagged BtADH. Elutes were reduced to 2.5 ml using a spin concentrator. Then, elutes were loaded on to a PD-10 desalting column (Amersham Biosciences, UK) for imidazole removal and it was collected by sodium phosphate buffer (50 mM, pH 7.5). Concentrated purified enzyme was stored in 50% (v/v) glycerol at -20°C until use.

3.2.6. Protein Analysis

10% SDS-PAGE analysis was performed on Mini-PROTEAN® 2-D electrophoresis system (Bio-RAD) in order to evaluate the purity of the produced enzyme. After the electrophoresis, proteins in the gel were visualized by Coomassie Brilliant Blue R250 staining. The concentration of purified was determined using Bradford Assay [35].

3.2.7. Activity Assay of Soluble and Immobilized BtADH

The activity of BtADH was measured spectrophotometrically using 2-propanol as the substrate. The standard reaction mixture in a total volume of 10 ml contained (1mg) soluble or (100mg) immobilized enzyme, 0.5 mM NAD^{+} , and 0.5 M 2-propanol in Tris-Cl buffer solution (50 mM, pH 8.5) in case of soluble enzyme and in sodium phosphate buffer solution (50 mM, pH 8.0) in case of immobilized enzyme. The reaction was initiated by addition of NAD^{+} , followed by incubation for 25 min with gentle shaking at 35°C and 30°C for soluble and immobilized enzyme, respectively. Aliquots (1.5 ml) from the reaction mixture

were centrifuged at 13000 rpm for 40 seconds. The absorbance of 800 μ L from the supernatant was measured at 340 nm. Unit activity of enzyme (IU) is defined by the amount of enzyme required for the conversion of 1 μ mol NAD⁺ to NADH per minute. The amount of NAD⁺ was calculated using Beer's law : $A = \epsilon L C$ where A = absorbance , L= Path length (1 cm) , and ϵ is the molar absorption coefficient of NAD⁺ at 340 nm = 6.22 mM⁻¹ cm⁻¹ (using a quartz cuvette).

Control reaction blanks were used in calculations for soluble and immobilized enzyme, In case of soluble enzyme, all the components of the reaction mixture were present except the enzyme and for the immobilized enzyme, all the components of the reaction mixture including Eupergit C 250 L were present except the enzyme.

3.2.8. Immobilization BtADH on Eupergit C 250 L

Immobilization was carried out by reacting 100 mg Eupergit C 250 L with 1 mg freshly prepared BtADH in 10 ml sodium phosphate buffer solutions (50 mM, pH 6.5) at +4°C with gentle shaking (150 rpm) for 3 hours. Then, the reaction mixture was washed with NaCl (10 ml, 1 M), sodium phosphate buffer solution (10 ml, 50 mM , pH 7.5), and 10 ml distilled water, respectively on a sintered glass filter by suction under vacuum. Immobilized enzyme was stored in sodium phosphate buffer (10 ml, 50 mM, pH 8) at +4°C until later use.

3.2.9. Optimization of BtADH Immobilization

Immobilization of enzymes on of Eupergit C 250 L is affected by different parameters such as amount of matrix, buffer concentration, pH, and duration of immobilization [25]. Thus, optimization of these different parameters was carried out by keeping all the immobilization parameters fixed except for one parameter as following:

- **Effect of matrix amount**

The effects of the amount of Eupergit C 250 L on both immobilization and activity yields were tested by reacting 1 mg soluble BtADH with different amounts of the matrix (50-1000 mg).

- Effect of pH

The effects of pH on both immobilization and activity yields were tested by carrying out the immobilization sodium phosphate buffer at different pH (6.0-8.0).

- Effect of buffer concentration

The effects of buffer concentration on both immobilization and activity yields were tested by carrying out the immobilization process in different concentrations of sodium phosphate buffer (25-800 mM).

- Effect of immobilization duration

The effects of immobilization duration on both immobilization and activity yields were tested by carrying out the immobilization for various amounts of time (1-12h).

3.2.10. Determination of the Substrate-Product Inhibition

In order to determine the extent of the substrate-product inhibition, the immobilized enzyme was incubated in 2-propanol (0.5 M) and acetone (0.34 mM) for different periods of time (25-200 minutes) simulating 6 batch reactions. The effect of incubation on the activity was determined.

3.2.11. Optimization of the Substrate Concentration

A study was carried out to determine the optimum substrate concentration that would achieve the highest activity yield and the minimum activity loss due to product inhibition. Simply, the reaction was carried out using different substrate concentrations (0.1, 0.4, 0.6, 0.8, 10, and 500 mM) and the immobilized enzyme was further used in 6 consecutive batch reactions. After every single reaction immobilized enzyme was washed with sodium phosphate buffer solution (10 ml, 50 mM, pH 7.5) on a sintered glass filter by suction under vacuum and collected carefully in order to avoid any loss in the immobilized enzyme amount.

3.2.12. Determination of Optimal pH and Temperature

- Optimum pH

To study the effect of pH on the activity of BtADH, the activity of both soluble and immobilized BtADH was determined by the standard assay conditions described above using 50 mM buffer of different pH values. The used buffers were sodium phosphate pH (6.0-7.5), Tris-Cl pH (8.0-9.0), glycine-sodium hydroxide pH (10.00), and potassium chloride-Sodium hydroxide pH (12.00). The pH at which enzyme showed the highest activity was taken as the control (100%) activity for the calculation of the remaining percent activity for both the soluble and immobilized BtADH.

- Optimum Temperature

To study the effect of temperature on the activity of BtADH, The activity assay was performed at different temperatures (15-50°C). The temperature at which enzyme exhibited highest activity was taken as the control (100%) activity for the calculation of the remaining percent activity for both the soluble and immobilized BtADH.

3.2.13. Reusability of the Immobilized Enzyme

The reusability of the immobilized BtADH was tested by carrying out 12 batch experiments in triplicate. After each assay reaction immobilized enzyme was washed with sodium phosphate buffer solution (10 ml, 50 mM , pH 7.5) on a sintered glass filter by suction under vacuum and collected very carefully in order to avoid any loss in the immobilized enzyme amount. The activity determined in the first assay was considered as control (100%) for the calculation of remaining activity.

4. RESULTS AND DISCUSSION

4.1. Investigating the Structural Features of BtADH

Generally, covalent immobilization is based on reactions between the matrix functional groups and the reactive residues abundant on the enzyme surface such as; lysine, glutamic acid, and histidine. In order to determine the possibility of immobilizing BtADH, a study was carried out to investigate the structural features of the enzyme. Different sequence alignments were performed and resulted in, a total of 4982 templates that match the BtADH sequence. A homo dimer alcohol dehydrogenase (PDB ID: 4eex) showed the highest sequence similarity (64.60%) with BtADH (Figure 4.1). This enzyme was used later as a template for the construction of BtADH homology model.



Figure 4.1: Sequence alignment of BtADH and 4eex.

After Visualizing the enzyme 3-D structure, it seems to be a homodimer that consists of two interacted dimers. Each one of them contains two zinc atoms, one catalytic and the other one is structural and shows its active site in opposite direction (Figure 4.2).

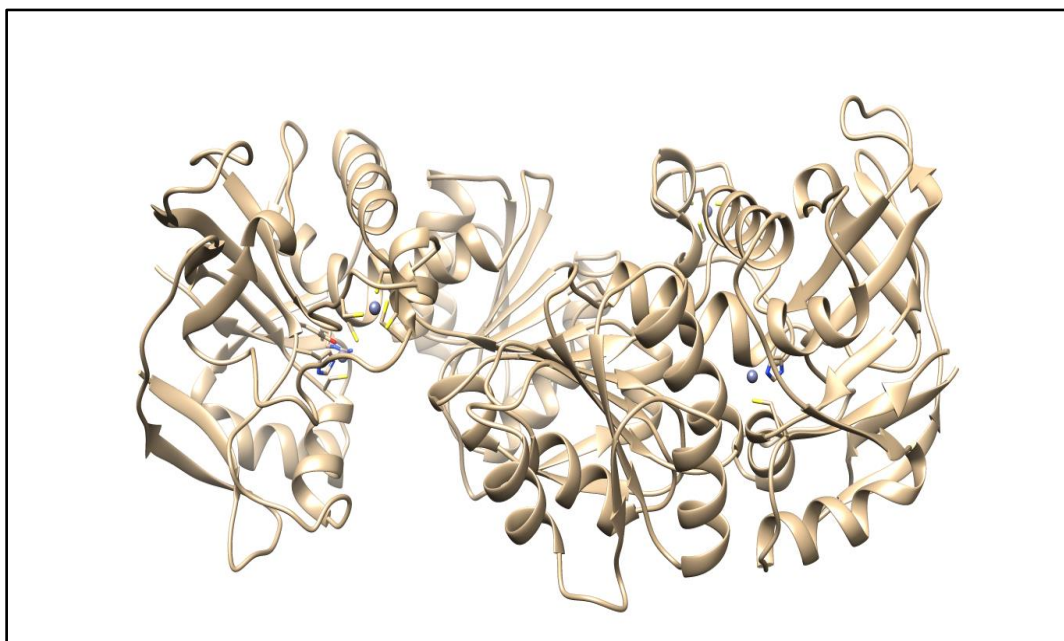


Figure 4.2: BtADH homology model.

