การตรึงฮอร์สเรดิชเปอร์ออกซิเดสบนบีดไคโตซานเพื่อการขจัดฟีนอล

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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IMMOBILIZATION OF HORSERADISH PEROXIDASE ON CHITOSAN BEADS FOR PHENOL REMOVAL

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ธนินท์ธร เนติธีระศักดิ์: การตรึงฮอร์สเรดิชเปอร์ออกซิเดสบนบีดไคโตซานเพื่อการขจัดฟีนอล (IMMOBILIZATION OF HORSERADISH PEROXIDASE ON CHITOSAN BEADS FOR PHENOL REMOVAL) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.มัญชุมาส เพราะสุนทร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.กฤษณา ศิรเลิศมุกุล, 88 หน้า

งานวิจัยนี้มีวัตถุประสงค์เพื่อหาตัวค้ำไคโตซานที่เหมาะสมเพื่อใช้ตรึงฮอร์สเรดิชเปอร์ออกซิเดส (HRP) เพื่อใช้ขจัดฟีนอล ตัวค้ำที่ถูกนำมาใช้คือ เมมเบรนไคโตซาน สเปรย์ดรายด์ไคโตซาน บีดไคโตซานชนิดแห้ง ที่มีความเข้มข้น 2%, 3%, 5% (w/v) และ บีดไคโตซานชนิดเปียก ที่ความเข้มข้น 3% (w/v) เมื่อนำไปศึกษาด้วย Scanning electron microscopy (SEM) พบว่าบีดไคโตซานเข้มข้น 3% (w/v) ทั้งสองชนิดมีความเหมาะสมที่จะนำไปศึกษาการตรึงเอนไซม์โดยใช้ กลูตารัลดีไฮด์เป็นตัวเชื่อม ทำการหาภาวะที่เหมาะสมของตัวแปรต่างๆที่มีผลต่อการตรึงเอนไซม์ เช่น ความเข้มข้นของตัวกระตุ้นและเวลาในการบ่ม พบว่าต้องกระตุ้นบีดไคโตซานชนิดแห้งด้วยสารละลายกลูตารัลดีไฮด์เข้มข้น 1.5% (v/v) ที่อุณหภูมิห้องเป็นเวลา 8 ชั่วโมง ในขณะที่บีดไคโตซานชนิดเปียกจะถูกกระตุ้นด้วยกลูตารัลดีไฮด์เข้มข้น 1.0% (v/v) ที่อุณหภูมิห้องเป็นเวลา 10 ชั่วโมง ตัวค้ำทั้งสองชนิดใช้เอนไซม์ในการตรึง 2.5 ยูนิต และใช้เวลาในการบ่มกับเอนไซม์ 2 ชั่วโมง หลังจากทำการตรึงแล้วพบว่าเอนไซม์ตรึงมีความสามารถในการทำปฏิกิริยาดีที่สุดที่ pH 5.5 และที่อุณหภูมิ 25ºซ ซึ่งเหมือนกับเอนไซม์อิสระ เอนไซม์อิสระและเอนไซม์ตรึงมีความเสถียรที่ pH 5.5 ทำการเปรียบเทียบประสิทธิภาพของเอนไซม์ตรึงบนตัวค้ำทั้งสองในชุดปฏิกิริยาแรกจะบ่มกลูตารัลดีไฮด์ (1.5%, v/v) กับตัวค้ำ 8 ชั่วโมง จากนั้นจึงบ่มกับสารละลาย HRP (10 ไมโครกรัม) ในสภาวะกวนและไม่กวน ส่วนในอีกชุดของการทดลองจะทำการบ่มกลูตารัลดีไฮด์กับสารละลาย HRP ก่อนเติมลงไปบนตัวค้ำทั้งสอง พบว่าการบ่มกลูตารัลดีไฮด์ (1.5%, v/v) กับตัวค้ำ 8 ชั่วโมง ก่อนบ่มเอนไซม์กับตัวค้ำนาน 2 ชั่วโมง มีประสิทธิภาพการตรึงดีที่สุด จากนั้นนำการตรึงเอนไซม์รูปแบบนี้มาใช้ในการขจัดฟีนอล พบว่าเอนไซม์ตรึงสามารถขจัดฟีนอลสูงสุดที่อุณหภูมิ 25°ซ, pH 10 และเมื่อนำเอนไซม์มาใช้ซ้ำพบว่าเอนไซม์ตรึงสามารถขจัดฟีนอลได้ถึง 36% หลังการใช้งาน 5 ครั้ง เมื่อศึกษาความเสถียรของการเก็บรักษาของเอนไซม์ตรึงและเอนไซม์อิสระนาน 1 เดือน พบว่าเอนไซม์ตรึงบนบีดชนิดเปียกมีความเสถียรต่อการเก็บรักษาทั้งที่อุณหภูมิ 25 และ 4°ซ สูงกว่าเอนไซม์อิสระ

สาขาวิชา…เทคโนโลยีชีวภาพ…….....ลายมือชื่อนิสิต……

ปีการศึกษา……2556.....……...ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก……..……

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: CHITOSAN / GLUTARALDEHYDE / HORSERADISH PEROXIDASE / PHENOL

THANINTHORN NETHITHEERASUK: IMMOBILIZATION OF HORSERADISH PEROXIDASE ON CHITOSAN BEADS FOR PHENOL REMOVAL. ADVISOR: ASST. PROF. MANCHUMAS PROUSOONTORN, Ph.D., CO-ADVISOR: KRISANA SIRALERTMUKUL, Ph.D., 88 pp.

This research aims to search for the appropriate chitosan for horseradish peroxidase (HRP) immobilization for phenol removal. The supports used were chitosan membrane, spray dried chitosan, 2%, 3%, 5% (w/v) of dry chitosan beads and 3% (w/v) of wet chitosan beads. Scanning electron microscopy (SEM) showed that 3% (w/v) of both chitosan beads were appropriate to be used for the immobilization of enzymes by covalent linkage using glutaraldehyde. The immobilization parameters such as concentration of coupling agent and coupling time were optimized. It was determined that the optimum conditions for enzyme immobilization were to activate dry support with 1.5% (v/v) of glutaraldehyde for 8 hours and 1.0% (v/v) of glutaraldehyde for 10 hours for wet support. The activated support was then incubated with the enzyme solution for 2 hours at room temperature, using 2.5 units for both supports. After immobilization, the optimum pH and temperature of immobilized HRP was found to be the same as that of the free enzyme (5.5 and at 25^{o}C). The pH stability of free and enzyme immobilized on both dry and wet chitosan beads was 5.5, whereas the thermal stability of immobilized HRP on dry beads was slightly higher than that of the immobilized HRP on wet beads. The performance of enzymes immobilized on both supports was compared. In the first set of the experiment, glutaraldehyde (1.5%, v/v, 8 hours) and HRP solution (10 µg) were allowed to react with the beads sequentially with and without agitation. In another set of experiments, glutaraldehyde and HRP solution were mixed prior to the addition to the supports with and without agitation. It was found that wet chitosan beads incubated with 1.5% (v/v) glutaraldehyde and HRP consecutively gave best immobilized activity at 2 hours incubation. Phenol removal by HRP immobilized on wet chitosan beads was highest at room temperature and at pH 10.0 Reusability of the immobilized enzyme for the removal of phenol after 5 repeated use still retained the ability to remove phenol up to 36%. The HRP immobilized on wet beads showed higher stability than the native enzyme when stored at both 25 and 4°C for 1 month.

Field of Study:……Biotechnology……..Student's Signature ……..…….

Academic Year:…….2013..……Advisor's Signature…….……..…….

Co-advisor's Signature..……...……...……

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Last but not least, this thesis will not be occurred if I didn't mention to the person who gave me birth; my parents, nothing in this world could express my great gratitude and infinite appreciation to them. They are the persons who are always beside me when I have a problem or when I need a shoulder. They are the persons who take care of me no matter how hard it is. But for my family, they are the ones who always support and understand me. There are no words that I could imagine to describe my parents' unlimited love throughout my life.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
4-AAP	4-aminoantipyrine
DMSO	Dimethyl sulfoxide
GA	glutaraldehyde
HCl	hydrochloric acid
HRP	horseradish peroxidase
kg	kilogram
l	liter
M	mole per liter (molar)
mg	milligram
ml	milliliter
mM	millimolar
MW	molecular weight
NaOH	sodium hydroxide
nm	nanometer
^{o}C	degree Celsius
OD	optical density
SEM	scanning electron microscopy
TMB	3, 3', 5, 5' tetramethylbenzidine
U	unit

CHAPTER I

INTRODUCTION

1.1 Phenol

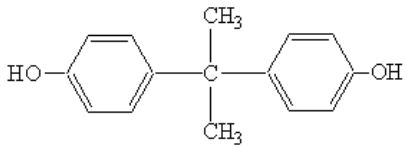
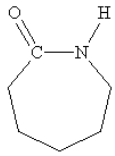
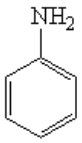
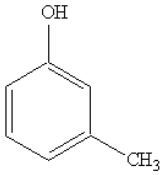
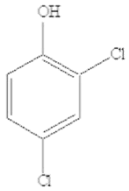
1.1.1 Phenolic

Phenol or phenolic compounds (C_6H_6O) are chemical compounds consisted of aromatic hydrocarbon group bonded directly to hydroxyl group (-OH). The physical properties of phenol are listed in Table 1. It has been produced since 1860's for the use as an antiseptic until the end of 19th century, phenol had widespread use in industries. Scientists used phenol as a component in the synthesis of aspirin and dye which was one of the first high explosives, picric acid (Mishra K., 2013). Today, phenols have been commonly used in chemical industries and consumer products (Table 2), for example antioxidants, plastics, pesticides, dyestuffs, explosives, drugs, stabilizers and detergents (Rice-Evan *et al.,* 1998). When the population increases the demand for consumer goods industries have increased. As a result, the production and release of waste associated with phenolic compounds has also been increased. Many of these compounds pass through conventional biological wastewater treatment systems untreated and have been found in environment animal and human (Jensen *et al.,* 1996).

Table 1. Physical properties of phenol (Kütt *et al.*, 2008)

Properties	**Characteristics**
Molecular formula	C_6H_6O
Molar mass	94.11 g mol^{-1}
Appearance	Transparent crystalline solid
Odor	Sweet and tarry
Density	1.07 g/cm^3
Melting point	40.5 °C, 314 K, 105 °F
Boiling point	181.7 °C, 455 K, 359 °F
Solubility in water	8.3 g/100 mL (20 °C)
Acidity (pKa)	9.95 (in water),
	29.1 (in acetonitrile)
λmax	270.75 nm
Dipole moment	1.7 D

Table 2. The intermediates and products of phenol

Intermediate	Products
Bisphenol A 	Used to produce epoxy resins for paints coatings and polycarbonate plastics, familiar in CDs and domestic electrical appliances.
Caprolactam 	Used in the manufacture of nylon and polyamide plastics, including carpets, clothing and fishing nets.
Phenylamine (Aniline) 	Used as an antioxidant in rubber manufacture and as an intermediate in the production of herbicides, dyes and pigments.
Alkylphenols 	Used in the manufacture of surfactants, detergents and emulsifiers, and also in insecticide and plastics production.
Chloro-phenols 	Used in medical antiseptics and bactericides such as TCPs (2,4,5-trichlorophenol and 2,4,6-trichlorophenol) and Dettol (Olaniran and Igbinosa., 2011).

1.1.2 Toxicity of phenol

Phenol is a chemical compound that can be accumulated in soil, air and water. This chemical can absorb through skin easily with high toxicity and cause harm to tissues, gastrointestinal, respiratory and nervous system. The disease that can be caused by phenol are mutagenicity, carcinogenicity and dysgenesis (Kar *et al.,* 2010). Therefore, these compounds need to be degraded or removed to an acceptable level before being released to the environment. The standard level of phenol contaminated in wastewater that has been approved is in the range of 0.1-0.5 mg/l.

1.2 Horseradish peroxidase

Horseradish peroxidase (HRP), found in horseradish, is used extensively in biochemistry applications primarily for its ability to amplify a weak signal and increase detectability of a target molecule. It occurs as a large family of isoenzymes. Isoenzymes (or isozymes) are different molecular forms of the same enzyme, which catalyze the same biochemical reaction but have distinct, physical, chemical and kinetic properties arising from small differences in their amino acid sequence.

In 1958, Paul isolated five different forms of peroxidase from horseradish roots using ion-exchange chromatography on carboxymethyl cellulose (CMC). The labeling of enzymatic forms were used the capital letter A, B, C, D and E. The research group of Morita (Aibara *et al.,* 1982) isolated and characterized five neutral (B1, B2, B3, C1 and C2), and six basic isoenzymes (E1, E2, E3, E4, E5 and E6) (Aibara *et al.,* 1981). The difference of neutral and basic isoenzymes is the amount of carbohydrate content, where the neutral isoenzymes have more carbohydrate content than the basic isoenzymes.

Peroxidase catalyzes the oxidation of electron donor substrates, for example aromatic amines and phenol, by H_2O_2. The reaction is a three-step cyclic process. First, HRP is oxidized by H_2O_2 and then reduced back to native form in two steps involving the two formation enzyme intermediates, Compounds I and II (Figure 1) (Everse., 1998).

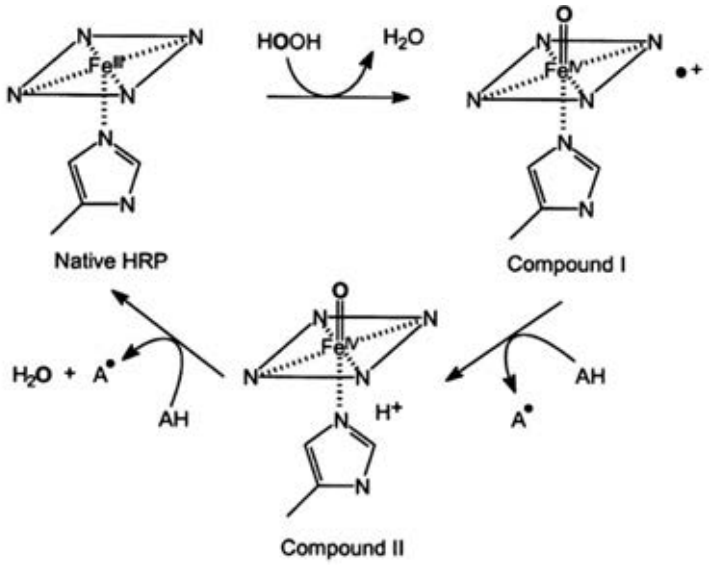


Figure 1. Reaction cycle of HRP, showing the enzyme intermediates, Compounds I and II (Everse., 1998).

1.2.1 HRP activity assay

H_2O_2 is the natural substrate of HRP which is used to monitor HRP activity. It can be used in colorimetric and fluorimetric assays. The oxidation form of substrate was colored product, which can be monitored by spectrophotometric technique. The lists of the most common substrates of HRP is shown in Table 3.

Table 3. Common substrates for HRP (Ana *et al.*, 2003).

Common name	Synonym	Detection method
ABTS	2,2′-Azino-di(3-ethylbenzothiazolin-6-sulfonate)	Spectrophotometric
Benzidine	4,4′-Diaminobiphenyl	Spectrophotometric
TMB	3,3′,5,5′-Tetramethylbenzidine	Spectrophotometric
DAB	3,3′-Diaminobenzidine	Spectrophotometric
Guaiacol	2-Methoxyphenol	Spectrophotometric
Pyrogallol	1,2,3-Trihidroxybenzene	Spectrophotometric
Phenol	Hydroxybenzene	Spectrophotometric
p-Cresol	4-Methylphenol	Spectrophotometric
o-Dianisidine	3,3′-Dimethoxybenzidine	Spectrophotometric
p-Toluidine	1-Amino-4-methylbenzene	Spectrophotometric
Tolidine	3,3′-Dimethylbenzidine	Spectrophotometric
Hydroquinone	1,4-Dihydroxybenzene	Spectrophotometric
Resorcinol	1,3-Dihydroxybenzene	Spectrophotometric
Catechol	1,2- Dihydroxybenzene	Spectrophotometric
4-Aminoantipyrine	4-Amino-2,3-dimethyl-1-phenyl-3-pyrazolinone	Spectrophotometric
p-Anisidine	1-Amino-4-methoxyphenol	Spectrophotometric
o-Phenylenediamine	1,2-Diaminophenol	Spectrophotometric
Luminol	3-Aminophthalhydrazide	Fluorimetric
Ferulic acid	4-Hydroxy-3-methoxycinnamic acid	Fluorimetric
Caffeic acid	3,4-Dihydroxycinnamic acid	Fluorimetric

1.2.2 Applications of HRP

HRP is an enzyme that can be used in a variety of applications, which are useful to human and environment.

1.2.2.1 Biosensors

Biosensors can analyze a biological component with a suitable transducer which converts a biological signal into an electrical signal. Therefore, biosensor is a very important tool in medicine, quality control, food and environmental monitoring and research (Chaubey *et al.*, 2002).

HRP is the reducer of H_2O_2 and also some organic peroxides, HRP-based biosensors were used to control and monitor these peroxides in pharmaceutical, environmental and dairy industries (Somasundrum *et al.*, 1996), in bleaching

operations in the textile and paper industries (Gundogan-Paul *et al.,* 2002), in air and water ozonization processes and in food products (Mulchandani and Rudolph., 1995).

1.2.2.2 Immunoassays

Immunoassays are used to detect and quantify antigens and antibodies that HRP conjugates have been used as markers in immunoassays, such as enzyme-linked immunosorbent assays (ELISA), Western-blotting and immune-histochemistry (IHC) techniques (Kumada *et al.,* 2002).

1.2.2.3 DNA detection

Some specific nucleic acid sequences using complementary DNA probes for detection. HRP was conjugated to complementary molecules which can be linked to the DNA probe, such as biotin or digoxigenin(Renz and Kurz, 1984). Chromogenic or chemiluminescent substrates are used to generate a colored product or light emission, signaling the hybridization event.

1.2.2.4 Microarrays

Microarray is a collection of microscopic spots attached to a solid surface. The common microarray has been known as biochip such as DNA chip. A DNA microarray contains a specific DNA sequence, known as probes that are used to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminesence-labeled targets to determine relative abundance of nucleic acid sequences in the target. HRP can catalyze a large number of electron-transfer reactions with natural and synthetic substrates. These HRP-based

or associated microarrays (or biochips) may be used in various applications such as expression analysis, recombination and gene mapping and mutation analysis (Moody *et al.*, 2001).

1.2.2.5 Bioremediation and wastewater treatment

HRP can catalyze the free-radical formation of a diversity of aromatic pollutants followed by spontaneous polymerization which can be used in bioremediation and wastewater treatment. For example, phenol, substituted-phenols (chlorophenols, methylphenols, naphthol) and azo dyes which are hazardous compounds and can be found in a variety of wastewaters from different industrial origins (textile, petrochemical, paper, chemical) (Wagner and Nicell, 2001). The removal of phenolic compounds from synthetic model and real industrial effluents uses HRP together with H_2O_2. Some representative studies are shown Table 4.

Table 4. Examples of analytes that can be monitored using HRP as sensing probe (Ana *et al.*, 2003)

Wastewater	Major pollutants	Observations
Coal derived	Phenols	
Foundry	Phenol	PEG stabilises HRP, 97–99% removal
Petroleum refinery	Phenol	PEG, chitosan stabilise HRP
Kraft pulping	Phenol, catechols, etc.	lignin derivatives in the effluent stabilise HRP
Model	Chlorophenols	HRP immobilised on magnetite
Model	Pentachlorophenol	PEG and chitosan ineffective as stabilisers, residual toxicity higher
Model	Azo dyes (Remazol, Cibacron)	
Model	Phenol and chlorophenols	Lower toxicity with chitosan Higher toxicity with PEG

1.2.3 Mechanism

The mechanism of HRP and phenol is initiated the mechanism of native HRP and H_2O_2 that one of the oxygen of H_2O_2 binds to Fe III of HRP, another oxygen of H_2O_2 binds to Arg38 of HRP, and the hydrogen of this oxygen is bonded to His42 of HRP (Figure 2) (Baek *et al.,* 1992; Rodriguez-Lopez *et al.,* 1996). From this mechanism occurs the compound, Compound 0, which is the intermediate and unstable.

The O-O bond of H_2O_2 is cleaved, a fast intramolecular electron transfer then occurs in the heme active site, which Fe III changes to Fe IV and native HRP changes to Compound I.

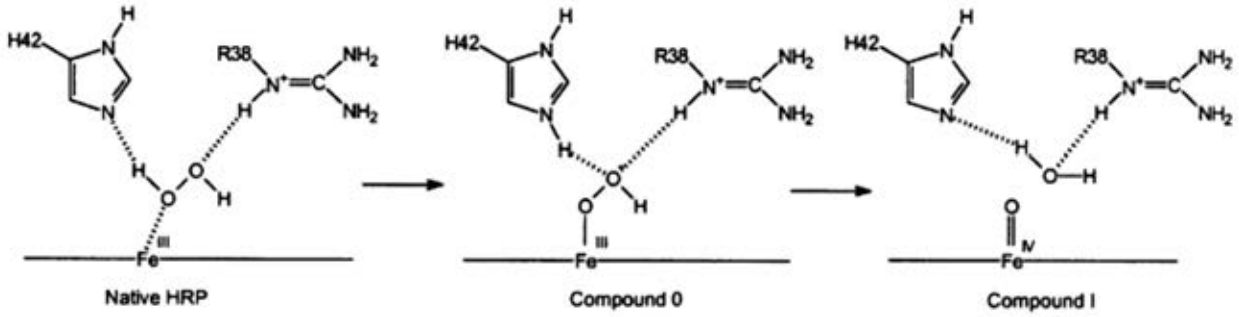


Figure 2. Mechanism of Compound I formation

1.2.3.1 Mechanism of Compound I and II reduction

From Compound I and phenol reaction, the photon of phenol is transferred by the protein group which is bound to His42 of HRP. Thus, Compound II and the radical phenolic substrate were occurred that Compound II is the initial reaction to the next step (Figure 3).

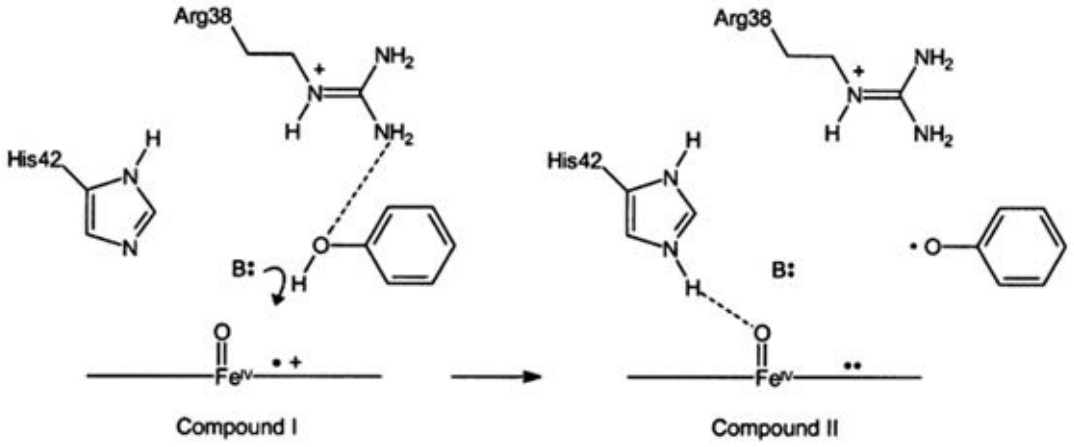


Figure 3. Reduction of Compound I by a phenol substrate molecule. B: represents a protein group that mediates the abstraction of the phenolic proton to His42 (Ana *et al.*, 2003).

The reaction of Compound II is occurred by a similar mechanism which is the photon of another phenolic substrate which is transferred to the ferryl oxygen by the protein group (Figure 4) (Azevedo *et al.*, 2003).

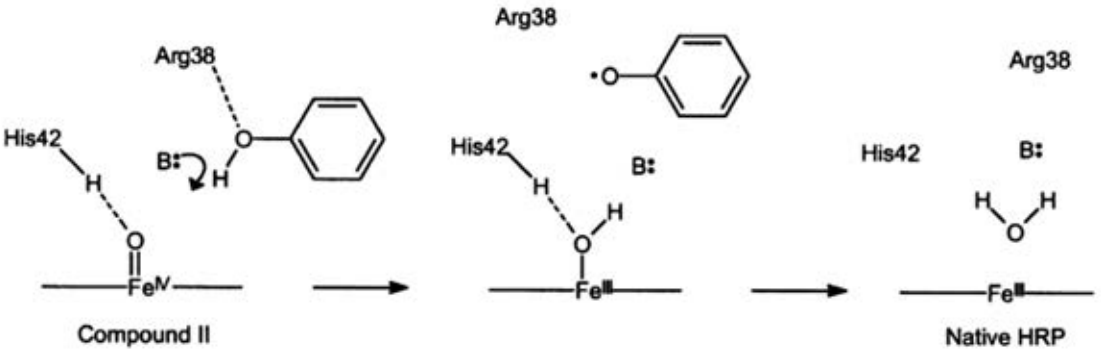


Figure 4. Mechanism of reduction of Compound II.

The final destination, both photon and electron are accepted by the ferryl oxygen and after it accepts two photon, the ferryl heme iron (Fe IV) is reduced to the ferric state (Fe III), to form water molecule and release from the heme iron.

According to the previously described, the reaction between HRP and phenol is the reduction of pollution such as the wastewater in industries, but the using of enzyme is directly that its property was low stable and its cost was expensive. The reuse of enzyme is required. The enzyme must immobilize on a suitable support for comfortable reuse. Therefore, the reuse of the immobilized enzyme requires a suitable support. However, enzyme is not stable in comparison with chemical catalysts and rather expensive. Thus, the using of enzyme is developed by the enzyme immobilization with supports.

1.3 The supports

The important elements of enzyme immobilization are enzyme, support and immobilization method. The support is necessary for the efficiency of immobilized enzyme, activity and stability of the enzyme. No any type of support can be used for every enzyme immobilization. However, the properties of an ideal support are large surface area, high porosity, appropriate ratio of hydrophilicity and hydrophobicity of the carrier matrix, chemical and thermal stability, insolubility, mechanical stability and rigidity, form and size of support, resistance to microbial attack and regenerability (Tischer and Wedekind, 1999).

1.3.1 Types of supports

The main types of support are divided into two types, organic and inorganic supports, when they are classified by its chemical characteristics. And each type can

classify to be natural and synthetic supports. In this thesis show the natural organic supports such as cellulose, agarose, agar, collagen, gelatine, chitin and chitosan, which chitosan is the support that we are interested because it was formed easily, cheap and biodegradable in nature.

However, chitosan was produced from the reaction between chitin and sodium hydroxide solution. Chitosan, which is polysaccharide or carbohydrate, has amino group and its cost is cheap for enzyme immobilization that amino group helps chitosan to crosslink with crosslinking agent. Chitin is by-product of citric acid fermentation industry, pharmaceutical manufacturing and fishery (crab and shrimp). Therefore, chitosan is chosen to be need as a support for enzyme immobilization.

1.3.2 Chitosan support

The bond of chitosan is a linear polymer of α(1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose, the deacetylated form of chitin, is a mucopolysaccharide having structural characteristics similar to glycosaminoglycans with a chemical formula $(C_6H_{11}O_4N)_n$. Advantage of chitosan were included biodegradable, low cost and non-toxic (Krajewska, 2004). So, it is preduced in different parts as a solution, powder fiber and bead forms. Chitosan is also available in various applications such as anticholesterol and fat-binding, membrane separations and immobilization of enzyme (Manrich *et al.,* 2008).

In previous study, for immobilized enzyme on chitosan supports were compared the efficiency between immobilization of enzyme on chitosan supports and enzyme soluble. Durgun *et al.* (2007) studied immobilization of pepsin on chitosan beads found that immobilization of enzyme on chitosan beads is increasing efficiency of pepsin, when it was compared with enzyme soluble. The same way Yi *et al.* (2007)

reported the efficiency of immobilization of ω-transaminase from *Vibrio fluvialis* on chitosan beads. The result shown that the immobilization of enzyme on chitosan supports can maintains efficiency of enzyme was more than free enzyme. Therefore, we had an interest to study the immobilization of HRP on chitosan supports for increasing efficiency of phenol removal of HRP.

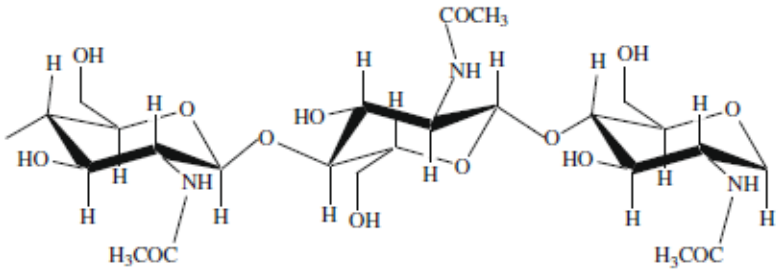


Figure 5. Structure of chitin (Jayakumar *et al.*, 2010).

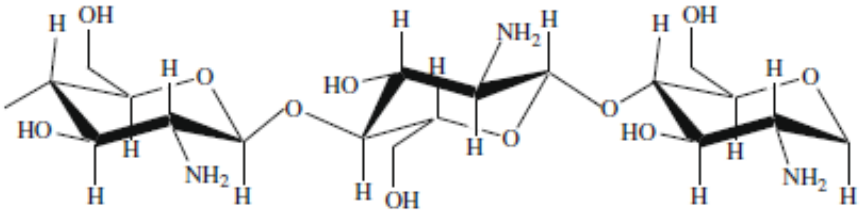


Figure 6. Structure of chitosan (Jayakumar *et al.*, 2010).

1.4 Enzyme immobilization

Immobilization of enzyme is basically undertaker either for the purpose of basic research or for the use in technical process of commercial interest. Studies on immobilized enzyme and their applications particularly in the biochemical, biomedical, pharmaceutical and food industries continue to expand. To obtain

maximum possible service from the catalytic potential of the enzyme, it remain challenging to develop the most appropriate carrier together with the correct immobilization method (Chase and Yang, 1998). That is, in general, the protein structure can be altered by immobilization thereby changing the properties of the enzyme. Stearic hindrance due to immobilization may also interfere with the contact between the enzyme and substrate. The degree of change depends on the method of immobilization, the nature of the support and coupling agent and the specificity of the reactive group. Hence, immobilization can be achieved in many ways, but it always affects enzyme activity to some extent (Chellapandian, 1998). The availability of large number of support material and the mode of attaching the enzymes to the carrier leave virtually no bioactive species without a feasible rate of immobilization. It is, thus, important that the choice of support material and immobilization method over the free bioactive agent should be well justified (Arica *et al.,* 2004).

Numerous methods for achieving immobilization of enzymes are available, each involving a different degree of complexity and efficiency. Various methods used to date may be subdivided into two general classes. These methods open up possibilities of efficient industrial applications that are chemical method, where covalent bonds are formed with the enzyme, and physical methods, where weaker interactions between support and enzyme exist (Kenedy, 1990; Chaplin and Bucke, 1990).

The detailed descriptions of different procedures along with applications were reported by various authors (Balcão *et al.,* 1996; Costa *et al.,* 2001; Yağar and Sağiroğlu, 2002; Park *et al.,* 2005). Adsorption and entrapment are included in physical immobilization. Covalent bonding and cross-linking are encountered in

chemical immobilization. Characteristics of the immobilization of enzymes by the covalent bonding are briefly summarized below.

Covalent bonding of enzymes to solid matrices is the most widely employed technique in the development of enzyme engineering in order to stabilize enzymes bonding to support. Covalent attachment involves derivation of the support to provide a chemical group capable of reacting with suitable coupling reagent and subsequently reacting with available functional group of the proteinaceous backbone of enzyme. This method has the advantages of good retention of activity in use, high stable linkages thus inhibiting leakage and sometimes rendering excellent accessibility and thermal stability (Veilleux and Duran, 1996; Jia, 2002). Stability of enzyme might be achieved through the multiple point covalent attachment that the enzyme rigidity would be greatly increased. Hence, any conformational change on the enzyme structure becomes greatly prevented (Fernández-Lafuente *et al.,* 1999). The function groups which can take part in the covalent bond formation to the support are N-terminal group, C-terminal group, aspartate β and glutamate γ-carboxylate groups, cysteine thiol groups and tyrosine phenol groups and the most common reactive group, lysine ε-amino. In addition, the carbohydrate residues of glycoproteins can also provide centers for immobilization (Jurgensen *et al.,* 1981; Gregorius *et al.,* 1995; Nahar *et al.,* 2001; Bílková *et al.,* 2002; Martin *et al.,* 2003; and Chiou and Wu, 2004).