By investigating the enzyme structure, it was found that the enzyme active site consists of four critical sites as following;

- Structure Zinc binding site (Cys91, Cys94, Cys97, Cys105)
- Catalytic zinc binding site (Cys39, His60, Cys147)
- Substrate binding site (Cys39, Thr41, Phe50, His60, Trp86, Cys147, Pro264, Gly288)
- NAD⁺ binding site (Cys39, His40, Tyr41, Cys147, Tyr151, Leu175, Gly174, Gly173, Gly171, Asp195, Val194, Lys200, Ala245, Pro264)

BtADH was found rich of both lysine and glutamic acid residues at its surface which make it profoundly suitable for the covalent immobilization. BtADH dimeric structure contains 48 lysine residues; 46 of them are on the

surface and only two exists at the active site. In addition, it also contains 44 glutamic residues; all of them exist at the surface of the enzyme (Figure 4.3).

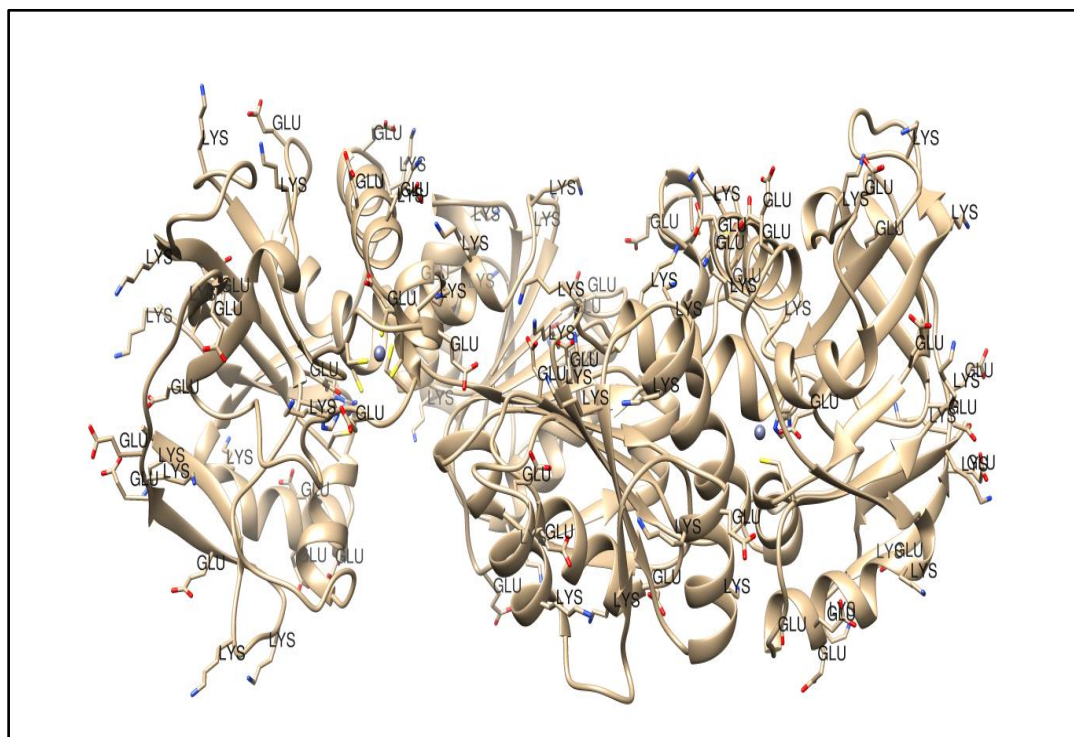


Figure 4.3: BtADH surface lysine and glutamic acid residues.

In order to show all the positive, negative, and neutral residues at the surface of the enzyme, the surface potentials were calculated and visualized (Figure 4.4).

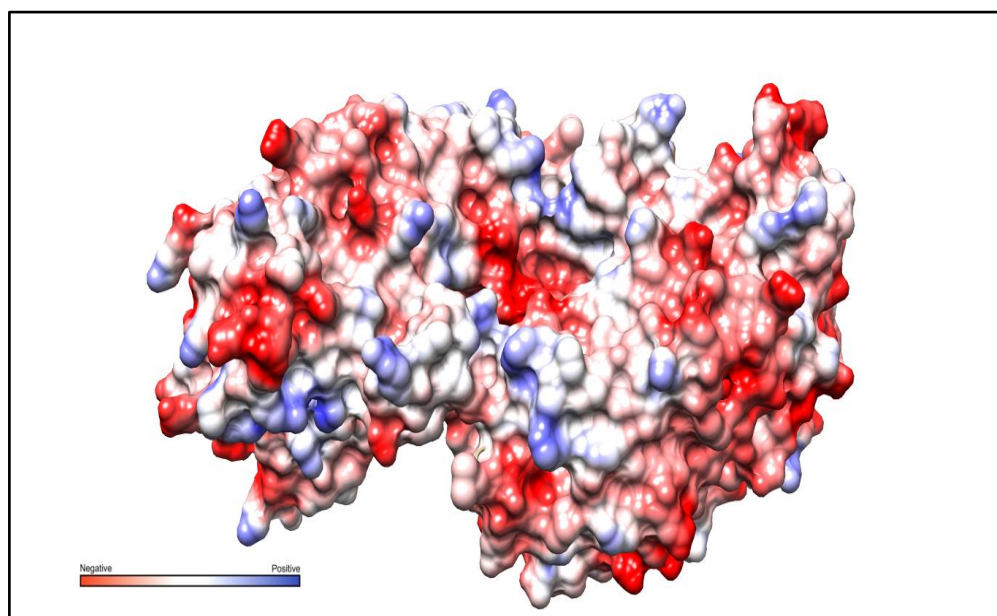


Figure 4.4: Electrostatic potential surfaces of BtADH.

4.2. Overexpression and Purification of BtADH

The pET14b-BtADH plasmid was isolated from the *E. coli* Top10 stock and transformed to *E. coli* BL21 (DE3) host cells. The recombinant BtADH was expressed in auto induction media. The enzyme was purified using Nickel column affinity chromatography and PD-10 desalting columns, resulting in 60 mg pure enzyme. The expression and purification of BtADH were confirmed by 10 % SDS-Page analysis (Figure 4.1).

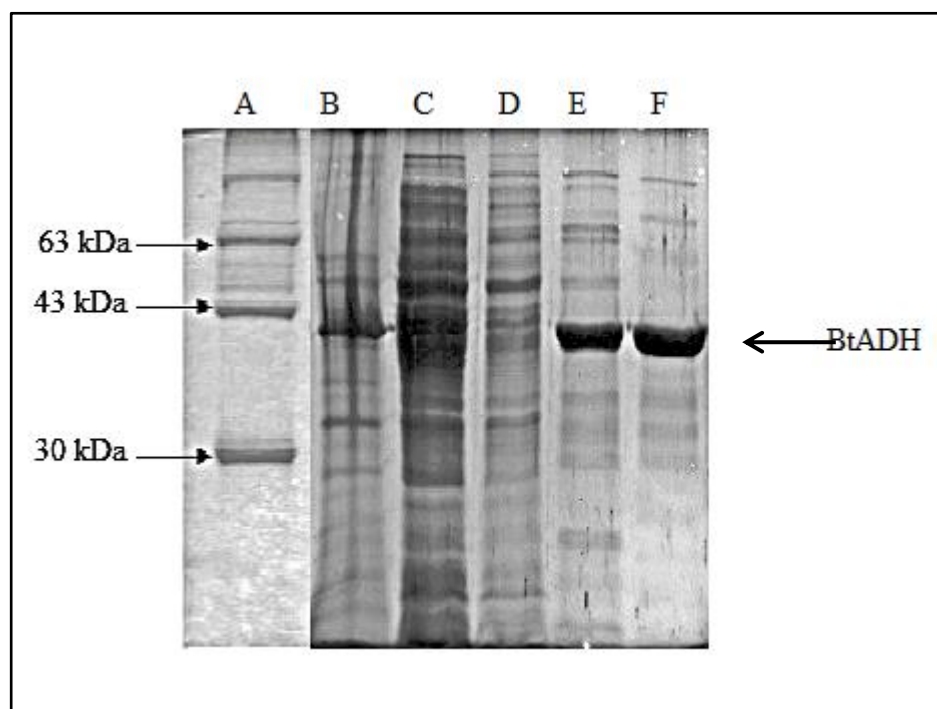


Figure 4.5: SDS-Page analysis of BtADH purification: (A) molecular mass standards; (B) insoluble fraction of *E. coli* BL21 (DE3), (C) cell free extract; (D) fraction eluted with 20 mM imidazole; lane (E) and (F) purified his-tagged BtADH eluted with 100 and 400 mM imidazole, respectively.