The covalent immobilization may result in better biomolecular activity, reduced nonspecific adsorption and greater stability against temperature, denaturants, and organic solvents in several cases (Nahar *et al.,* 2001; and Bayramoğlu *et al.,* 2004). Since covalent attachment is a chemical method, whereby the immobilization is dependent on the formation of stable covalent bonds, the binding force is expected

to be strong and thus leakage of the enzyme does not happen even when washed with salt solutions of high ionic strength (Chibata, 1978). This is also ideal for mass production and commercialization (Scouten *et al.,* 1995). There are of course some disadvantages to this method that are complication, time consuming and expensive because of more chemical reagents involved in the preparation. Generally, covalent bonding lowers the immobilized enzyme activity compared to that from free soluble enzyme (Jia, 2002). It is also possible of activity loss due to the chemical modifying near the active site of enzyme (Braun *et al.,* 1996). So it is highly desirable to have some knowledge of the amino acid residues that are essential for catalysis or binding so as to avoid complete inactivation of the enzyme upon immobilization (Gemeiner, 1992; Goel, 1994; Unlig, 1998). Furthermore, the carrier could not be easily regenerated after enzyme deactivation because of tight binding force between enzymes and carriers. However, in some cases, immobilized enzyme can show higher activity than that of free enzyme. Noda *et al.* (2001) proposed that immobilized β-amylase activity was higher than that of the free β-amylase on the alkaline side (pH 7.0-10.0).

1.4.1 HRP immobilization

The immobilization of HRP has been widely reported. It has been successfully immobilized on various carriers by different methods. Torab *et al.* (2007) studied on the co-immobilization of HRP and cholesterol oxidase for covalent attachment on perlite surface. The co-immobilized enzyme retained 65% of its initial activity after 20 consecutive reactor batch cycles. Bayramoglu *et al.* (2008) presented enzymatic removal of phenol in enzyme reactor by immobilization of HRP on magnetic beads.

Found that the immobilized HRP retained 79% if the activity of free enzyme and the immobilized HRP for the degradation of phenol was successfully in the enzyme reactor. The HRP immobilized onto modified chitosan beads (Monier *et al.,* 2010). The efficiency of the immobilization was investigated by examining the relative enzymatic activity of free enzyme before and after the HRP immobilization. The immobilization result in stabilization of enzyme over a broader pH range. The activity of immobilized HRP decreased slowly against time when compared to that of the HRP solution and could retain 65.8% residual activity after 6 consecutive cycles.

From the all report, we have an interest to study the efficiency of immobilization of HRP on chitosan supports for phenol removal, which glutaraldehyde is the crosslinker. Glutaraldehyde is an organic compound with formula $CH_2(CH_2CHO)_2$ (Migneault *et al.,* 2004). The structure of glutaraldehyde has two carbonyl groups that they can react with amino groups of chitosan and HRP by covalent bond, which they are the strong bonds. Since, it has the strong covalent bonds, thus, the HRP immobilization on chitosan supports has stability (Sakuragawa *et al.,* 1998). And another reasons of glutaraldehyde is used to be extensively crossliker is that the properties of glutaraldehyde are low toxicity, easy to use, low cost and good stability (Migneault *et al.,* 2004).

1.5 Objectives of this research

Due to the fact that HRP has high potential for removal of phenol, a hazardous waste from various industries. However, an enzyme in solution form cannot be recovered from solution with their activity retained. Therefore, an effort to find a support that is biodegradable in nature and is suitable for enzyme immobilization is an increasing interest for use development of an immobilized enzyme for the removal of phenol and thus, reusability. Hence, this research aims to study the immobilization of HRP and evaluate the usefulness of the immobilized HRP for phenol removal. Characterization of immobilized enzyme for its biochemical properties and investigation of the optimum conditions for phenol removal will also be performed.

These demonstrate may create the fundamentals for subsequent large-scale phenol removal in industrial application.

The objectives of this research were:

i) To determine for appropriate support for immobilization of HRP

ii) To study the immobilization of HRP by covalent coupling method

iii) To determine the optimum conditions for covalent attachment of HRP

iv) To characterize biochemical properties of the immobilized HRP

v) To study efficiency of immobilized HRP for phenol removal and reusability

CHAPTER II

MATERIALS AND METHODS

2.1 Equipment

Analytical balance : Model AB204-S, Mettler Toledo, Switzerland

Autopipette : Gilson, USA

Centrifugal mill : Model ZM-1, Retsch, USA

Filter papers : Whatman, Japan

Hypodermic needle : size 26Gx1″, Nipro, UK

Incubator : Gallenkamp, Genway, England

Magnetic stirrer : Model CH-1E, Clifton/Nickel Electro, UK

Parafilm : Parafilm, USA

pH meter : Model S20-KS SevenEasy Mettler Toledo, Switzerland

Roller mixer : Model BTR5, Ratck Instruments Pty Ltd, Australia

Scanning electron microscopy : Model XL30CP, Philips, UK

Single syringe pump : Model NE-100, New Era Pump Systems

Spray drier : Model L-8, Ohkawara Kakohki, Japan

Syringe : Capacity 10 ml, Nipro, UK

UV/vis spectrophotometer : Model DU-530, Beckman Coulter, USA

Vortex : Model FB-15024, Fisher Scientific, USA

Water bath : Model WNB7, Memmert, Germany

2.2 Chemicals

3, 3', 5, 5' tetramethylbenzidine : Sigma Aldrich, USA

4-aminoantipyrine : Sigma Aldrich, USA

Acetic acid : Carlo Erba Reagents, Italy

Ammonium hydroxide : Sigma Aldrich, USA

Chitosan flake shrimp : MW 200,000 - 250,000, Biolife, USA

Citric acid : Carlo Erba Reagents, Italy

Dimethylsulfoxide : Sigma Aldrich, USA

Dipotassium phosphate : Univar, New Zealand

Ethanol : Carlo Erba Reagents, Italy

Formaldehyde : Sigma Aldrich, USA

Glutaraldehyde : Sigma Aldrich, USA

Glycine : Sigma Aldrich, USA

Horseradish peroxidase type VI : Sigma Aldrich, USA

Hydrochloric : Analar Normapur, UK

Phenol : Carlo Erba Reagents, Italy

Potassium dihydrogen phosphate : Sigma Aldrich, USA

Potassium ferricyanide : Sigma Aldrich, USA

Silver nitrate : Sigma Aldrich, USA

Sodium bicarbonate : Carlo Erba Reagents, Italy

Sodium hydroxide : Carlo Erba Reagents, Italy

Sulfuric acid : Sigma Aldrich, USA

Tris (hydroxymethyl) – aminomethane : Sigma, USA

Tri-sodium citrate dehydrate : Scharlau, Spain

Urea hydrogen peroxide : Sigma Aldrich, USA

2.3 Preparation of chitosan supports

2.3.1 Preparation of chitosan membrane

To make the membrane, a 1.0% (w/v) chitosan solution was prepared in 1.0% (v/v) aqueous acetic acid on shaker for overnight at room temperature and filtered. The chitosan solution was then cast in rimmed plastic dish with a dimension of 11x11 cm and completely dried in a clean room at room temperature for 72 hours. After drying, the chitosan membrane was neutralized in 4% (w/v) NaOH which was dissolved in 50% ethanol solution for 1 hour and was dried at room temperature. After that the membrane was washed with distilled water to remove residual sodium hydroxide. To ensure a complete neutralization, chitosan membrane was washed with distilled water until the pH was neutral and dried at room temperature for 12 hours (Lim *et al.,* 1998). Finally, the membrane was milled with 0.25 mm blade number from centrifugal mill. The membrane powder was subsequently visualized by scanning electron microscopy (SEM). The samples were coated with gold particles prior to the analysis.

2.3.2 Preparation of spray dried chitosan

Spray dried chitosan was prepared by dissolving 1.0% (w/v) chitosan in 1.0% (v/v) aqueous acetic acid. The solution was shaken on shaker overnight at room temperature and filtered. Chitosan solution was then spray dried and the chitosan spray dried chitosan was subsequently visualized by SEM.

2.3.3 Preparation of wet and dry chitosan beads

Chitosan flake was dissolved in 1.0% (v/v) aqueous acetic acid to make 2.0, 3.0 and 5.0% (w/v). The solution was shaken for 12 hours at room temperature and

filtered. The chitosan solution was added dropwise from a 26 gauge syringe needle into 10% (w/v) NaOH that was dissolved in 50% (v/v) ethanol, where the chitosan precipitated immediately to form gelatinous beads. The chitosan beads were washed with distilled water until the pH was neutral. The chitosan beads were stored in distilled water at 4^{o}C until use.

For dry chitosan beads, the preparation was the same as that of chitosan wet beads. After the gelatinous beads were formed, the beads were allowed to dry at room temperature for 48 hours. Chitosan beads were then subsequently visualized by SEM.

2.4 Immobilization of horseradish peroxidase

2.4.1 Activation of support material

Fifty milligrams of chitosan dry beads were reacted with 5 ml of 0.3% (v/v) glutaraldehyde (GA) in deionized water under mild agitation for 2 hours at room temperature. The concentration of GA of 0.3% (v/v) was used according to Sakuragawa *et al.*(1999). For activation of chitosan wet beads, after optimization, GA concentration of 1.5% (v/v) was allowed to react for 2 hours with 150 mg wet beads, which is equivalent to the surface area of the dry beads. The beads were subsequently washed with deionized water several times to remove residual GA.

2.4.2 Enzyme coupling

Amount of horseradish peroxidase (HRP) of 2.5 units was dissolved in 4 ml of phosphate buffer (0.1 M, pH 7.0) before being transferred to activated support, from section 2.4.1. The mixture was gently stirred at room temperature for 1 hour. The unbound HRP was removed by 0.1 M phosphate buffer, pH 7.0 (5 times). All eluates

were collected and analyzed for HRP activity as described in section 2.6. The enzyme was stored in phosphate buffer, pH 7.0 until further use.

2.5 Optimization of HRP immobilization

The optimum conditions for covalent immobilization of HRP were investigated by varying some of the conditions described in sections 2.4.1 and 2.4.2. The GA concentration, range from 0.1-3.0% (v/v), and incubation time of GA range from 0.5-12 hours, were tasted. Various HRP concentrations applied to the support were also investigated in the range of 0.025-25 units. The coupling time between the enzyme and the support was also examined during 0.5-12 hours. The immobilized enzyme activity and the protein content were determined by the method mentioned in section 2.6 and 2.7, respectively. The best condition for HRP immobilization was selected.

2.6 Enzyme assay

The free and immobilized enzyme activities were determined by colorimetric assay (Appendix A) using 1.0 mg of 3, 3', 5, 5' tetramethylbenzidine (TMB) was dissolved in dimethyl sulfoxide (DMSO) 100 µl. The TMB solution was added dropwise under gentle shaking to 10 ml of 0.1 M citrate buffer pH 5.5 and incubated at room temperature for 10 minutes. After that, 1.0 mg of urea hydrogen peroxide was added and 1 ml of this substrate solution was added into the enzyme solution. The reaction was stopped by the addition of 2.0 M of sulfuric acid (H_2SO_4). The reaction rate was determined by measuring the absorbance at 450 nm with a UV-VIS spectrophotometer. It the reaction was not stopped, the absorbance at 650 nm was then read (Bos *et al.*,1981).

2.7 Protein determination

2.7.1 Silver staining

Silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins immobilized on the beads (Appendix B).

Two grams of dry beads and 6 grams of wet beads under 3 conditions, which includes chitosan beads, chitosan beads crosslinked with GA and chitosan beads immobilized with HRP were put into 10 ml of silver staining solution for 15 minutes at room temperature. The excess stain was removed by thoroughly washed with distilled water. This was then further developed with developer for 5 minutes and washed thoroughly with in distilled water.

2.8 Calculation of the immobilization yield

The efficiency of the immobilized enzyme was usually expressed as the activity retention after immobilization. The immobilization yield of the immobilized HRP was calculated by equation as follow:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme activity (U)} \times 100}{\text{Free enzyme activity used (U)} - \text{Unbound enzyme activity (U)}}$$

2.9 Characterization of the biochemical properties of the free and immobilized HRP

2.9.1 Effect of pH on the activity of free and immobilized HRP

The effect of pH on the activity of free and immobilized HRP was determined with pH ranging from 3.0 to 11.0. Citrate buffer (0.1 M, pH 3.0-6.0), phosphate buffer (0.1 M, pH 6.0-7.0), Tris-HCL buffer (0.1 M, pH 7.0-9.0), glycine-NaOH buffer (0.1

M, pH 9.0-11.0) were used (Appendix C). The enzyme activity was measured by the method described in section 2.6.

2.9.2 Effect of temperature on the activity of free and immobilized HRP

The temperature dependence on the activity of free and immobilized HRP was determined in 0.1 M citrate buffer at pH 5.5. The activity was measured in the temperature ranging between 20 and 70^{o}C by a method described in section 2.6.

2.9.3 pH stability

The effect of pH on the stability of the free and immobilized enzyme was examined after pre-incubating enzyme sample in buffers at different pH values (the buffer solution used was the same as described in section 2.9.1) at room temperature for 60 minutes. The residual activity was measured under the standard condition as described in section 2.6.

2.9.4 Thermal stability

The thermal stability of the immobilized enzyme was compared to that of the free enzyme. Both of the enzyme samples were incubated in 0.1 M phosphate buffer pH 7.0 and exposed to different temperature (20-70^{o}C) for 20 minutes. After incubation, the residual activity for both forms of enzyme was measured under standard condition as described in section 2.6.

2.10 Performance comparison between immobilized HRP on wet and dry chitosan beads

Performance comparison between immobilized HRP on wet and dry beads were examined under the following conditions. Fifty milligrams of dry beads and same 30 milligrams of wet beads were reacted with 5 ml of 1.5% (v/v) of GA and incubated for 8 hours at room temperature. Excess of GA was removed with distilled water. HRP solution (2.5 units dissolved in 4 ml of phosphate buffer, pH 7.0) were subsequently added and incubated for 2 hours, followed by washing with the same buffer. The reaction was perlorwed with and without stirring. For comparison, another set of experiments were performed where the immobilized enzymes were allowed to react with GA prior to the addition to the chitosan beads. The mixture was allowed to incubate for 2-12 hours before being washed with the same buffer. Again, the reaction proceeded under stirred and unstirred condition. The remaining activity was measured as described in section 2.6.

2.11 The storage stability of free and enzyme immobilized on wet beads

Both free and immobilized HRP were stored in 0.1 M phosphate buffer pH 7.0 at 4ºC and room temperature for 1 month. The remaining activity was measured every 2 days as described in section 2.6 and compared to the value at time zero.

2.12 Analysis for the optimum conditions of free and immobilized enzyme for phenol removal

This section involves the study of the removal of phenol using free and immobilized enzyme. The effect of phenol concentration, pH and temperature on the removal of phenol was investigated.

The residual concentration of phenol was determined by colorimetric assay. Phenolic compounds within a sample solution volume of 1.0 ml was reacted with 4-aminoantipyrine (4-AAP) (2.0 mM, 1.0 ml) and potassium ferricyanide reagent (6.0 mM, 1.0 ml). The latter two reagents were prepared in sodium bicarbonate buffer solution (0.1 M, pH 10.0). The reaction rate was measured at 510 nm after incubation at room temperature for 6 minutes (Bayramoglu and Arica, 2008). Absorbance readings were converted to concentration of phenol using the standard calibration curve (Appendix D).

2.12.1 Calculation of the phenol concentration

The efficiency for phenol removal of free and immobilized enzyme was calculated using equation as follow:

$$\text{Reduction (\%)} = \frac{\text{(Initial phenol concentration-Concentration of phenol residual) x100}}{\text{Initial phenol concentration}}$$

2.12.2 Effect of phenol concentration on the enzymatic removal of phenol by free and immobilized enzyme

The concentration of phenol was varied from 0.1-2.0 mM. One milliliter of phenol solution was added to free and immobilized HRP. The reaction was started by the addition of H_2O_2 (4.0 mM, 1 ml) then the reaction mixture was incubated for 15 minutes. After a predetermined time, the sample was withdrawn and assayed for phenols as described in section 2.12.

2.12.3 Effect of temperature on the enzymatic removal of phenol by free enzyme and immobilized enzyme

The influence of temperature on the removal efficiency of phenol by free and immobilized HRP was determined in 0.1 M sodium bicarbonate buffer pH 10.0. The activity was measured in the temperature ranging between 20-70ºC. The reaction was started by the addition of H_2O_2 (4.0 mM, 1 ml) and incubated for 15 minutes before the residual phenol was assayed as described in section 2.12**.**

2.12.4 Effect of pH on the enzymatic removal of phenol by free and immobilized enzyme

The effect of pH on the activity of free and immobilized HRP for the removal of phenol was determined by the addition of 1 ml of 0.5 mM phenol solution to free and immobilized enzyme sample. These experiments were carried out over the pH range of 3.0-11.0. The buffer used were citrate buffer (0.1 M, pH 3.0-6.0), phosphate buffer (0.1 M, pH 6.0-7.0), Tris-HCl buffer (0.1 M, pH 7.0-9.0), glycine-NaOH buffer (0.1 M, pH 9.0-11.0) and sodium bicarbonate buffer (0.1 M, pH 9.0-11.0). The reaction mixture was incubated as described in section 2.12.2. The amount of phenolic compound was determined by a method described in section 2.12 except that 4-AAP and potassium ferricyanide reagents were prepared in sodium bicarbonate buffer solution (0.5 M, pH 10.0).

2.13 The reusability of the immobilized enzyme

The reusability of the immobilized enzyme for the removal of phenol was studied in repeated batch experiments up to 5 times in phenol (0.5 M, 1 ml) and the reaction was started by the addition of H_2O_2 (4.0 mM, 1 ml). At the end of each cycle,

the immobilized enzyme was separated and the supernatant was assayed for phenol as described in section 12.2. The immobilized enzyme beads were then thoroughly washed with phosphate buffer (0.1 M, pH 7.0) before subjected to the next cycle.

CHAPTER III

RESULTS

3.1 Appropriate form of chitosan

In this study, the suitability of support for enzyme immobilization was investigated. Four models of supports were studied including chitosan film, chitosan microsphere or spray dry chitosan and chitosan beads at concentrations of 2%, 3% and 5% as described in section 2.3. Chitosan morphology was examined using scanning electron microscopy (SEM). Figure 7A shows a form of chitosan film that has been crushed by the microcentrifugal mill. It can be seen that the size of the particles was not uniform. Figure 7B was spray dried chitosan particles, most of which were shaped like a sphere. However, the particle size was still not uniform and not separated into single particle. The shape of chitosan beads at 2% (w/v) (Figure 7C) were biconcave and they were not uniform in size and shape. In contrast, chitosan beads that were prepared at concentration of 3% (w/v) (Figure 7D) and 5% (w/v) (Figure 7E) were found to be suitable for enzyme immobilization. Both models were consistent in shape and size, which was an advantage that cannot be found in other forms. Nevertheless, chitosan beads at concentration of 3% (w/v) were chosen because they showed the same properties as those of 5% (w/v) but less amount of chitosan powder was used. Wet chitosan beads at 3% (w/v) were also prepared (Figure 7F). Wet chitosan beads also showed regularity in size and shape as found in

dry chitosan beads. Thus, wet chitosan beads at 3% (w/v) were also used for further study.

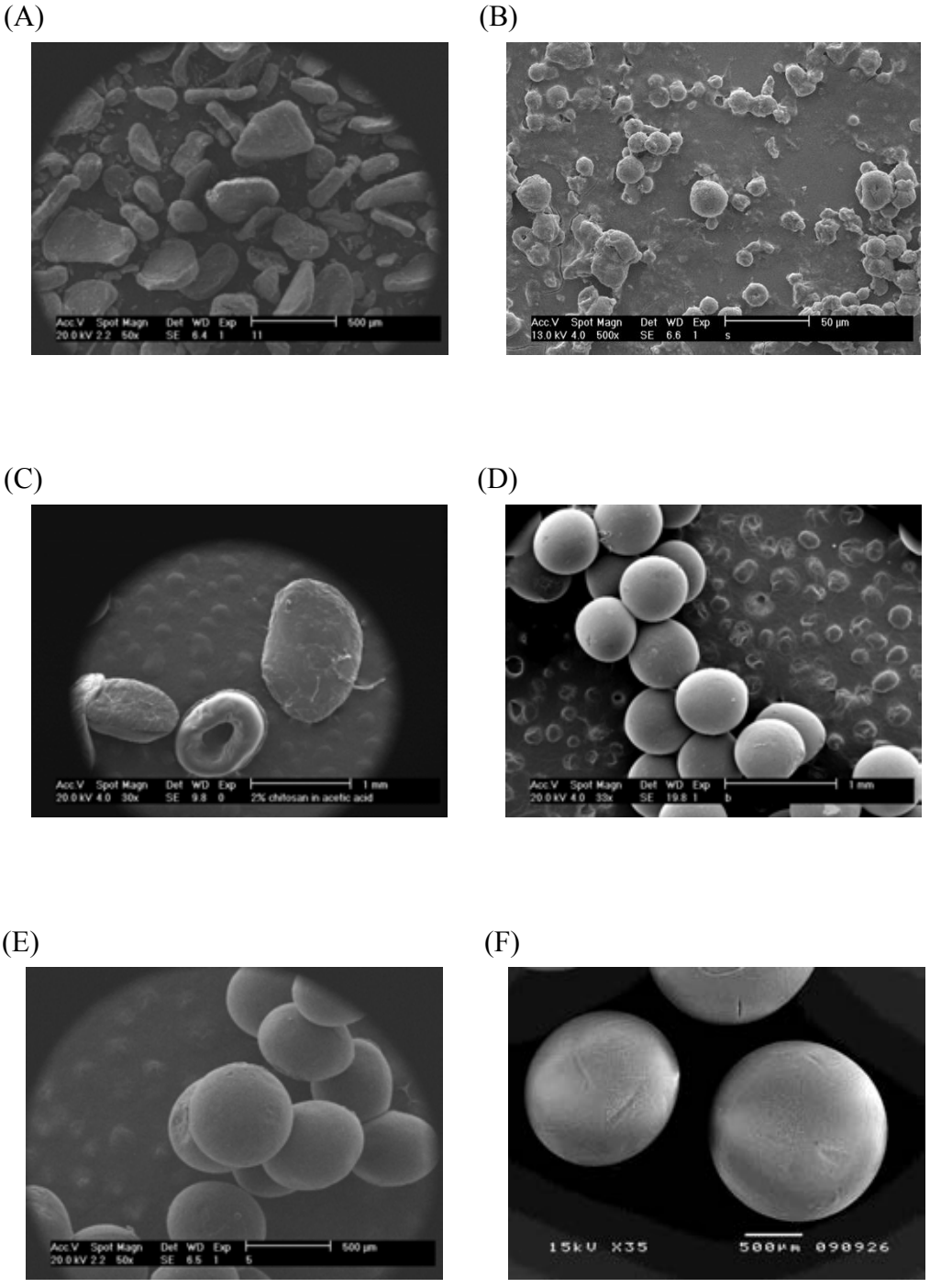


Figure 7. SEM images of chitosan film (A), chitosan spray dry (B), dry chitosan beads at 2% (C), 3% (D), 5% (E), (v/v) and wet chitosan beads at 3% (v/v), (F).

SEM images of the surfaces of dry (Figure 8A) and wet (Figure 8D) chitosan beads, dry (Figure 8B) and wet (Figure 8E) chitosan beads activated with glutaraldehyde (GA) and dry (Figure 8C) and wet (Figure 8F) chitosan beads immobilized with HRP were investigated.

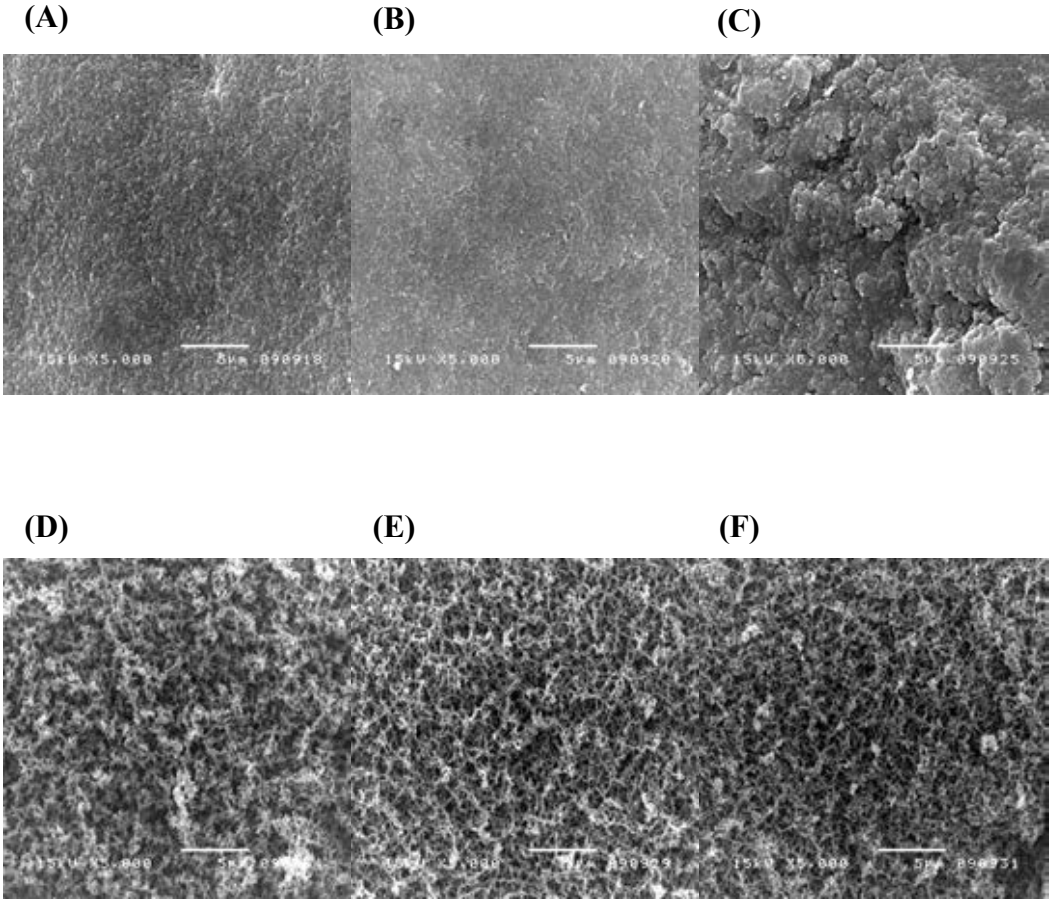


Figure 8. SEM images of (A) dry chitosan beads, (B) dry chitosan beads-GA, (C) dry chitosan beads-GA-HRP, (D) wet chitosan beads, (E) wet chitosan beads-GA and (F) wet chitosan beads-GA-HRP.

3.2 Optimization of the immobilization conditions

The influence of several parameters (GA concentration, time of incubation, enzyme concentration and coupling time) on the immobilization of HRP has been studied.

3.2.1 Effect of glutaraldehyde concentration

The dry chitosan (50 mg) and wet chitosan supports (30 mg) were activated with different concentrations of GA in 0.1 M phosphate buffer pH 7.0 for 2 hours and incubated with HRP solution (2.5 U) at room temp for 1 hour. Results of the effect of GA on the immobilized enzyme activity are shown in Figure 9. It can be seen that the immobilized enzyme activity was maximum when 1.5% (Figure 9A) and 1.0% (v/v) (Figure 9B) of GA concentration was used to activate dry chitosan and wet chitosan beads, respectively. In addition, when concentration of GA was higher, the immobilized enzyme activity tended to decrease slightly. Thus, 1.5% and 1.0% (v/v) concentration of GA were selected for the activation of dry and wet supports, respectively.

3.2.2 Effect of reaction time of glutaraldehyde

To investigate the influence of reaction time of GA on dry chitosan and wet chitosan beads, the beads were treated with 1.5% (v/v) or 1.0% (v/v) of GA, respectively, with different reaction time varying from 0.5-12 hours. The activated support was then incubated with HRP solution, followed by immobilized enzyme activity assay as mentioned previously. The results can be seen in Figure 10A that the

incubation time between the dry chitosan beads and GA at 8 hours showed the outstanding value of the immobilized HRP activity. For the wet chitosan beads, the immobilized enzyme activity was comparable at all reaction time used, although it was found to be maximum at 10 hours of incubation (Figure 10B).

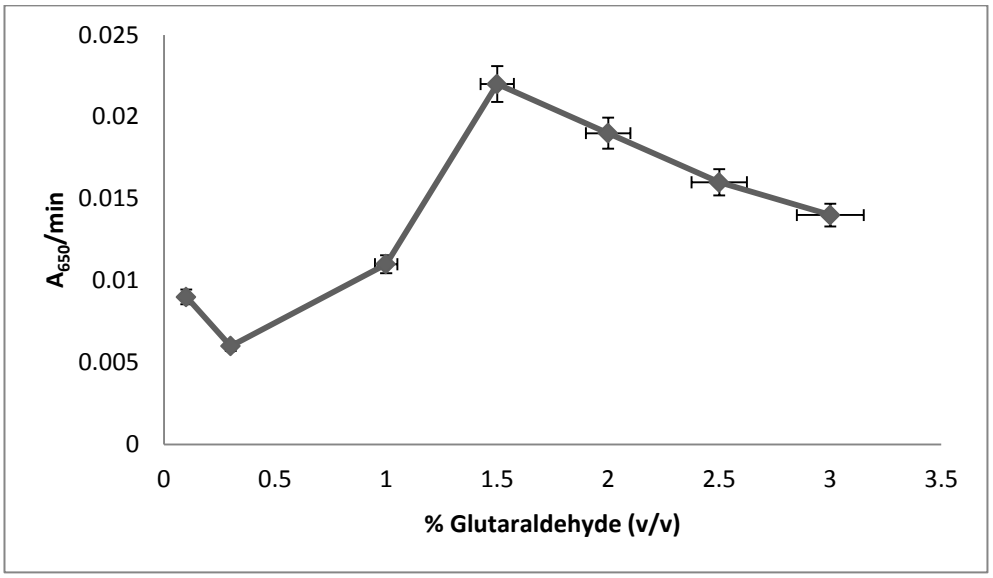
3.2.3 Effect of enzyme concentration

To obtain high immobilization yield, the reaction was carried out by varying the amount of enzyme added to dry and wet chitosan beads. As the amount of HRP increased in both supports, the activity of the immobilized HRP also increased although the immobilization yield was found to decrease (Figure 11). At 2.5 μg/ml of HRP is the appropriate concentration for both supports.

3.2.4 Effect of coupling time

The appropriate coupling time to efficiently immobilize HRP on dry and wet chitosan supports were estimated for different time period (2-12 hours). The activated chitosan were incubated with HRP solution at room temperature. The results in Figure 12 concluded that coupling time of 2 hours between HRP and both supports gave highest immobilized activity. The prolongation of coupling time did not show much difference in the immobilized activity.

(A)



(B)

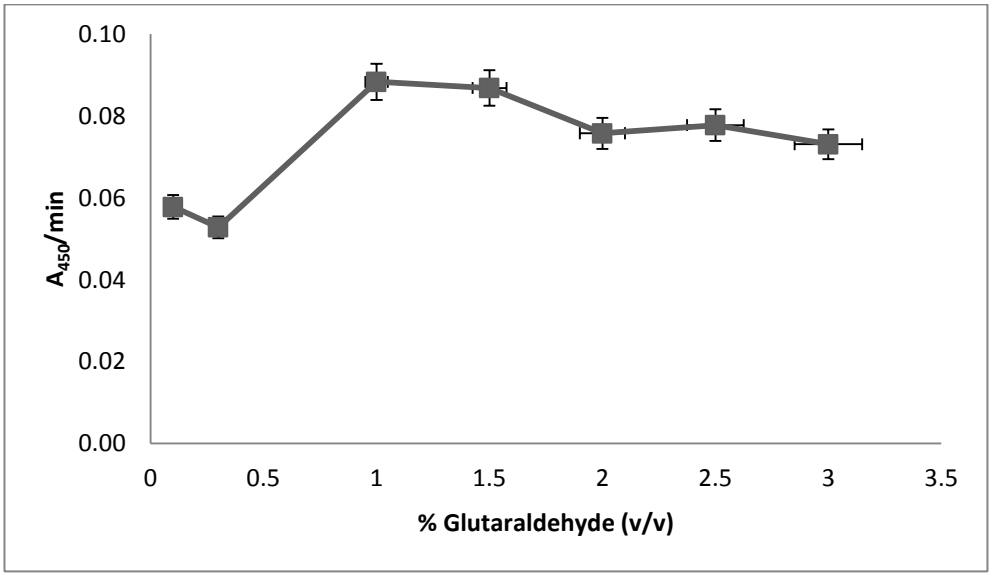
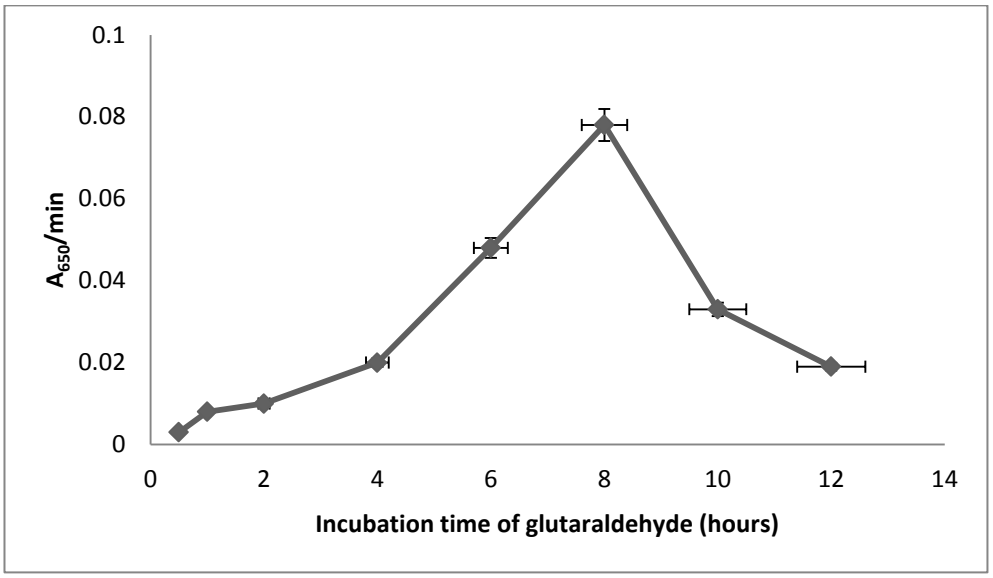


Figure 9. Influence of glutaraldehyde concentration on the amount of HRP immobilized on dry (A) and wet chitosan beads (B). The surface was activated with GA for 2 hours and incubated with 2.5 U of HRP at room temperature for 1 hour. Results shown were average values of triplicate experiments.