4.3. Immobilization of BtADH on Eupergit C 250 L

BtADH immobilization on Eupergit C 250 L was optimized, resulting in 100% immobilization and 85% activity yields. Immobilization yield is defined as the ratio of the amount of soluble enzyme immobilized to the amount of free enzyme used in immobilization process. Activity yield is defined as the ratio of the activity of immobilized enzyme to the activity of soluble enzyme immobilized.

4.4. Optimization of BtADH Immobilization on Eupergit C 250 L

Immobilization of enzymes on of Eupergit C 250 L is affected by various such parameters as amount of matrix, buffer concentration, pH, and duration of immobilization. Thus, we carried out a study for the optimization of the immobilization process. Eupergit C 250 L contains epoxy functional groups that react with different protein groups (amino, thiol and phenolic) that are abundant on the enzyme surface forming covalent multipoint attachment under mild experimental conditions [23]. First, the optimal amount of Eupergit C 250 L was determined for 1 mg of soluble enzyme. As can be seen in (Table 4.1), the highest activity yield (85.02%) was obtained for 100 mg Eupergit C 250 L. Lower activity yields were obtained using higher matrix amounts this could be due to multiple attachments and crosslinking reactions with groups (amino, thiol and phenolic) associated with enzyme active site residues.

Table 4.1: Effect of matrix amount on BtADH immobilization.

Matrix amount (mg)	Immobilization yield ^a (%)	Activity yield ^b (%)	Immobilized enzyme activity (IU)	Amount of protein in filtrate (mg)
50.00	89.17	27.5	2.66	0.11
100.00	100.00	85.02	8.81	0.00
200.00	100.00	64.00	6.18	0.00
300.00	100.00	53.72	5.10	0.00
500.00	100.00	42.10	4.10	0.00
800.00	100.00	32.82	3.20	0.00
1000.00	100.00	18.97	1.83	0.00

The tendency of enzyme residues to react with the matrix epoxy groups depends mainly on the pH of the immobilization medium. For example, sulfhydryl and carboxylic groups react with epoxy groups in acidic pH; however, lysine react with epoxy groups at neutral and alkaline conditions [36] . As shown in (Table 4.3) the highest activity yield was obtained at pH 6.5.

Table 4.2: Effect of pH on BtADH immobilization.

pH	Immobilization yield (%)	Activity yield (%)	Immobilized enzyme activity (IU)	Amount of protein in filtrate (mg)
6.00	100.00	55.84	5.39	0.00
6.50	100.00	85.02	8.81	0.00
7.00	100.00	29.34	2.83	0.00
7.50	100.00	25.39	2.45	0.00
8.00	100.00	20.55	1.98	0.00

The ionic strength of buffer solutions has a strong influence on immobilization of enzymes on hydrophobic materials [25]. Generally high buffer concentrations have been used in immobilization in order to decrease the solubility of enzymes. Decreasing the solubility of enzymes results in better precipitation on the matrix surface leading to higher activity yields. However, in our case the activity yield fell dramatically at high buffer concentration as shown in (Table 4.2). This is could be due to the interference of phosphate ions with the divalent catalytic metal ion (Zn^{+2}), reducing the activity of the enzyme. In addition, the high buffer concentrations may also complex with the substrate decreasing its recognition by the enzyme.

Table 4.3: Effect of buffer concentration on BtADH immobilization.

Buffer Concentration (mM)	Immobilization yield (%)	Activity yield (%)	Immobilized enzyme activity (IU)	Amount of protein in filtrate (mg)
25	94.17	70.24	6.78	0.0583
50	100	85.02	8.81	0.00
100	100	28.36	2.74	0.00
200	100	25.61	2.47	0.00
300	100	25.03	2.42	0.00
500	100	24.61	2.38	0.00
800	100	23.00	2.22	0.00

The duration of the immobilization has an important effect on both activity and immobilization yields. A short immobilization period may be not enough for binding all of enzyme to matrix. On the other hand, long immobilization period

could result in multiple crosslinking leading to lower activity yields. Thus, the immobilization period was optimized. Usually immobilization of enzymes onto Eupergit C 250 L takes long time that may last for days [24]. However in our case the highest activity yield (85.02%) was obtained in only three hours (Table 4.4). This is mainly due to the presence of large number of both lysine and glutamic acid residues (46 Lys and 44 Glu) that play vital role during the immobilization process. The activity yeild started to drop sharply as the immobilization duaration increased. This can be explained by the multiple crosslinking between the enzyme and the matrix.

Table 4.4: Effect of coupling time on BtADH immobilization.

Time(h)	Immobilization yield (%)	Activity yield (%)	Immobilized enzyme activity (IU)	Amount of protein in filtrate (mg)
1	87.36	47.67	4.60	0.13
2	95.46	57.90	5.59	0.05
3	100.00	85.02	8.81	0.00
4	100.00	55.22	4.58	0.00
9	100.00	34.22	3.30	0.00
12	100.00	18.60	1.80	0.00

4.5. Optimization of the Substrate Concentration

The most important parameter to evaluate the efficiency of immobilized enzymes is their reusability in multiple batch reactions with minimum activity loss. In the activity assay 500mM 2-propanol was used. The activity of immobilized BtADH fell sharply starting from the second batch reaction. Immobilized enzyme lost about 60% of its initial activity in 2 batch reactions. This activity loss increased to 80% of the initial activity in 6 batch reactions as shown in (Figure 4.2). This sharp loss in activity makes the whole process inefficient.

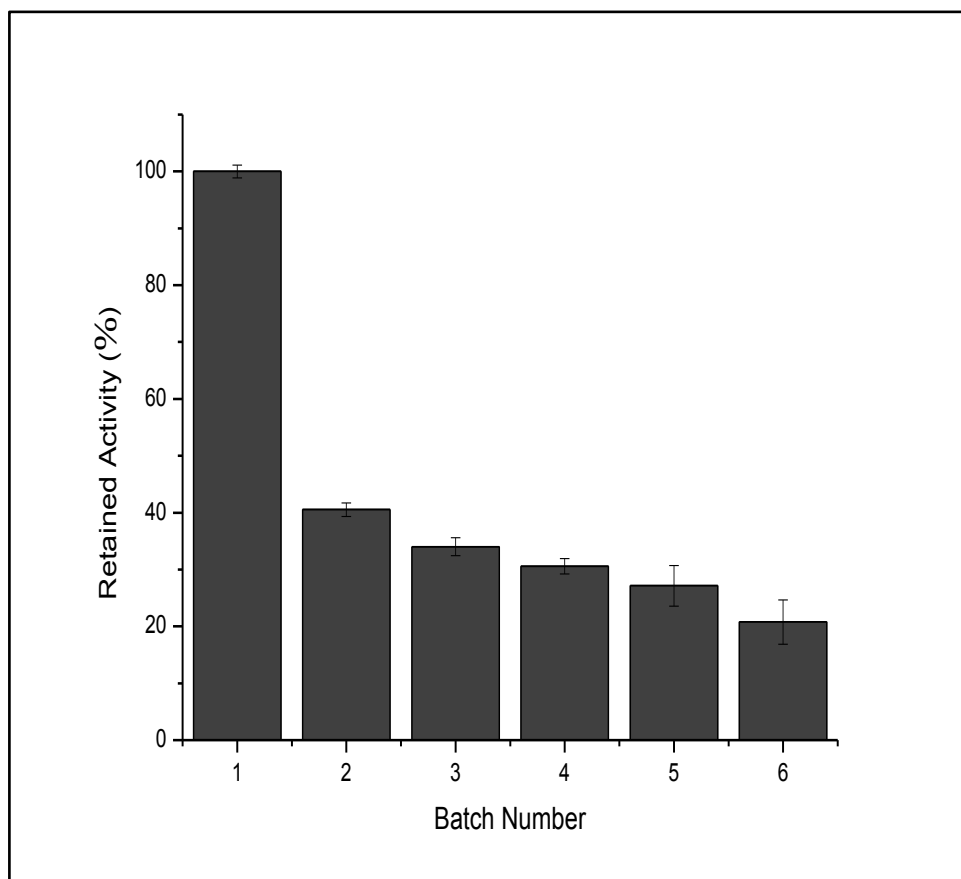


Figure 4.6: Effect of reusability on Immobilized BtADH activity using 500 mM 2-propanol.