(A)



(B)

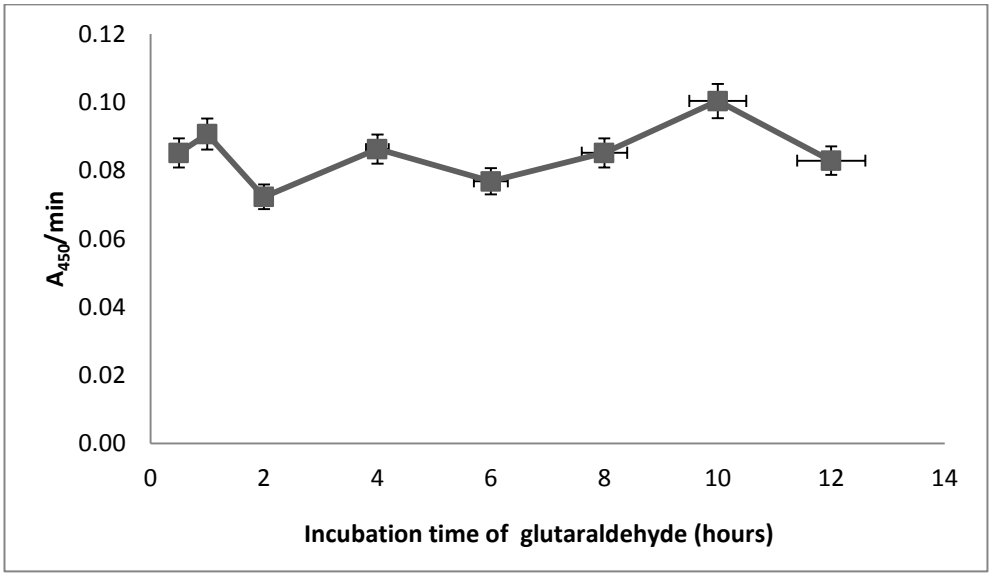


Figure 10. Effect of coupling time between glutaraldehyde solution with dry (A) and wet chitosan beads (B). The surface was activated with 1.5% v/v of GA for 2 hours and incubated with 2.5 U at room temperature for 1 hour. Results shown were average values of triplicate experiments.

3.2.5 Protein determination

A staining test derived from the silver staining assay was used to investigate protein on the polymeric support. The results are shown in Figure 13. The protein immobilized on the beads were explored by immersing dry and wet chitosan beads in silver nitrate solution. The immobilized HRP on dry beads showed less change in colour in comparison with that immobilized on wet chitosan beads. Thus, HRP immobilized on wet chitosan beads showed higher amount of protein bound.

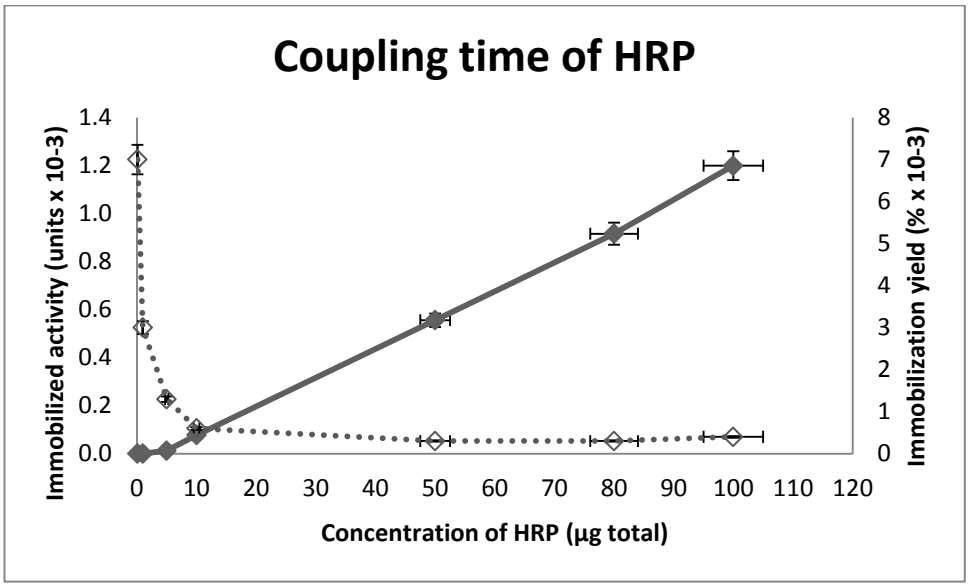
3.3 Biochemical characterization of the free and immobilized HRP

Properties of catalysts, such as optimum temperature, thermal stability, pH optimum, pH stability and storage stability of immobilized HRP was studied and compared with those of the free enzyme. All the activities were investigated on dry and wet chitosan supports.

3.3.1 Optimum temperature

Effect of temperature on the activity of free and immobilized HRP on dry and wet chitosan beads was determined by incubating the reaction mixture at temperature ranging from 20ºC to 70ºC in 0.1 M citrate buffer, pH 5.5 as described in section 2.9.2. As can be seen in Figure 14, all enzyme preparations, either free or immobilized on dry or wet chitosan supports showed similar value of the optimum temperature (25ºC). When the temperature was higher than 40ºC, the activity of the free and immobilized enzymes on either dry or wet chitosan beads was dramafically decreased.

(A)



(B)

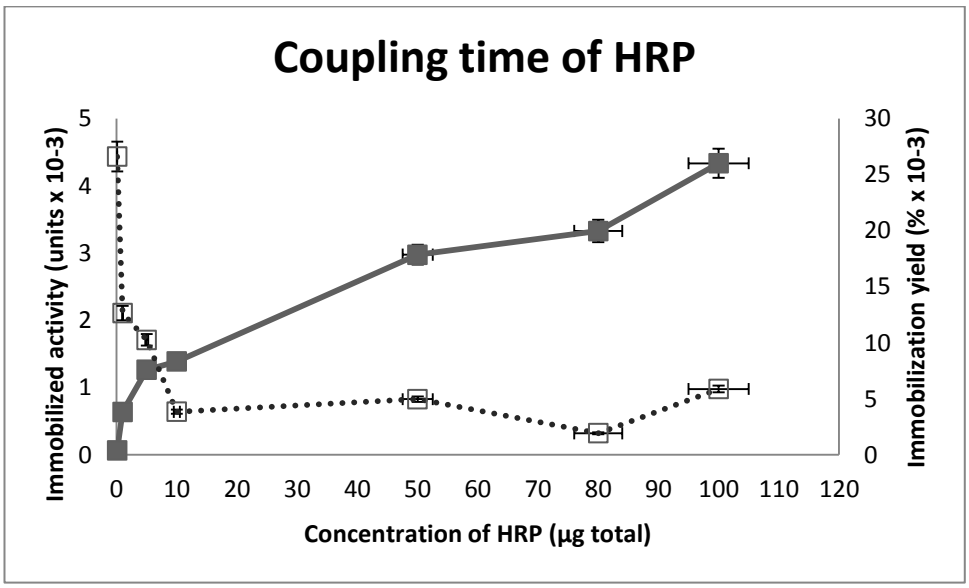
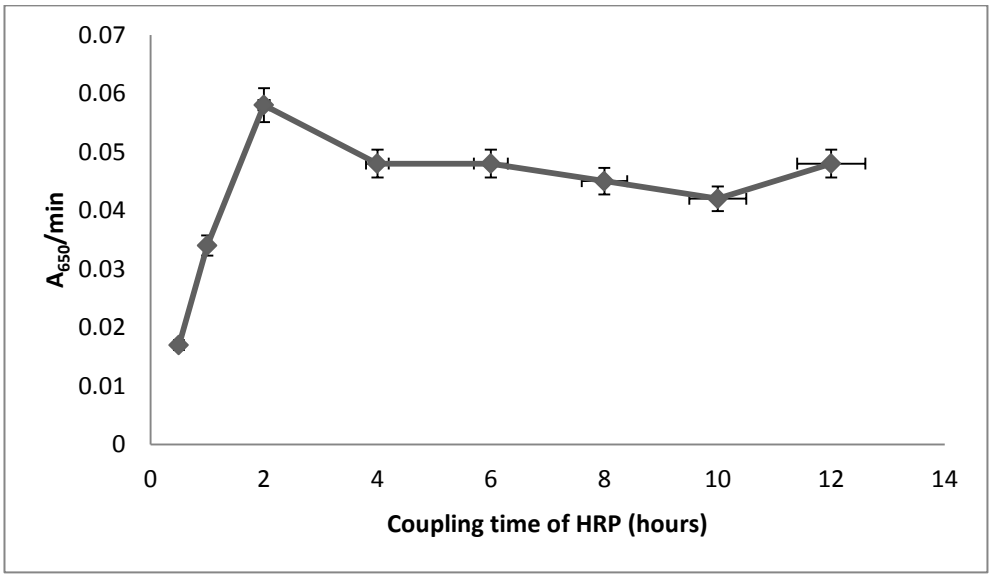


Figure 11. Effect of the amount of HRP applied with exhibited activity (—) and immobilization yield (···) on dry (A) and wet chitosan beads (B). GA (1.5% v/v) was used to activate the surface for 8 hours and HRP at different amount was added and incubated for 2 hours. Results shown were average values of duplicate experiments

(A)



(B)

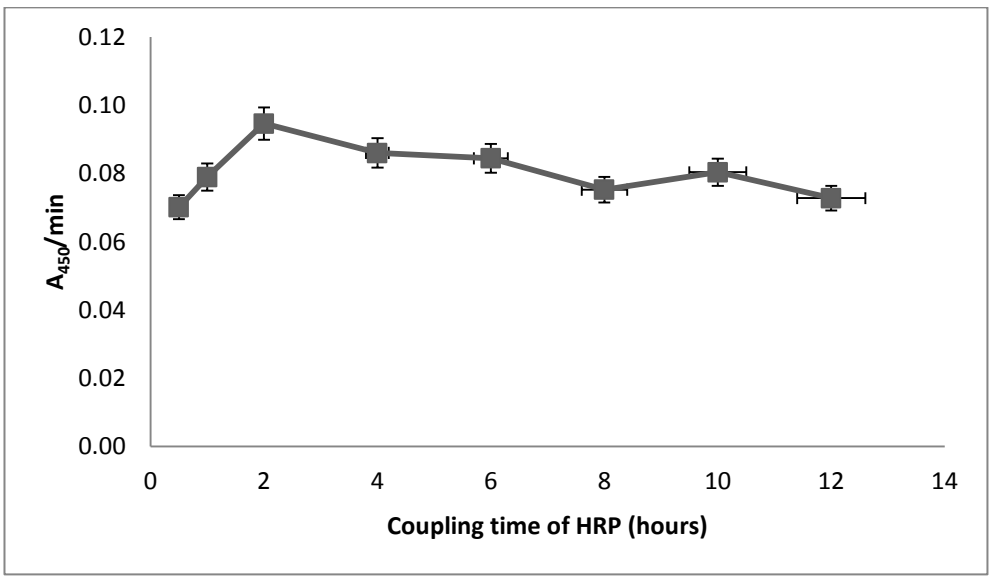


Figure 12. Effect of coupling time between dry (A) and wet chitosan beads (B) on the immobilized HRP activity. GA (1.5% v/v) at different incubation time (0.5-12 hours) was used to activate the surface for 8 hours before HRP (2.5 U) was added. Results shown were average values of triplicate experiments

(A)

(a) **(b)** **(c)** **(d)**

(B)

(a) **(b)** **(c)** **(d)**

Figure 13. Digital photograph of dry and wet chitosan beads before (A) and after (B) being dipped into silver nitrate solution. (a) dry chitosan beads-GA, (b) dry chitosan beads-GA-HRP, (c) wet chitosan beads-GA and (d) wet chitosan beads-GA-HRP.

3.3.2 Thermal stability

The thermal stability of free and immobilized HRP on dry and wet chitosan supports was compared. The free and immobilized enzyme samples were incubated in 0.1 M phosphate buffer pH 7.0 at different temperature (20-70ºC). The residual activities were examined under standard conditions at room temperature. The enzyme immobilized on dry chitosan beads exhibited higher thermostability than the native enzyme. On the contrary, HRP immobilized on wet chitosan beads showed lower thermostability than the soluble enzyme (Figure 15). In addition, HRP immobilized on both dry and wet chitosan beads showed the values of relative activity higher than 70% until the temperature was above 40ºC, the relative activity of enzyme immobilized on wet beads tends to decrease faster than the enzyme on dry beads.

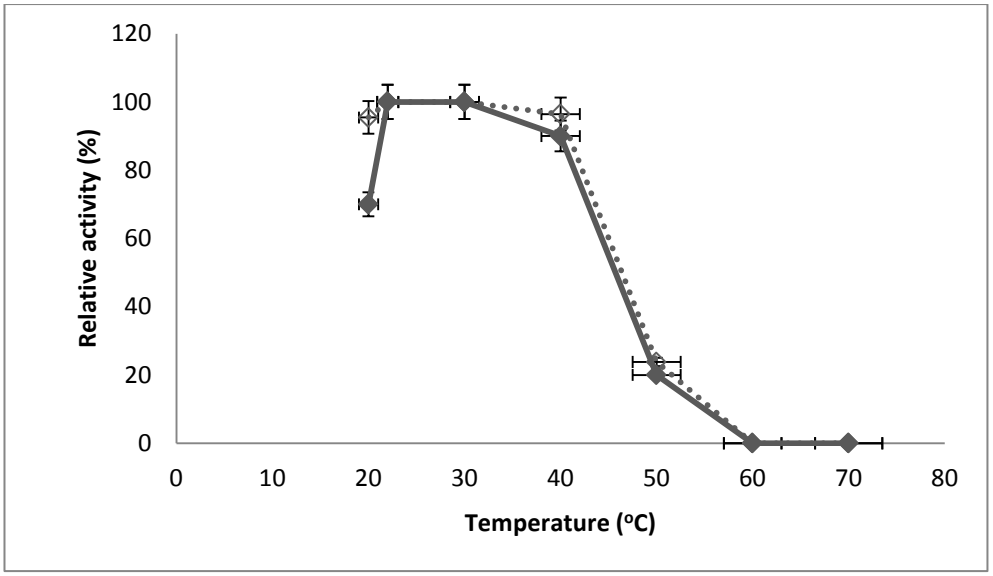
3.3.3 pH optimum and pH stability

The effect of pH on the activity of free and immobilized HRP on dry and wet chitosan supports were determined as mentioned in section 2.9.1. Reaction rate of all enzyme preparations was measured at various pH values (3.0-11.0) and calculated for relative activity which was expressed in percentage. The optimum pH of free and immobilized enzyme on both dry and wet chitosan beads were found to be the same at pH 5.5 (Figure 16).

The pH stability was compared and investigated for free and immobilized enzyme on wet and dry chitosan supports. All conditions were exposed to different pH values (3.0-11.0) for 1 hour at room temperature before the enzyme activity was determined under standard condition (0.1 M citrate buffer, pH 5.5 at room

temperature) as described in section 2.6. The result showed that free and immobilized HRP on dry chitosan beads were stable over pH values of 5.0-7.0. At the same time, free and immobilized HRP on wet chitosan beads were stable over the pH range 5.0-11.0 (Figure 17).

(A)



(B)

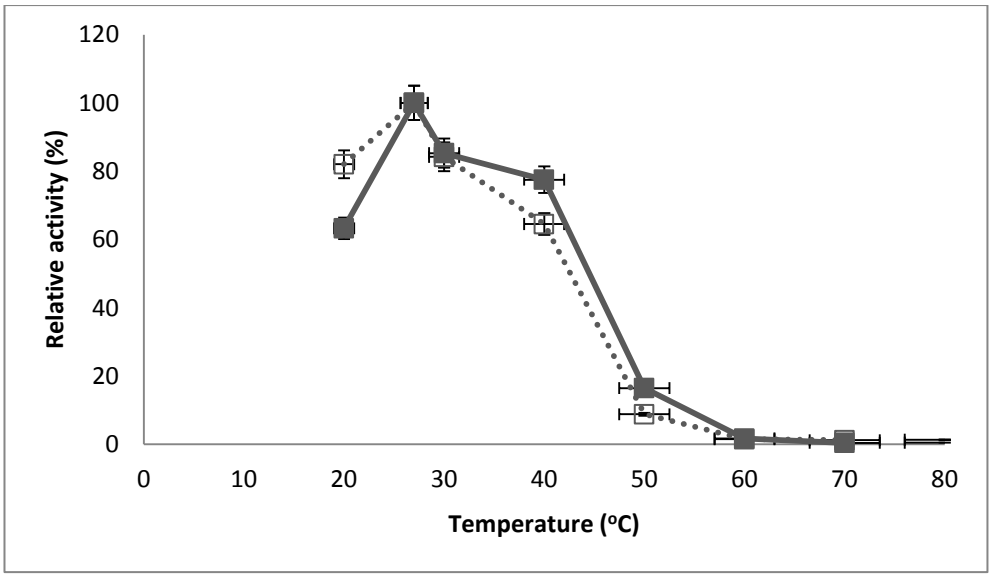
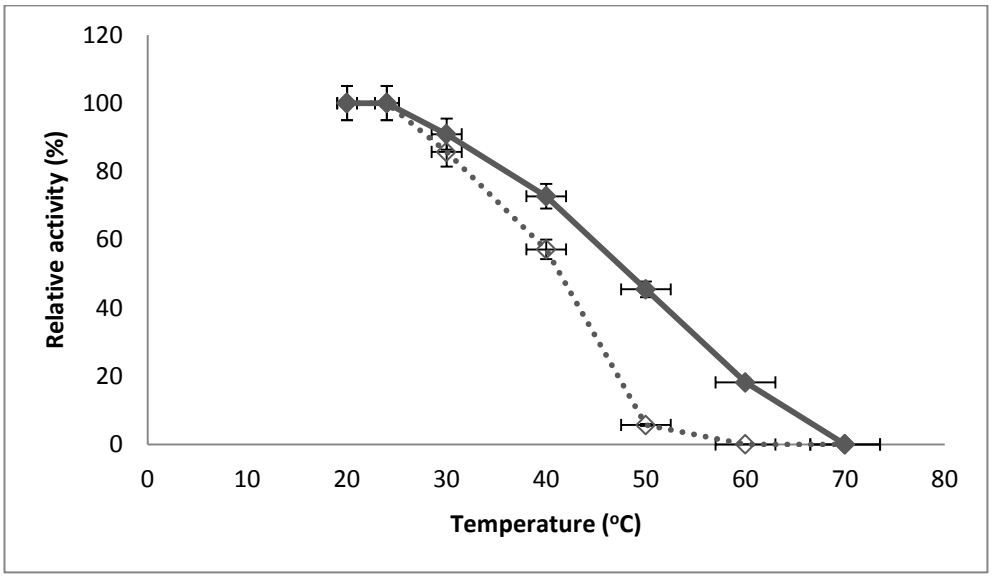


Figure 14. Effect of temperature on the activity of free (····) and immobilized HRP (—) on dry (A) and wet chitosan beads (B). The reaction was operated at temperature ranging between 20ºC-70ºC in 0.1 M citrate buffer pH 5.5. Results shown were average values of triplicate experiment.

(A)



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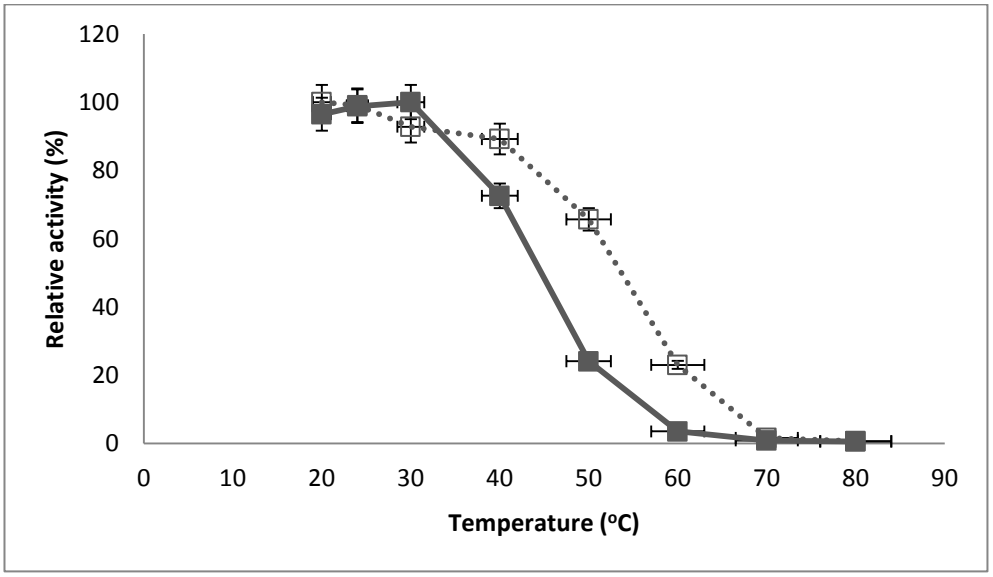
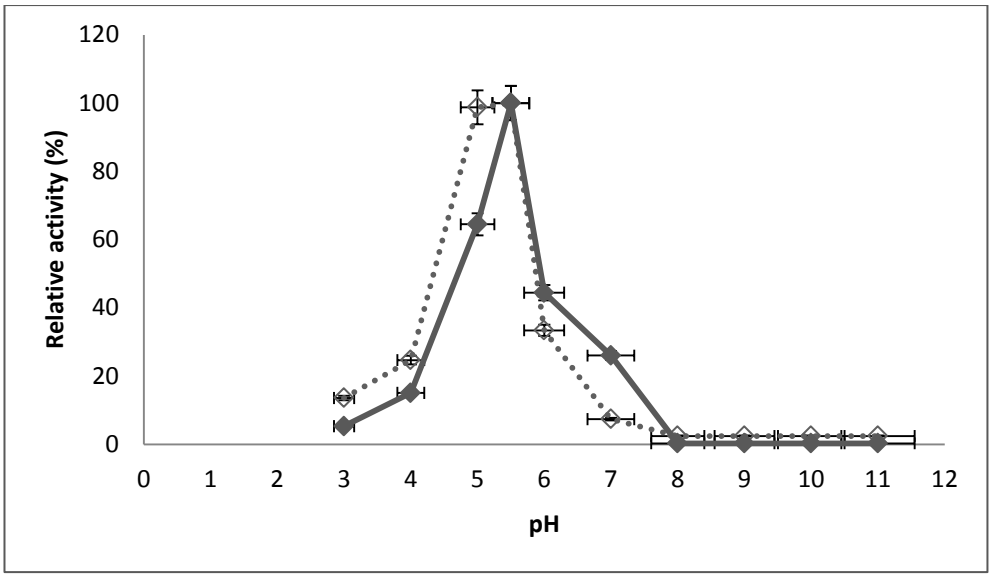


Figure 15. Effect of temperature on the stability of free (⋯) and immobilized HRP (—) on dry (A) and wet chitosan beads (B). All enzyme samples were incubated in 7.0 M phosphate buffer pH 7.0 at various temperature between 20ºC to 70ºC for 20 minutes before residual activities were assay at room temperature. Results shown were average values of triplicate experiments.

(A)



(B)

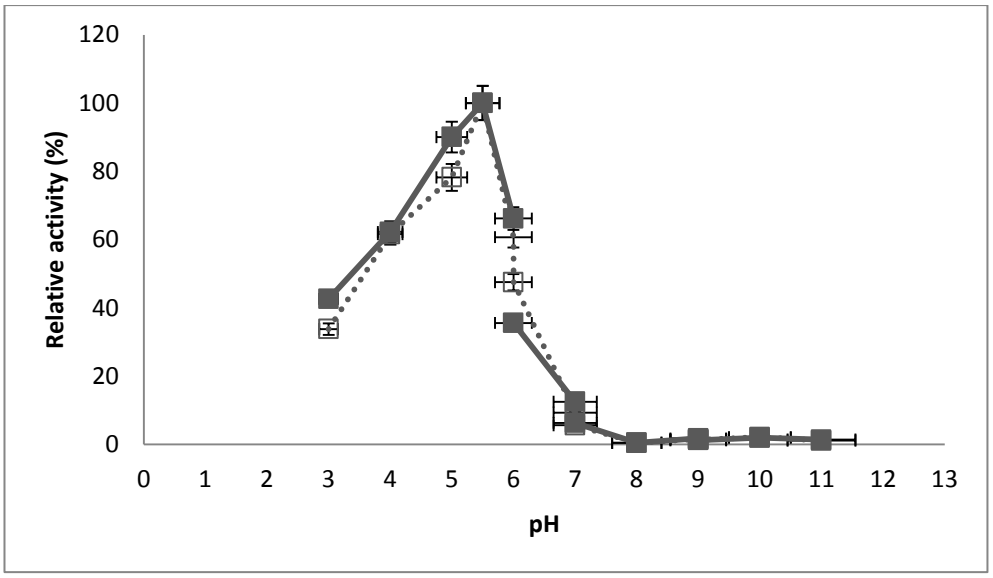
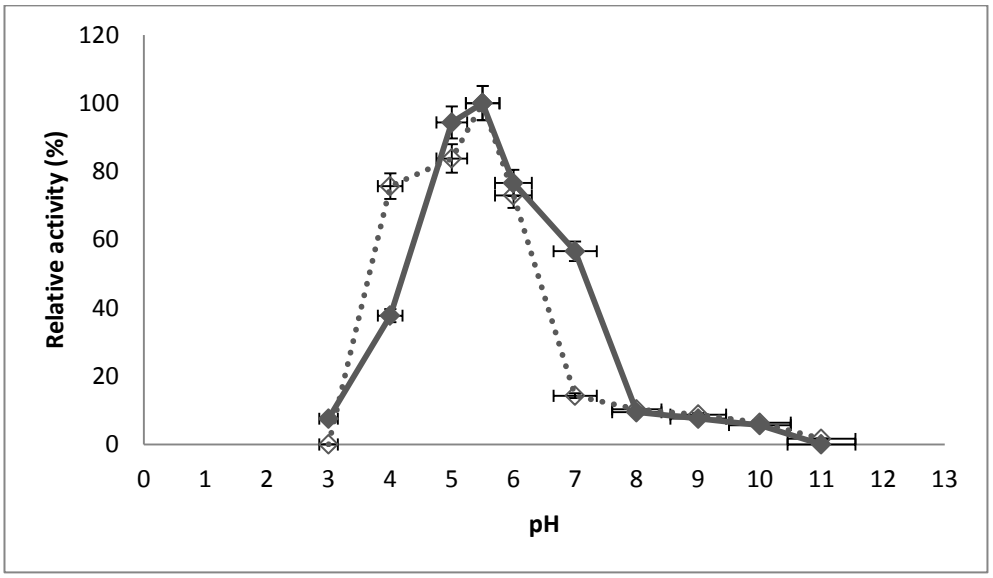


Figure 16. Effect of pH on the activity of free (⋯) and immobilized HRP (—) with dry (A) and wet chitosan beads (B). The reaction was carried out at different pH values (3.0-11.0) in 0.1 M buffer solution. The results shown were average values of duplicate experiments at room temperature.

(A)



(B)

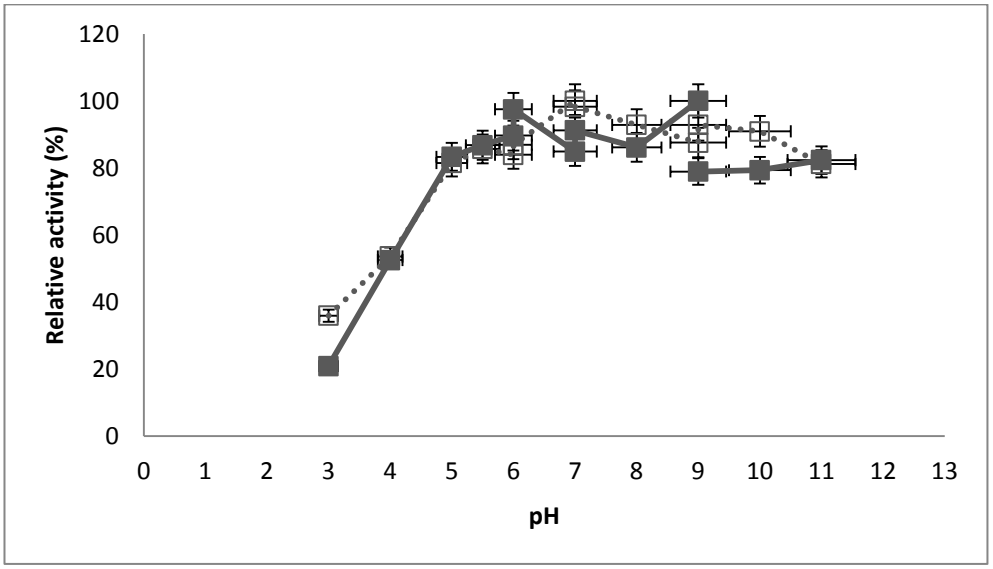


Figure 17. pH stability of free (⋯) and immobilized HRP (—) on dry (A) and wet chitosan beads (B). Enzyme samples were preincubated at different values of pH (3.0-11.0) for 1 hour at room temperature. The residual activity was investigated under standard condition (pH 5.5). The results shown were average values of duplicate experiments.

The summary of optimal conditions for HRP immobilized on dry and wet chitosan beads is shown in Figure 18 and 19 respectively. Under these optimal conditions, dry chitosan beads were activated with 1.5% (v/v) GA for 8 hours and wet chitosan beads were activated with 1% (v/v) GA for 10 hours. The amount of enzyme added to dry and wet chitosan beads were 10 µg with coupling time of 2 hours.

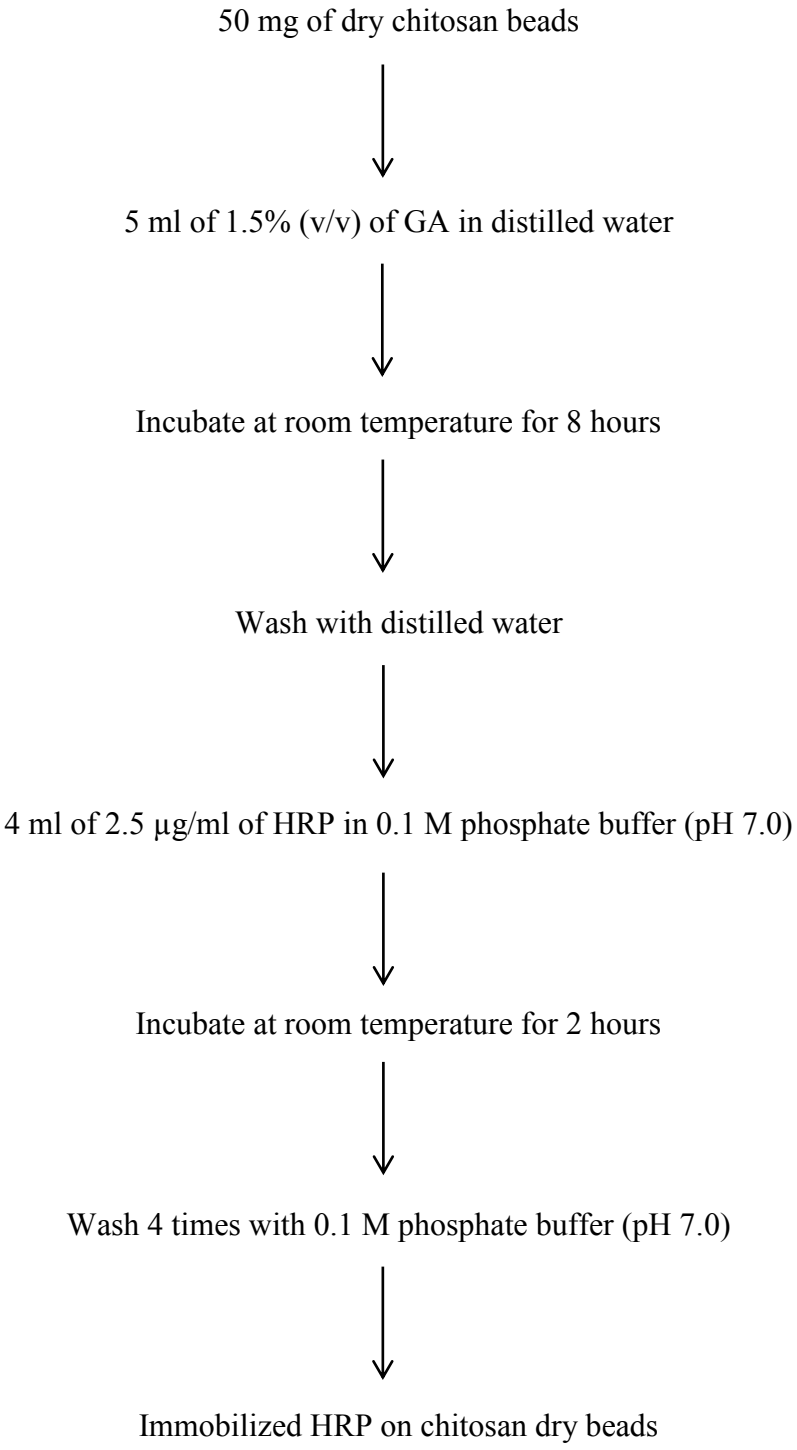


Figure 18. The optimal conditions for HRP immobilization on dry chitosan beads.