Organic solvents in most cases have a negative effect on the activity of different enzymes [37],[38],[39]. For example, multiple exposures of alcohol dehydrogenases to acetone decrease their activities strongly [40]. This inactivation happens due to the binding of solvent to enzyme hydrophobic regions disturbing the enzyme 3-D structure [41]. Since substrates and products are organic solvents, we observed a sharp inactivation of the enzyme. Thus, a study was carried out to determine the extent of this inhibition by incubating the immobilized enzyme in 2-propanol and acetone for different periods of time. As shown in Figure 4.3, the product, acetone, (0.34 mM) has a fatal effect on the enzyme activity; immobilized enzyme lost 70% and 94% of its initial activity after the first and the fifth batch reactions, respectively. On the other hand, the substrate, 2-propanol (0.5 M) had less inhibition effect on the enzyme, resulting in 33% activity loss after 8 batch reactions.

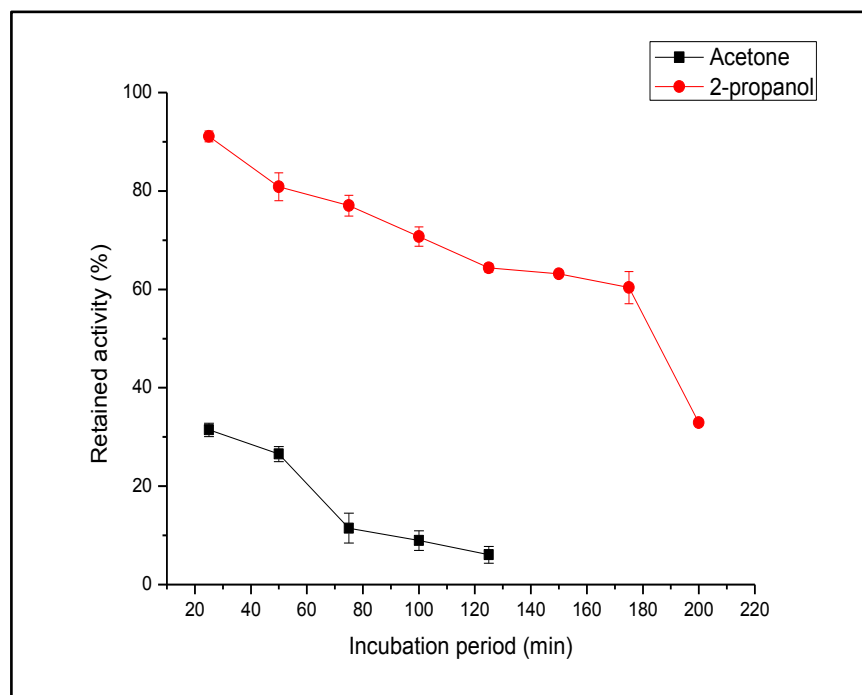


Figure 4.7: Effects of acetone and 2-propanol on the activity of immobilized BtADH.

The inactivation of enzyme occurs in a dose-dependent manner [inactivation]. Thus; a study was carried out to determine the substrate concentration that would achieve the highest activity yield and the minimum activity loss due to detrimental organic solvent effect. 6 consecutive reactions were carried out using different substrate concentrations (0.1, 0.4, 0.6, 0.8, 1.0, and 5.0 mM). Using 1 mM 2-propanol was not enough to avoid the inhibition effect. As shown in Figure 4.4, the immobilized enzyme retained 82.34% and 69.78% of its initial activity after first and sixth batch reactions, respectively. The immobilized enzyme retained 85.68% and 73.18% of its initial activity after first and sixth batch reactions, respectively in 0.8 mM 2-propanol (Figure 4.5).

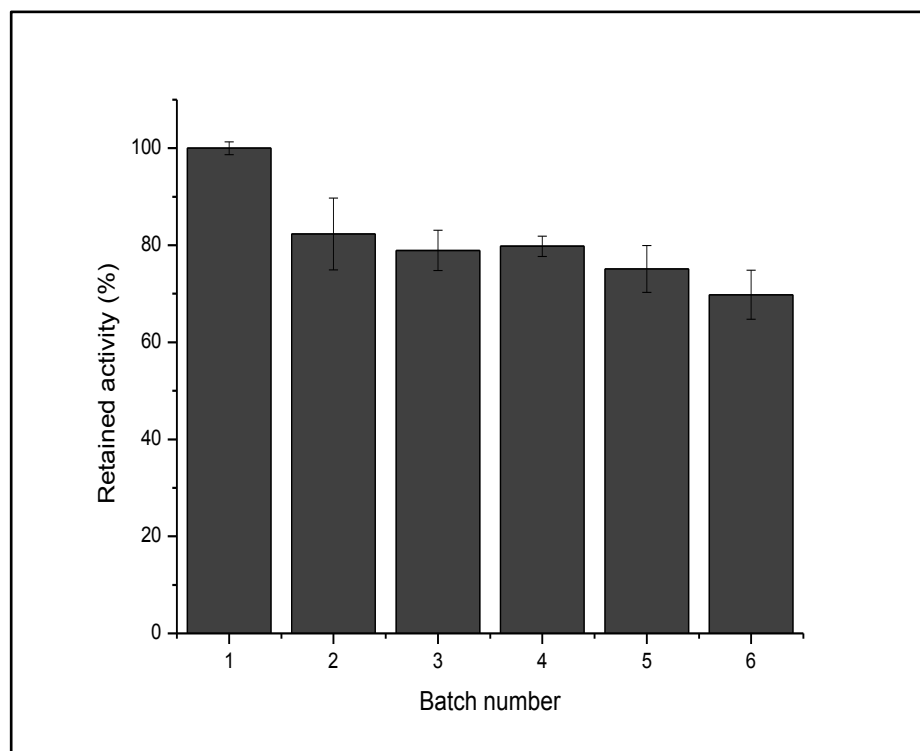


Figure 4.8: Effect of the reusability on Immobilized BtADH activity using 2-propanol (1mM).

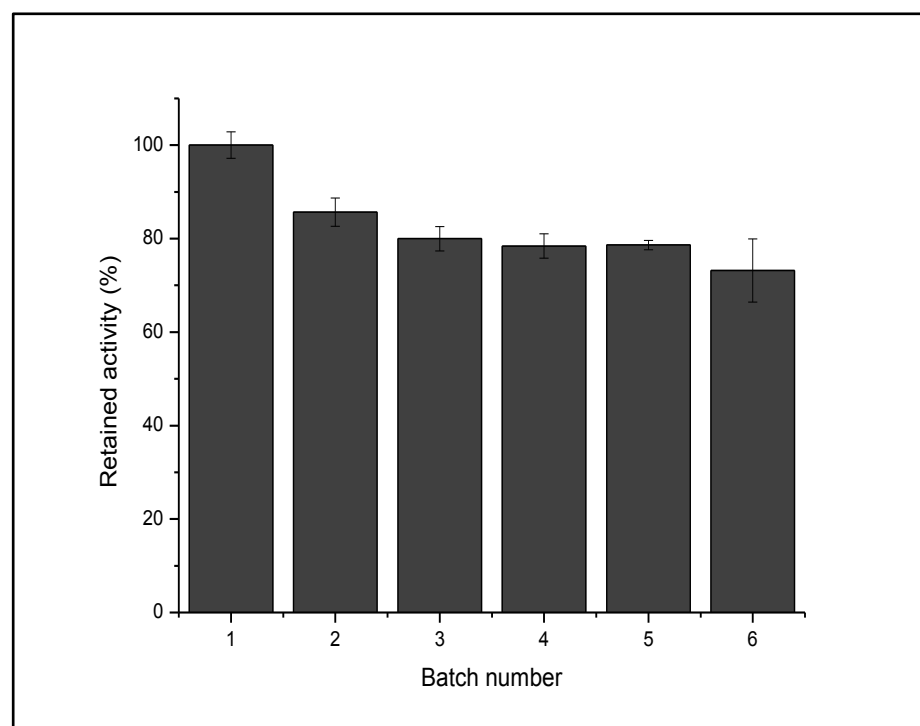


Figure 4.9: Effect of the reusability on Immobilized BtADH activity using 2-propanol (0.8mM).

As seen in (Figure 4.7), decreasing the substrate concentration to 0.6 mM improved the reusability of the immobilized BtADH. Thus, enzyme retained 89.56% and 83.73% after one and six batch reaction, respectively.

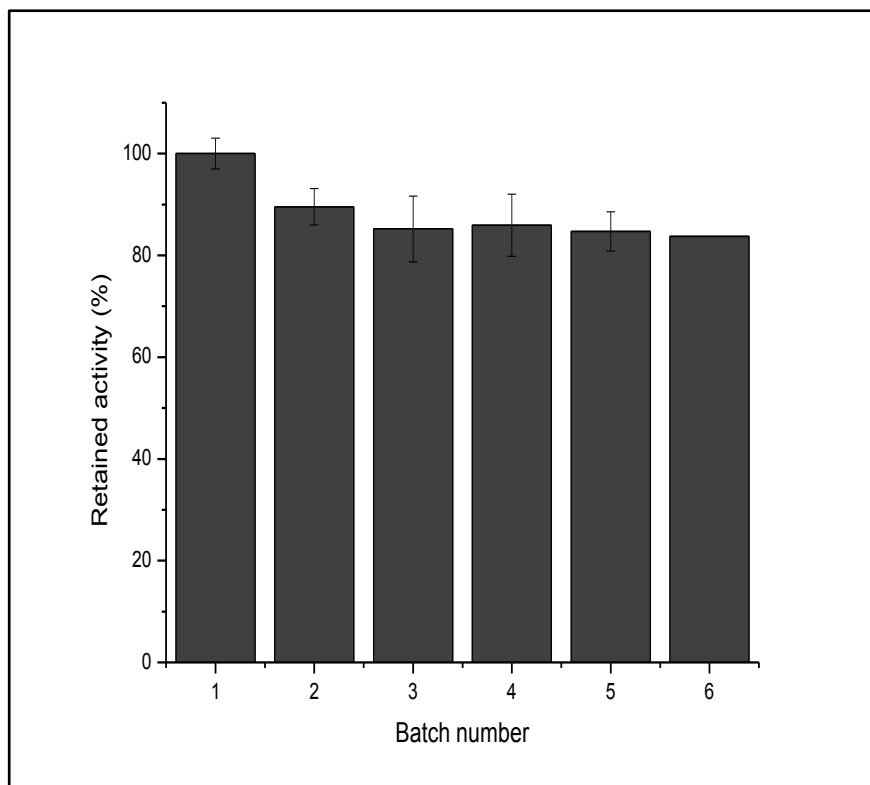


Figure 4.10: Effect of the reusability on Immobilized BtADH activity using 2-propanol (0.6mM).

As seen in Figure 4.8, 0.4 mM 2-propanol is the optimum concentration that has lower detrimental effect on the reusability and the highest activity yield. Immobilized BtADH retained 89.20% of its initial activity after six batch reactions. Aiming to reach 100% retained activity; we used 0.1 mM 2-propanol (Figure 4.9). It was found that low concentration, 0.1 mM 2-propanol had positive effect on the immobilized enzyme in consecutive batch reactions; however, it resulted in, lower activity yield.

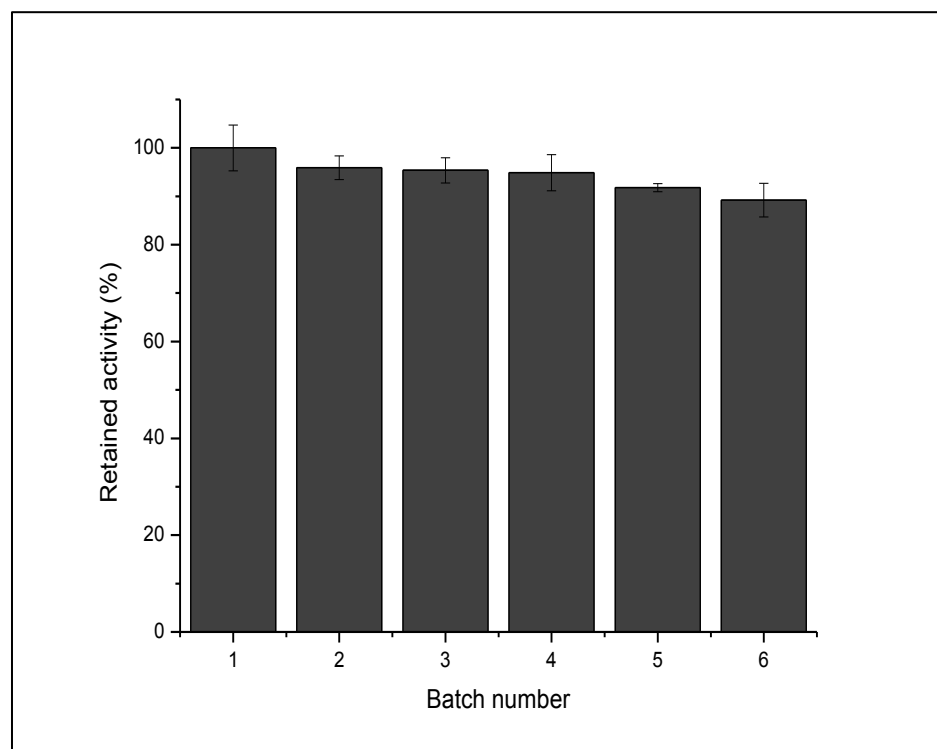


Figure 4.11: Effect of the reusability on Immobilized BtADH activity using 2-propanol (0.4mM).

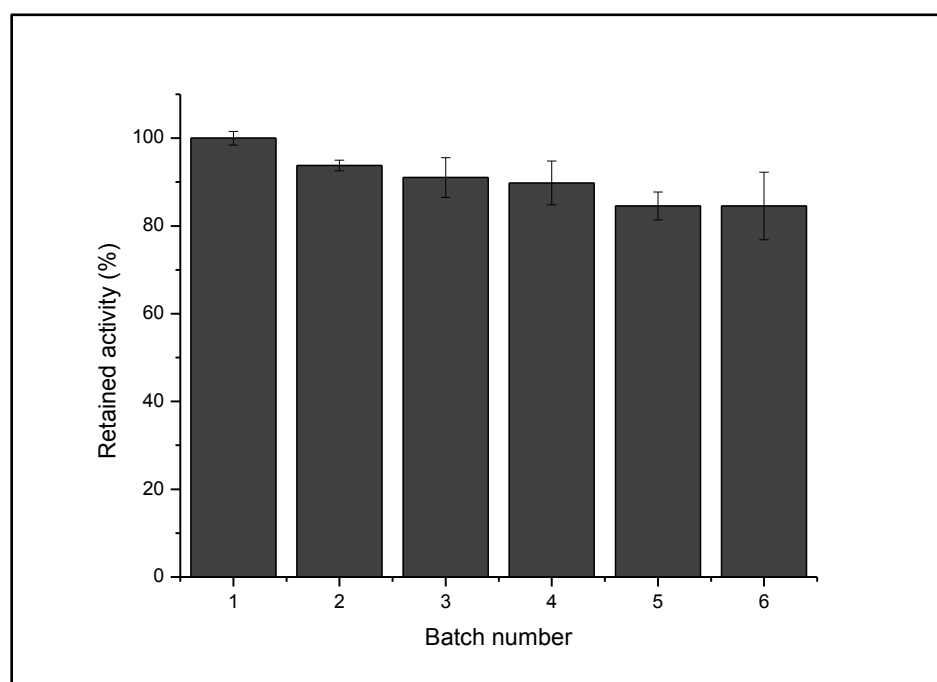


Figure 4.12: Effect of the reusability on Immobilized BtADH activity using 2-propanol (0.1mM).

4.6. Determination of the optimal pH and temperature

The optimal pH and temperature of both soluble and immobilized enzyme were determined by changing the activity assay conditions individually (pH from 6.00 to 12.00; temperature from 15 to 50 °C). As seen in figure 4.10, the optimal pHs were 8.00 and 8.50 for immobilized and soluble enzymes, respectively.