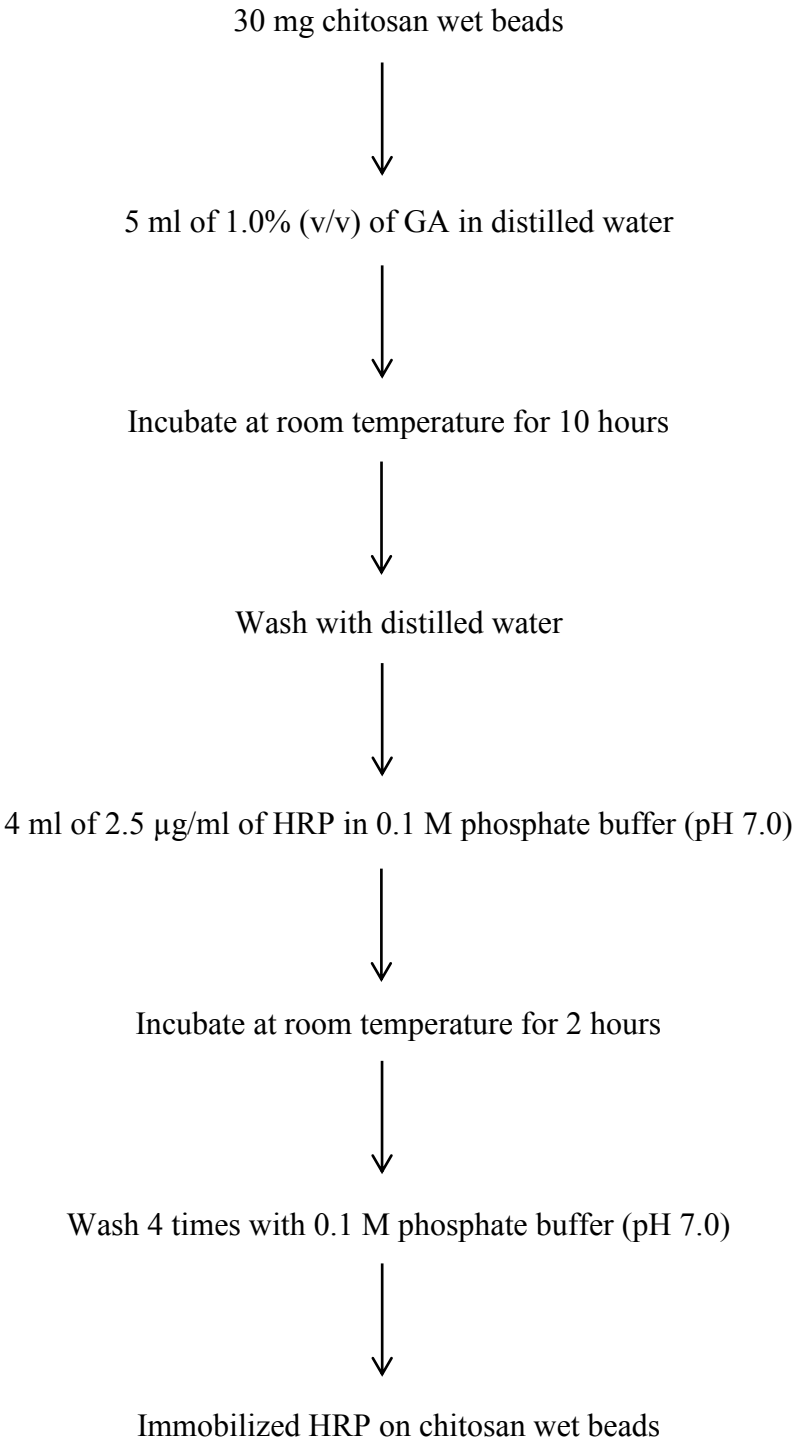


Figure 19. The optimal conditions for HRP immobilization on wet chitosan beads.

Table 5. Summary of properties of free and immobilized HRP on dry and wet chitosan beads

Parameters	Free HRP	Immobilized HRP on dry support	Immobilized HRP on wet support
pH optimum	5.5	5.5	5.5
pH stability (25ºC, 60 min)	5.0-7.0	5.0-7.0	5.0-11.0
Temperature optimum (ºC)	25	25	25
Temperature stability (20 min, ºC)	20-40	20-40	20-40

3.4 Performance comparison between immobilized HRP on wet and dry beads

After the optimization of HRP immobilization condition on both dry and wet chitosan beads were investigated, experiments to compare the performance of immobilized enzyme on both supports were explored. The experiments were carried out into 2 fashions. First, GA (1.5% v/v) was allowed to react with dry and wet chitosan beads without agitation for 8 hours at room temperature. After that the reaction mixture was incubated with 4 ml of 2.5 μg/ml of HRP for 2 hours at room temperature, also without agitation. In another condition, the experiment was set the same as previously described, except that the reaction was proceeded under agitation.

Form Figure 20, it can be seen that HRP immobilized on wet chitosan beads gave higher immobilization yield in comparison with that of dry chitosan beads. In addition, the immobilization yield was also found to be higher when the reaction mixture was stirred.

To achieve the highest immobilized activity, another set of experiments were performed. GA (1.5% v/v) and 4 ml of 2.5 μg/ml of HRP solution were allowed to mix before adding to the dry and wet chitosan supports with and without agitation at different reaction time. Immobilization of HRP onto wet chitosan beads gave mush higher immobilized activity than that on dry beads (Figure21).

Since immobilized HRP activity on wet chitosan beads was higher than that of dry chitosan beads, wet beads were then selected for further study. Various conditions were investigated. HRP was immobilized on the activated chitosan with and without agitation. In another set of experiment, GA and HRP solution was mixed together before adding to the chitosan beads at different incubation time, with and without agitation. The result in Figure 22 shows that when wet chitosan beads were incubated with a mixture of GA and HRP solution with agitation, the immobilized HRP activity

was found to be highest at 4 hours incubation. After that, it was dramatically decreased. However, when the wet chitosan beads were incubated with 1.5% (v/v) GA and 2.5 μg/ml of HRP consecutively, the best immobilized activity was achieved at 2 hours incubation and the decrease in rate of reaction was slower. Thus, HRP immobilized on wet chitosan beads was prepared by incubating GA first with wet chitosan beads prior to the incubation with HRP solution. The reaction with agitation was chosen to further study on the removal of phenol.

3.5 The storage stability of free and immobilized enzyme on wet chitosan beads

Storage stability of free enzyme and enzyme immobilized on wet chitosan beads was studied by storing the enzyme in 0.1 M phosphate buffer pH 7.0 at 4ºC and 25ºC for 1 mount. The remaining activities of both enzyme samples were determined every 2 days for 1 month as described in section 2.11. Results in Figure 23 show that at 4ºC and 25ºC, the immobilized enzyme retained higher activity than the native enzyme. Both free and immobilized enzyme samples were more stable at 4ºC as compared with that at room temperature 25ºC. After 30 days of storage, the immobilized enzyme stored at 4ºC retained 19% of its activity, while the native enzyme maintained only to 7% of its activity.

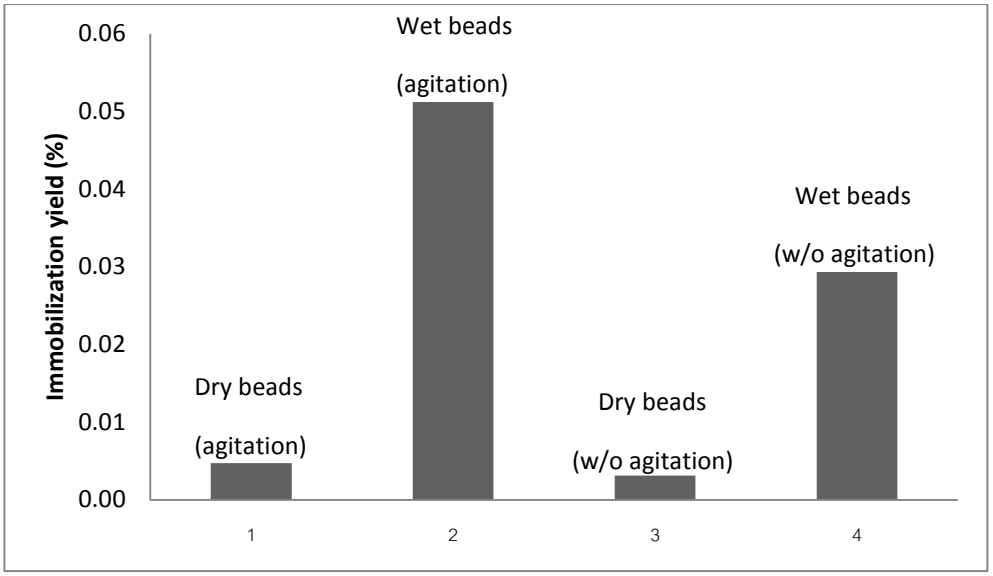


Figure 20. Immobilization yield on dry and wet chitosan beads with and without agitation. GA (1.5%) was allowed to react with dry and wet chitosan supports for 8 hours. After 4 ml of HRP solution (2.5 μg/ml) were added to the activated supports. The results shown were average values of triplicate experiments.

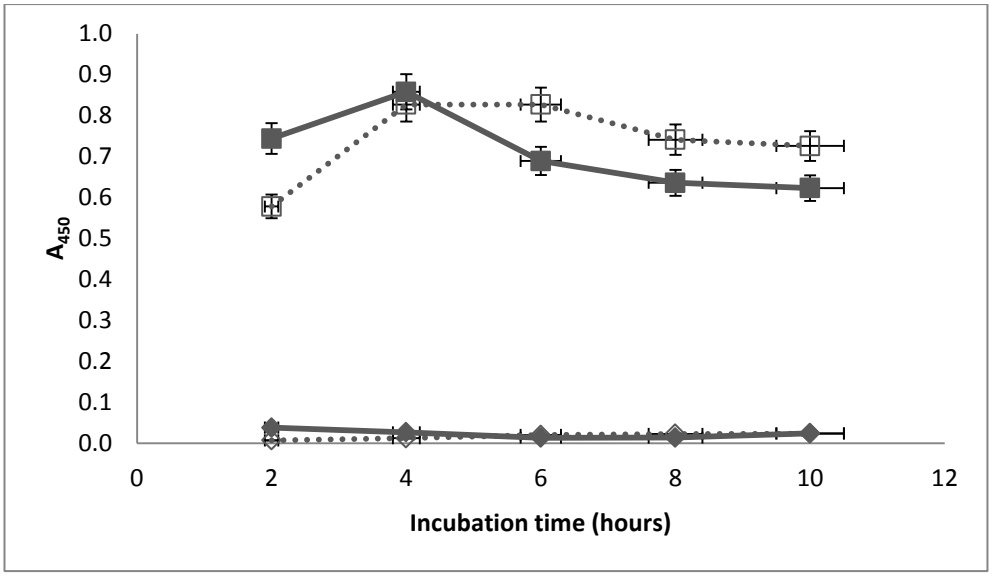


Figure 21. Immobilized activity on dry (◇) and wet chitosan beads (□), with (—) and without agitation (····). GA (1.5% v/v) was mixed with 4 ml of HRP solution (2.5 μg/ml), prior to the addition to the dry and wet chitosan beads of different incubation time. The results shown were average values of triplicate experiments

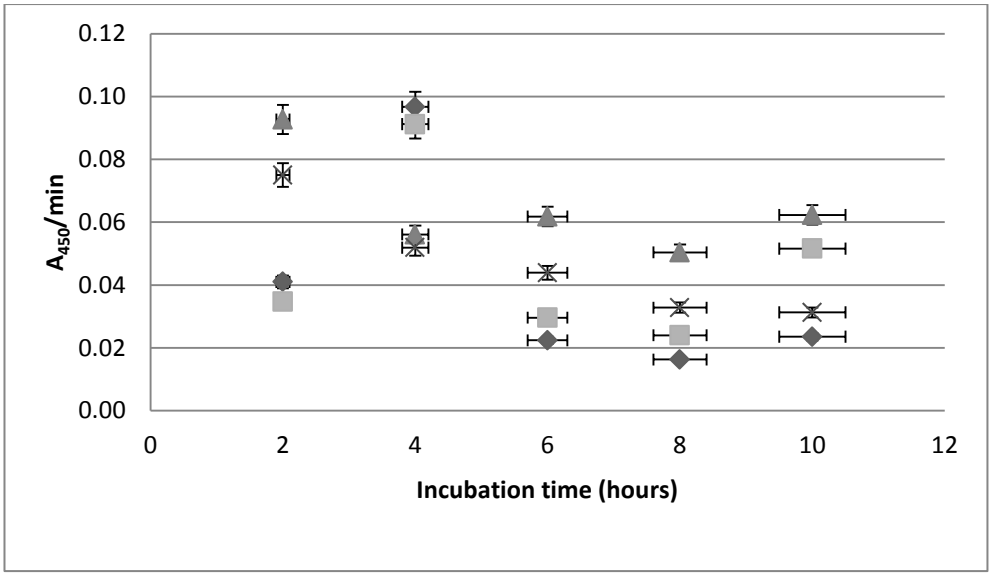
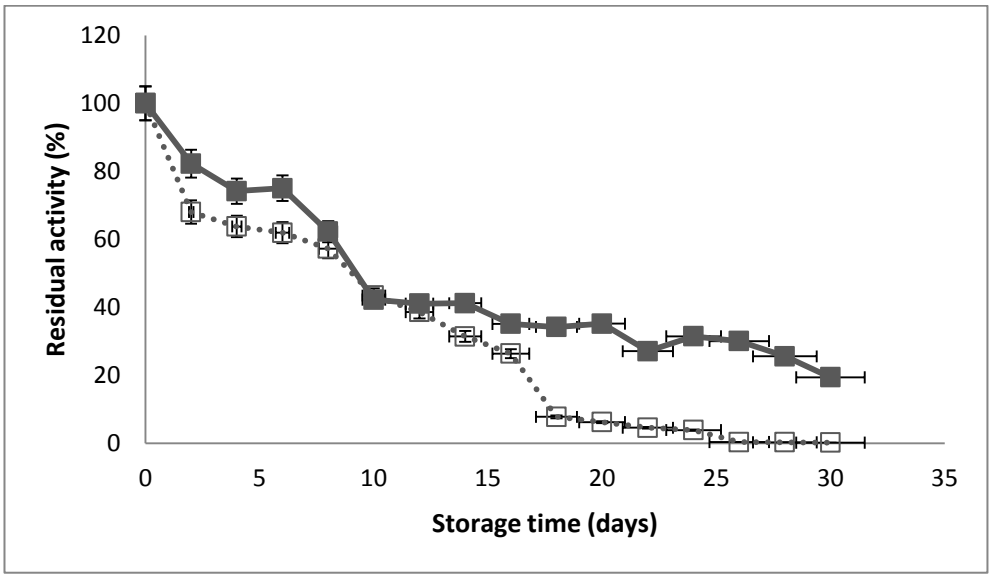


Figure 22. Comparison of immobilized HRP activity on wet chitosan beads under various conditions. GA (1.5% v/v) was first mixed with 4 ml of 2.5 µg/ml of HRP and was added to wet chitosan beads with (◆) and without (■) agitation. Another set of experiment was carried out where GA was first incubated with wet chitosan beads prior to the incubation with HRP solution. The reaction was carried out with (▲) and without agitation (**X**). The results shown were average values of triplicate experiments.