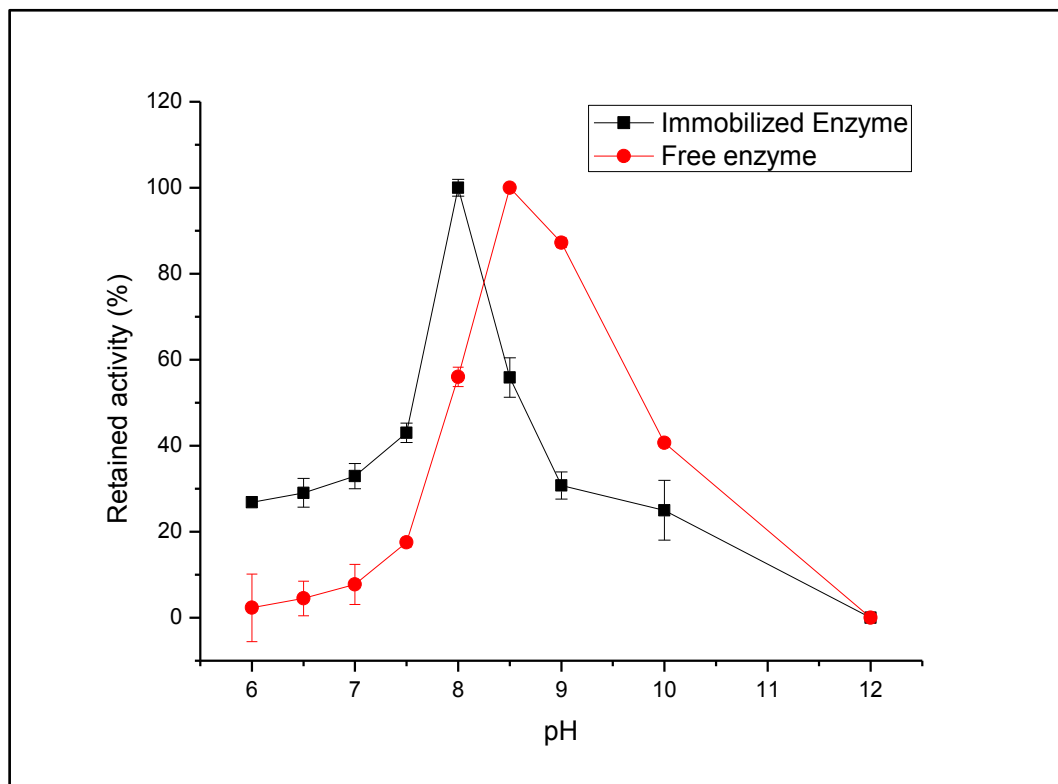


Figure 4.13: Effect of pH on the activities of soluble and immobilized enzymes.

The optimal temperatures were 35°C and 30°C for soluble and immobilized enzymes, respectively (Figure 4.11). Both soluble and immobilized enzyme showed the same activity-temperature behavior; increased temperature resulted in increase of the activity up to the optimum temperature. At higher temperatures, the denaturation of enzyme started resulting in a sharp decrease in activity.

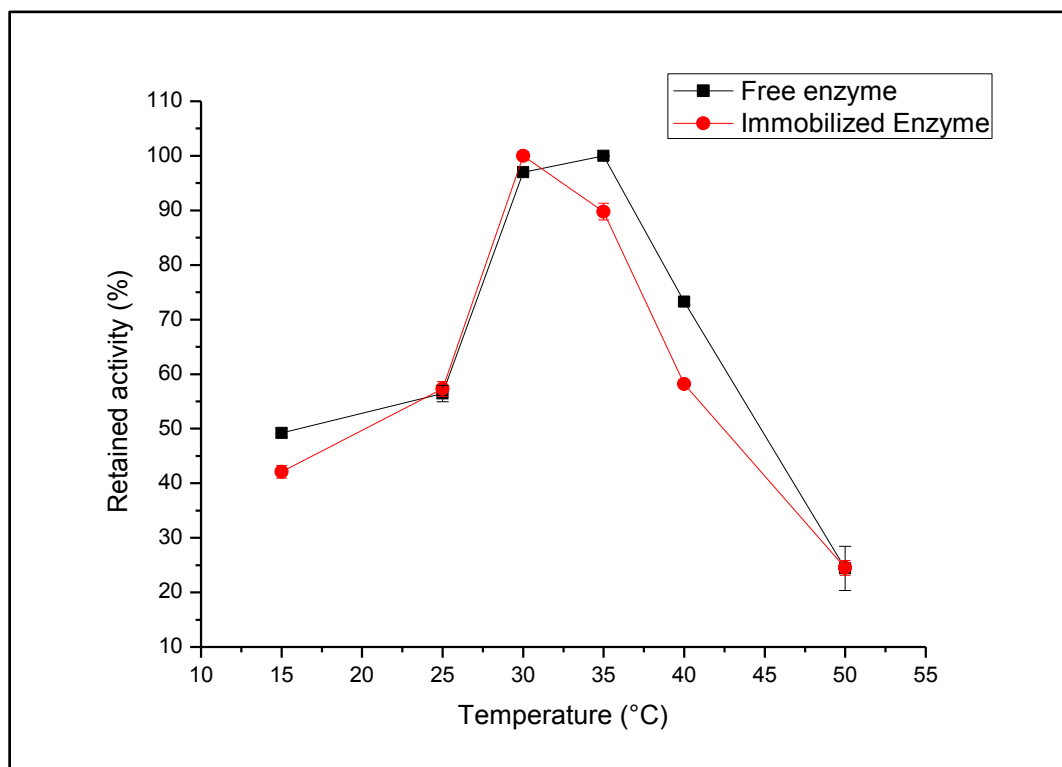


Figure 4.14: Effect of temperature on the activity of soluble and immobilized enzyme.

4.7. Reusability of the Immobilized Enzyme

Reusability is crucial for the economical usage of immobilized enzymes. Immobilized BtADH reusability was determined by carrying out 12 consecutive batch reactions using the standard activity assay. As shown in figure 4.11, the immobilized enzyme retained 88.77% of its initial activity in 12 subsequent uses. Some of this activity loss could be due to losing some of the immobilized enzyme during the filtration and washing processes.

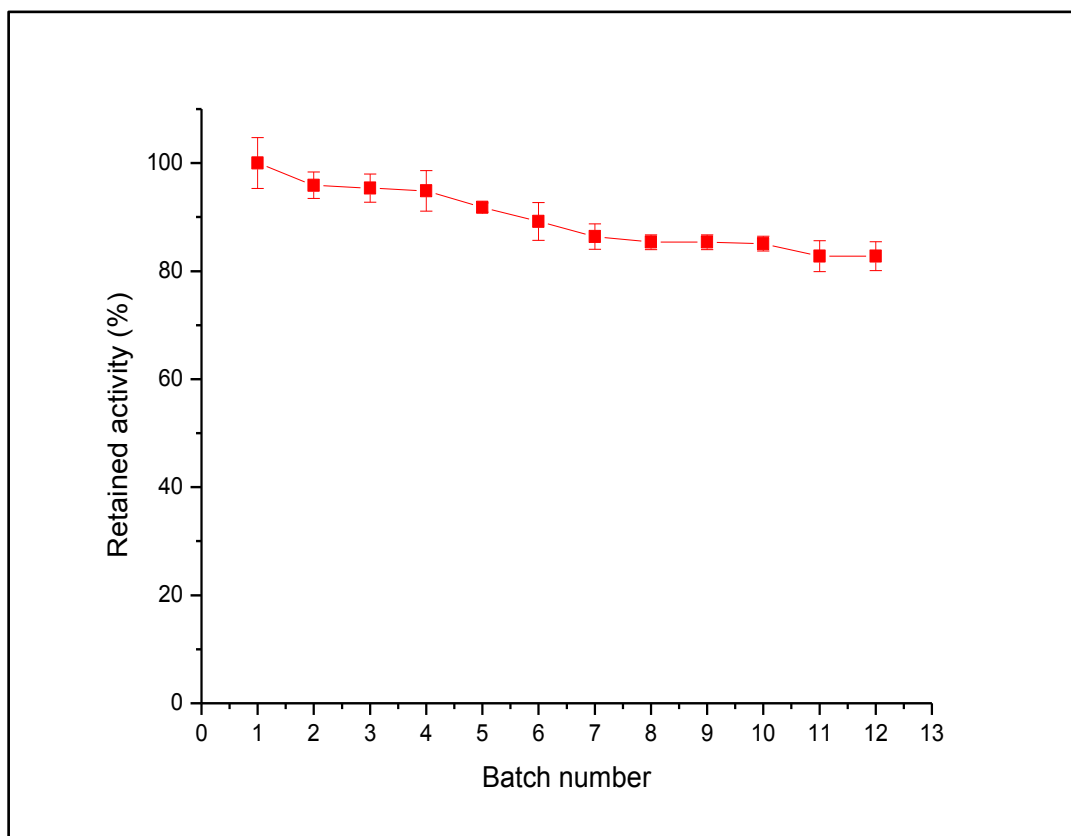


Figure 4.15: Effect of the reusability on the immobilized enzyme activity.

5. Future Work

The stability against different organic solvents is one of the desired properties of enzymes. During this thesis work, it was found that the immobilized enzyme is unstable against organic solvents such as; acetone and 2-propanol. To solve this problem, the following methods can be carried out in a future work

- **Multipoint covalent attachments immobilization:** Mateo et al. reported that favoring additional multipoint covalent attachments imparts higher conformational stability to the enzymes immobilized on epoxy activated supports [27]. This protocol is based on increasing the reactivity of the surface lysine residues through a three steps immobilization in which the enzyme is initially immobilized at mild conditions (pH 7.00) followed by incubation of the immobilized enzyme for long periods under more drastic conditions (pH 10.00) and blocking the extra epoxy groups to prevent any further interactions

which could lead to inactivation. This protocol could be applied to the enzyme used in this thesis work since it is rich in lysine residues on its surface.

- Site-directed mutagenesis: a study was carried out by Martinez et al. indicated that the substitution of charged residues on the enzyme surface provides stabilization of enzymes against organic solvents [42]. Briefly, a subtilisin E stability against polar organic solvent (dimethylformamide) was improved through substituting a charged amino acid residue, Asp 248, with three neutral amino acids (Asn, Ala, and Leu). The mutant was found three times more stable against high concentration of organic solvent (80% DMF) in comparison to the wild type. This kind of mutation can be applied to the enzyme used in this thesis work in order to improve its stability against different polar organic solvents including acetone and 2-propanol.
- Production using immobilized enzyme in a column reactor: in this protocol, the production is carried out in a column reactor containing the immobilized enzyme. This method can be a good solution of the substrate-product inhibition problems since it will minimize the exposure of the enzyme to both the substrate and the product.

REFERENCES

- [1] Straathof A. J., Panke S., Schmid A., (2002), "The production of fine chemicals by biotransformations", *Current Opinion in Biotechnology*, 13 (6), 548-556.
- [2] Ahuja S. K., Ferreira G. M., Moreira A. R., (2004), "Utilization of enzymes for environmental applications", *Critical Reviews in Biotechnology*, 24 (2-3), 125-154.
- [3] Singh R., Kumar M., Mittal A., Mehta P.K., (2016), "Microbial enzymes: industrial progress in 21st century", *3 Biotech*, 6 (2), 174.
- [4] Woodley J. M., (2008), "New opportunities for biocatalysis: making pharmaceutical processes greener", *Trends in Biotechnology*, 26 (6), 321-327.
- [5] Koeller K. M., Wong C. H., (2001), "Enzymes for chemical synthesis", *Nature*, 409 (6817), 232-240.
- [6] Tischer W., Kasche V., (1999), "Immobilized enzymes: crystals or carriers?", *Trends in Biotechnology*, 17 (8), 326-335.
- [7] Celik A., Aktas F., (2013), "A new NADH-dependent, zinc containing alcohol dehydrogenase from *Bacillus thuringiensis* serovar israelensis involved in oxidations of short to medium chain primary alcohols", *Journal of Molecular Catalysis B-Enzymatic*, 89, 114-121.
- [8] Kirk O., Borchert T. V., Fuglsang C. C., (2002), "Industrial enzyme applications", *Current Opinion Biotechnology*, 13 (4), 345-351.
- [9] Gong J. S., Shi J. S., Lu Z. M., Li H., Zhou Z. M., Xu Z. H., (2017), "Nitrile-converting enzymes as a tool to improve biocatalysis in organic synthesis: recent insights and promises", *Critical Reviews in Biotechnology*, 37 (1), 69-81.
- [10] Lutz S., (2010), "Biochemistry. Reengineering enzymes", *Science*, 329 (5989), 285-287.
- [11] Devaux-Basseguy R., Bergel A., Comtat M., (1997), "Potential applications of NAD(P)-dependent oxidoreductases in synthesis: A survey", *Enzyme and Microbial Technology*, 20 (4), 248-258.
- [12] Crabb D. W., Matsumoto M., Chang D., You M., (2007), "Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology", *Proceedings of the Nutrition Society*, 63 (1), 49-63.

- [13] Theorell H., Nygaard A., Bonnichsen R., (1955), "Studies on liver alcohol dehydrogenase. III. the influence of pH and some anions on the reaction velocity constants", *Acta Chemica Scandinavica*, 9 (7), 1148-1165.
- [14] Hayes J. E., Velick S. F., (1954), "Yeast alcohol dehydrogenase: molecular weight, coenzyme binding, and reaction equilibria", *Journal of Biological Chemistry*, 207 (1), 225-244.
- [15] Kavanagh K. L., Jörnvall H., Persson B., Oppermann U., (2008), "Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes", *Cell Mol Life Sci*, 65 (24), 3895-3906.
- [16] Theorell H., Mckinleymckee J. S., (1961), "Liver alcohol dehydrogenase I. kinetics and equilibria without inhibitors ", *Acta Chemica Scandinavica*, 15 (9), 1797-1810.
- [17] Jörnvall H., Jörnvall H., (1970), "Horse liver alcohol dehydrogenase on the primary structure of the ethanol-active isoenzyme", *European Journal of Biochemistry*, 13(3), 565-576.
- [18] Eklund H., Ramaswamy S., (2008), "Medium- and short-chain dehydrogenase/reductase gene and protein families : Three-dimensional structures of MDR alcohol dehydrogenases", *Cellular and Molecular Life Sciences*, 65 (24), 3907-3917.
- [19] Itoh N., Mizuguchi N., Mabuchi M., (1999), "Production of chiral alcohols by enantioselective reduction with NADH-dependent phenylacetaldehyde reductase from *Corynebacterium* strain, ST-10", *Journal of Molecular Catalysis B-Enzymatic*, 6 (1-2), 41-50.
- [20] Itoh N., Matsuda M., Mabuchi M., Dairi T., Wang J., (2002), "Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled within situregeneration of NADH", *European Journal of Biochemistry*, 269 (9), 2394-2402.
- [21] Sheldon R.A., (2007), "Enzyme immobilization: The quest for optimum performance", *Advanced Synthesis & Catalysis*, 349 (8-9), 1289-1307.
- [22] Ulker C., Gokalp N., Guvenilir Y., (2016), "Immobilization of *Candida antarctica* lipase B (CALB) on surface-modified rice husk ashes (RHA) via physical adsorption and cross-linking methods", *Biocatalysis and Biotransformation*, 34 (4), 172-180.
- [23] Sheldon R.A., van Pelt S., (2013), "Enzyme immobilisation in biocatalysis: why, what and how", *Chemical Society Reviews*, 42 (15), 6223-6235.

- [24] Gomez de Segura A., Alcalde M., Yates M., Rojas-Cervantes M.L., Lopez-Cortes N., Ballesteros A., Plou F.J., (2004), "Immobilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F on Eupergit C supports", *Biotechnology Progress*, 20 (5), 1414-1420.
- [25] Boller T., Meier C., Menzler S., (2002), "Eupergit oxirane acrylic beads: How to make enzymes fit for biocatalysis", *Organic Process Research & Development*, 6 (4), 509-519.
- [26] Mateo C., Grazu V., Palomo J.M., Lopez-Gallego F., Fernandez-Lafuente R., Guisan J.M., (2007), "Immobilization of enzymes on heterofunctional epoxy supports", *Nature Protocols*, 2 (5), 1022-1033.
- [27] Mateo C., Abian O., Fernandez-Lafuente R., Guisan J.M., (2000), "Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment", *Enzyme and Microbial Technology*, 26 (7), 509-515.
- [28] Keinan E., Hafeli E.K., Seth K.K., Lamed R., (1986), "Thermostable enzymes in organic-synthesis. 2. asymmetric reduction of ketones with alcohol-dehydrogenase from *Thermoanaerobium-Brockii*", *Journal of the American Chemical Society*, 108 (1), 162-169.
- [29] Liao M.H., Chen D.H., (2001), "Immobilization of yeast alcohol dehydrogenase on magnetic nanoparticles for improving its stability", *Biotechnology Letters*, 23 (20), 1723-1727.
- [30] Alsafadi D., Paradisi F., (2014), "Covalent immobilization of alcohol dehydrogenase (ADH2) from *Haloferax volcanii*: how to maximize activity and optimize performance of halophilic enzymes", *Molecular Biotechnology*, 56 (3), 240-247.
- [31] Bolivar J.M., Wilson L., Ferrarotti S.A., Guisan J.M., Fernandez-Lafuente R., Mateo C., (2006), "Improvement of the stability of alcohol dehydrogenase by covalent immobilization on glyoxyl-agarose", *Journal of Biotechnology*, 125 (1), 85-94.
- [32] Soni S., Desai J.D., Devi S., (2001), "Immobilization of yeast alcohol dehydrogenase by entrapment and covalent binding to polymeric supports", *Journal of Applied Polymer Science*, 82 (5), 1299-1305.
- [33] Sambrook J., Green R., (2001), "Molecular Cloning: a Laboratory Manual", 4th Edition, Cold Spring Harbor Laboratory Press.
- [34] Studier F. W., (2005), "Protein production by auto-induction in high density shaking cultures", *Protein Expression and Purification*, 41 (1), 207-234.

- [35] Bradford M., (1976), "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry*, 72, 248-254.
- [36] Barredo J. L., (2005), "Microbial enzymes and biotransformations", 1st edition, Humana Press.
- [37] Grazinoli-Garrido R., Sola-Penna M., (2004), "Inactivation of yeast inorganic pyrophosphatase by organic solvents", *Anais Da Academia Brasileira De Ciencias*, 76 (4), 699-705.
- [38] McKarns S. C., Hansch C., Caldwell W.S., Morgan W. T., Moore S. K., Doolittle D. J., (1997), "Correlation between hydrophobicity of short-chain aliphatic alcohols and their ability to alter plasma membrane integrity", *Fundamental and Applied Toxicology*, 36 (1), 62-70.
- [39] Stamatis H., Christakopoulos P., Kekos D., Macris B. J., Kolisis F. N. (1998), "Studies of the effect of organic solvents on the stability of -glucosidase from *Fusarium oxysporum*", *Journal of Molecular Catalysis B: Enzymatic*, 4 (4), 229-236.
- [40] Cosmidis N., Loukas M., Peppas V., Goulielmos G., Zouros E., (2002), "Effect of acetone feeding on alcohol dehydrogenase activity in the olive fruit fly, *Bactrocera oleae*", *Heredity*, 89 (6), 453-459.
- [41] Lopes D.H., Sola-Penna M., (2001), "Urea increases tolerance of yeast inorganic pyrophosphatase activity to ethanol: the other side of urea interaction with proteins", *Archives of Biochemistry and Biophysics*, 394 (1), 61-66.
- [42] Martinez P., Van Dam M.E., Robinson A.C., Chein K., Arnold F.H., (1991), "Stabilization of subtilisin E in organic solvents by site-directed mutagenesis", *Biotechnology and Bioengineering*, 39 (2), 141-147.

BIOGRAPHY

Mohamed Shehata was born in 28th April 1992 in Qalyubia governorate north Cairo, Egypt. He began his undergraduate studies in Chemistry in 2009 at Benha University, Egypt. After graduation in 2013, he moved to Turkey for Masters Studies through a Turkish Government scholarship. In 2014 he obtained a diploma in Turkish language from Kocaeli University, Turkey. In autumn 2014 he started his M. Sc. in biochemistry at the Graduate School of Natural and Applied Science in Gebze Technical University (formerly Gebze Institute of Technology, Gebze/Kocaeli).

APPENDICES

APPENDIX A: Protein Expression Media

LB (Luria-Bertani): peptone (10g), yeast extract (5g), and NaCl (5g) were dissolved in 950 ml H₂O and the pH was adjusted to 7.00 with NaOH. The medium volume was then adjusted to one litre and was autoclaved at 121°C for 15 minutes.

- LB Broth with agar: peptone (10g), yeast extract (5g), and NaCl (5g) were dissolved in 900 ml H₂O and the pH was adjusted to 7.00 with NaOH. 15 g agar was added and the final volume adjusted to one litre and was autoclaved at 121°C for 15 minutes.
- SOC medium: 20g peptone, 5g yeast extract, 2.5ml of 1M KCl, 2ml of 5M NaCl, and 10ml of 1M MgSO₄ were dissolved in water and autoclaved at 121°C for 15 minutes. Then, 10ml of 1M MgCl₂, and 20ml of 1M glucose were added and the final volume was adjusted to one litre. Stocks were stored at -20°C until later use.
- Studier medium: peptone (20g), yeast extract (5g), glucose (0.5g), glycerol (6g) and NaCl (5g), and lactose (2g) were dissolved in 950 H₂O and the pH of the medium was adjusted to 7.0 using NaOH. The final volume adjusted to one litre and was autoclaved at 121°C for 15 minutes.

APPENDIX C: Buffers and solutions

- TSS buffer for competent cell preparation
 - 10% poly ethylene glycol
 - 5% Dimethyl Sulfoxide
 - 20 mM MgCl_2
 - Solution was autoclaved at 121°C for 15 minutes.
- 100 mM PMSF (Phenylmethanesulfonyl fluoride)
 - 17.4 mg PMSF
 - 10ml isopropanol
 - Solution was stored at -20 °C
- 10% APS
 - 1 g ammonium persulfate
 - 10 ml H_2O
 - Solution was stored at 4°C
- SDS-PAGE stacking gel (5 ml)
 - H_2O (2.975 ml)
 - 0.5 M Tris-HCl, pH 6.8 (1,25 ml)
 - 10% (w/v) SDS (0.05 ml)
 - (30%/0.8% w/v) Acrylamide/Bis-acrylamide (0.67 ml)
 - 10% (w/v) ammonium persulfate (0.05 ml)
 - TEMED (0.005 ml)
- SDS-PAGE 10 % separating gel (10ml)
 - H_2O (3.8 ml)
 - 1.5 M Tris-HCl, pH 8.8 (2.6 ml)
 - 10% (w/v) SDS (0.1 ml)
 - (30%/0.8% w/v) Acrylamide/Bis-acrylamide (3.4 ml)
 - 10% (w/v) ammonium persulfate (100µl)
 - TEMED (10µl)
- Coomassie Blue staining solution (1 L)
 - 1 g Coomassie Blue R-250
 - 450 ml Methanol
 - 100 ml Glacial acetic acid

– 450 ml dH₂O

APPENDIX C: *Bacillus thuringiensis* serovar *israelensis* Alcohol dehydrogenase (BtADH) sequence

- Fasta format

```
MKAVVVNKNKSKANIEVVEKELRPLRSGEALVDVEYCGVCHTDLHVANH
DFGNTDGRILGHEGVGIVTKIADDDVNSLKIGDRVSIWWMFQSCGRCEYCV
TGRETFCREVKNAGYSVDGGMAEQCIVTADYAVKVPEGLDPAQASSITC
AGVTTYKAIKVSDIKPGQPIVIYGCCGLGNLAIQYAKNVFGAKVIAVDIND
DKLALAKEVGADMTINPISQGPADKIVQEELGGAYAAVVTAVSKVAFNS
AVDAVRACGKVVAVGLPVETMDLNIPRLVLDGIEVVGSLVGTRKDLAEA
FMFGAEGKVVPVQTCSLDKVQNVFEEMEQGRIQGRMVIDFKKHNCDC
K
```

- BtADH gene sequence

```
ATGAAAGCAGTAGTGGTTAATAAAAACAGCAAAGCAAACATC
GAAGTGGTTGAAAAAGAATTACGTCCGTTACGCTCAGGTGAA
GCGTTAGTAGATGTAGAGTATTGTGGAGTTTGCCACACTGATT
TACACGTTGCGAATCATGATTTTGGTAACACTGATGGCCGTAT
TCTTGGTCATGAGGGTGTAGGTATTGTTACGAAAATAGCTGAT
GATGTTAATTCGCTAAAGATAGGTGACCGTGTAAGTATTGCAT
GGATGTTCCAATCTTGTGGACGTTGTGAATATTGCGTAACTGG
TAGAGAAACATTTTGCCGTGAAGTTAAAAACGCAGGTTATTCA
GTAGATGGTGGTATGGCTGAACAATGTATCGTTACAGCTGATT
ATGCGGTAAAAGTACCAGAAGGATTAGATCCTGCTCAAGCAT
CATCAATTACATGTGCTGGTGTAACCTACATATAAAGCTATTAA
AGTATCAGATATTAAACCTGGTCAACCTATTGTAATCTATGGC
TGCGGTGGATTAGGTAACCTTAGCTATCCAATATGCTAAAAATG
TATTTGGTGCAAAGGTAATCGCAGTAGACATTAATGACGACA
AATTAGCCTTAGCGAAAGAAGTTGGTGCTGATATGACTATCAA
TCCAATTTCTCAAGGTCCTGCTGATAAAATTGTTCAAGAGGAG
CTTGGTGGCGCTTATGCTGCGGTAGTAACAGCAGTCTCTAAAG
TAGCCTTTAACTCAGCAGTTGATGCAGTACGTGCTTGCGGTAA
AGTAGTTGCAGTAGGGCTACCAGTAGAAACAATGGACTTAAA
CATCCCGCGACTTGTACTGGATGGAATTGAAGTAGTTGGTTCT
CTAGTTGGTACTCGTAAAGATTTGGAAGAGGCGTTTATGTTTG
GGGCAGAAGGAAAAGTAGTGCCGGTTGTTCAAACCTTGTTTCGC
TAGATAAAGTACAAAATGTATTCGAAGAAATGGAACAAGGTA
GAATTCAAGGGCGTATGGTAATTGATTTTAAAAAACATAATTG
TGATTGCAAATAA
```

APPENDIX C: Papers and Posters published during the thesis work

Shehata M., Aktas F., Celik A., (2015) “A Biocatalytic oxidation of cyclic alcohols by an alcohol dehydrogenase from bacillus thuringiensis serovar israelensis”, 27th National Chemistry Congress, 59, Çanakkale, Turkey, 23-28 August.

Shehata M., Aktas F., Celik A., Tanriseven A., (2016) “Immobilization of alcohol dehydrogenase from bacillus thuringiensis serovar israelensis on Eupergit C 250 L for production of alcohols and ketones”, 28th National Chemistry Congress, 92, Mersin, Turkey, 15-21 August.