(A)



(B)

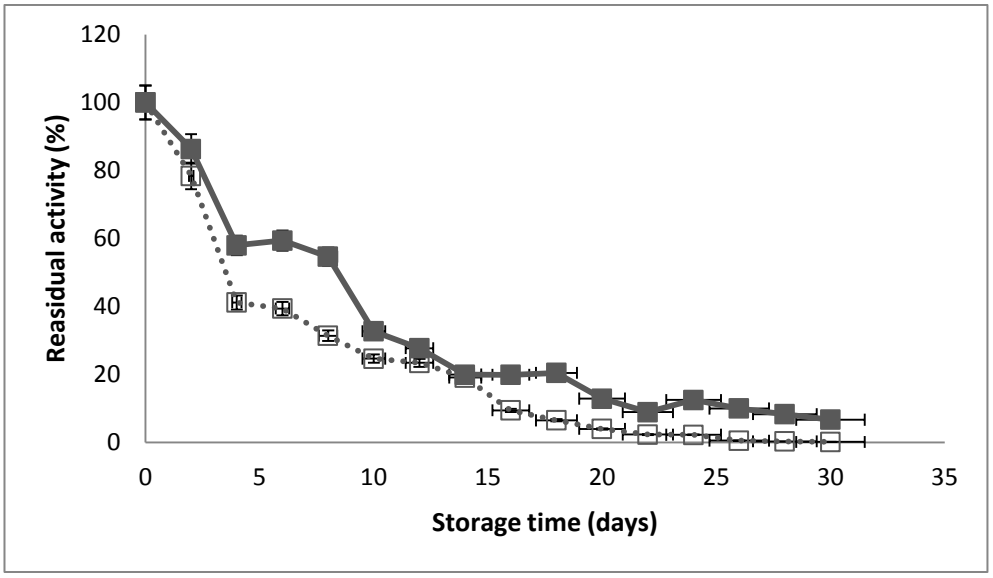


Figure 23. Storage stability of the free (⋯) and immobilized HRP (—) on wet chitosan beads at 4ºC (A) and room temperature (B). All enzyme samples were stored in 0.1 M phosphate buffer and the remaining activity was examined at designated time. The results shown were average values of duplicate experiments.

3.6 Analysis for the optimum conditions of free and immobilized enzymes for phenol removal

3.6.1 Effect of phenol concentration on the enzymatic removal of phenol by free and immobilized enzymes

The concentration of phenol was varied from 0.1-2.0 mM and each concentration was transferred to free and immobilized enzyme samples. The reaction was started by the addition of H_2O_2 solution. The reaction mixture was allowed to incubate for 15 minutes. After a predetermined time, the sample was withdrawn and assayed for phenols as described in section 2.12. The result in Figure 24 shows the efficiency of phenol removal of free and immobilized enzymes. When the concentration of phenol increased, the ability to remove phenol by free and immobilized enzyme was also increased. Although the concentration of phenol was increased to 2 mM, the free and immobilized enzyme was still able remove phenol up to 60%.

3.6.2 Effect of temperature on the enzymatic removal of phenol by free enzyme and immobilized enzymes

The effect of temperature on phenol removal by free and immobilized HRP on wet chitosan beads was compared. The free and immobilized enzymes were preincubated in 50 mM of phenol solution and 4 mM of H_2O_2 with 0.1 M sodium bicarbonate buffer pH 10.0 at different temperature (20-70ºC). After 15 minutes of incubation, the residual phenol was assayed in assay solution as explained in section 2.12. In Figure 25, it can be seen that the temperature between 20-60ºC, the activity to remove phenol by free and immobilized enzymes is similar. However, when

temperature rises above 60ºC, the immobilized enzyme showed slightly higher efficiency to remove phenol than free enzyme. From these experiments, both enzyme samples showed the highest phenol degradation rates at room temperature.

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3.6.3 Effect of pH on the enzymatic removal of phenol by free enzyme and immobilized enzymes

The effect of pH on the free and immobilized enzymes for phenol removal was determined by preincubating enzymes with 0.5 mM of phenol at different pH values (3.0-11.0), 0.1 M. The reaction was started by the addition of H_2O_2 solution and incubated for 15 minutes before being assayed for residual phenol. The results showed that the optimum pH for phenol degradation by free and immobilized enzymes was at pH 10 of sodium bicarbonate buffer, which was effective in the removal of phenol up to 97% and 98%, respectively. While the phenol removal by free and immobilized enzymes at pH 10 when glycine buffer was used was 94% and 95%, respectively (Figure 26). Thus, the phenol degradation by both free and immobilized enzymes was highest at pH 10 when bicarbonate buffer was used.

3.6.4 The reusability of the immobilized enzyme for phenol removal

After 0.5 mM of phenol was preincubated with the immobilized enzyme on wet chitosan beads and reaction was started by the addition of H_2O_2 and incubated for 15 minutes, phenolic compound in sample solution was assayed under standard condition as described in section 2.12. After the beads was used for the reaction with phenol, they were thoroughly washed with 0.1 M phosphate buffer before being subjected to the next cycle. The ability to reuse the enzyme for phenol removal is shown in Figure 27. The ability to remove phenol in the first round was set as 100%

of phenol removal efficiency. The immobilized enzyme lost activity after every use. However, phenol removal efficiency was still higher than 50% after being reused 3 times and in the fifth cycle, the immobilized enzyme still retained the ability to remove phenol up to 36%

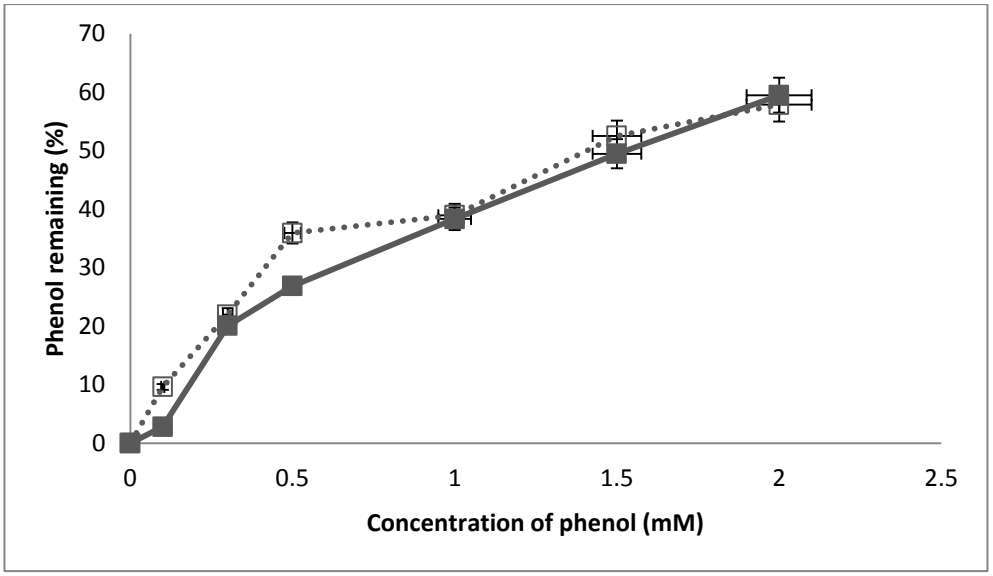

Figure 24. Effect of phenol concentration on the ability of phenol removal by free (⋯) and immobilized enzymes (—). The reaction mixture consisted of phenol (0.1-2.0 mM), free or immobilized enzyme and 4 mM of H_2O_2. After incubation for 15 minutes, the residual phenol was transferred to phenol assay solution. The results shown were average values of duplicate experiments.

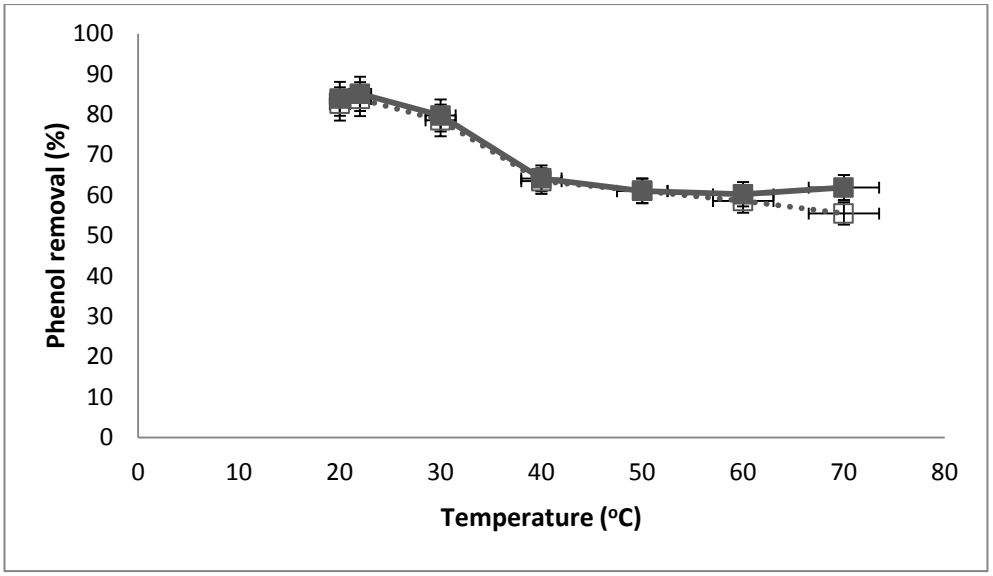

Figure 25. Effect of temperature on phenol removal by free (···) and immobilized enzymes (—). Reaction was preincubated at temperature ranging from 20-70ºC before the residual phenol was determined in phenol assay solution. The results shown were average values of duplicate experiments.

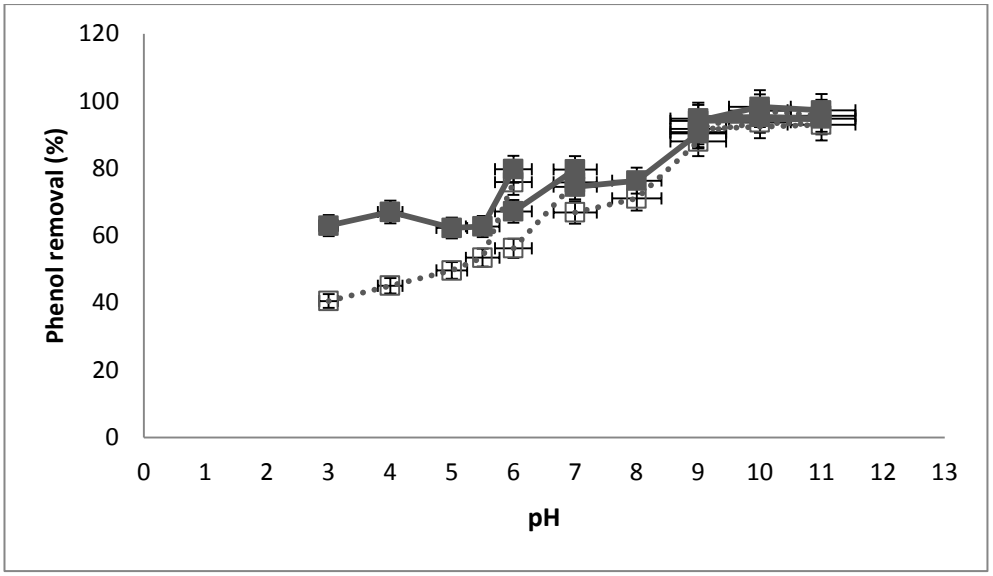

Figure 26. Efeect of pH on phenol removal by free (⋯) and immobilized HRP (—). Reaction was preincubated in buffer solution with pH range of 3.0-11.0**.** The residual phenol was determined in phenol assay solution. The results shown were average values of duplicate experiments.

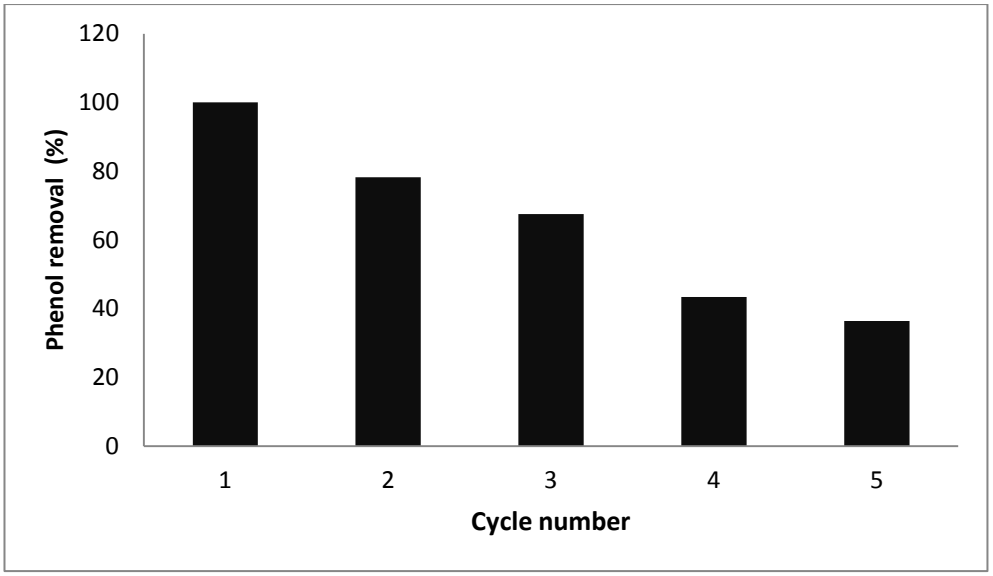

Figure 27. Reusability of the immobilized enzyme for the removal of phenol. The results shown were average values of duplicate experiments.

CHAPTER IV

DISCUSSIONS

Chitosan is a biodegradable organic compound in nature that has been found in a variety of forms. The chitosan structure is composed of reactive amino and hydroxyl groups (Dutta *et al.,* 2004). Chitosan presents advantages in relation to other materials, mainly due to its great versatility, relative low cost and broad availability (Krajewska, 2004) which make chitosan interesting in biological properties such as immunological, antibacterial and pharmaceutical (Bayramoglu *et al.,* 2008). Therefore, chitosan has drawn much attention to be used as a support for HRP immobilization using glutaraldehyde (GA) as a crosslinker (Monier *et al.,* 2010).

The forms of chitosan used in our study were chitosan membranes, spray dried chitosan, 2%, 3% and 5% (w/v) of wet and dry chitosan beads. The morphology of all forms of chitosan was investigated by scanning electron microscopy (SEM). The best support for enzyme immobilization was chosen and various parameters, such as GA and horseradish peroxidase (HRP) concentration, incubation time, pH and temperature which had an effect on the immobilized enzyme activity were investigated. Free enzyme and enzyme immobilized on chitosan support were then used for the removal of phenol to observe the efficiency of both enzymes in phenol removal. Concentration of phenol, pH and temperature were tested to find the optimum conditions for the enzyme in removing phenols. Finally, the reusability which is a very important factor when considering for commercial or industrial applications was also studied.

Results of the surfaces of dry and wet beads comparison between the native chitosan beads, chitosan beads were activated with GA and chitosan beads were immobilized with HRP shown in Figure 8. The typical of dry chitosan beads with relatively smooth surface is microscopically demonstrated in Figure 8A. Also, the activated dry chitosan beads show an observed increase in roughness (Figure 8B) which may be due to the insertion of GA chains onto the dry chitosan beads surface. On the other hand, the roughness of the surface increases greatly after HRP immobilization (Figure 8C) which may confirm the crosslinking of the enzyme on the dry chitosan beads. The wet chitosan beads (Figure 8D), wet chitosan beads was activated with GA (Figure 8E) and wet chitosan beads was immobilized HRP (Figure 8F) shown the similar surface due to the swelling of the chains in the wet chitosan beads, which is caused by the insertion of water molecules. Therefore, the GA and HRP can be inserted into the chains of wet chitosan beads. Of the above reasons for wet chitosan beads, wet chitosan beads activated with GA and wet chitosan beads immobilized with HRP has a similar surface.

4.1 Selecting an appropriate form of chitosan support

Initially, the types of chitosan used were chitosan film, chitosan microspheres and dry chitosan beads. The concentrations of chitosan beads, which were used in this study were 2%, 3% and 5% (w/v). The morphology of all chitosan supports was studied by SEM. It was found that chitosan films and chitosan microspheres were not suitable for enzyme immobilization because the particle of chitosan films was not regular after they were crushed by microcentrifugal mill. The uniformity of particle size of chitosan film could not be controlled.

For chitosan microspheres, it was also found that the particle size was not regular. This could be due to the fact that the sphere formed was incomplete since the density of chitosan was not enough (Ping *et al.,* 1999). In addition, at chitosan 2% (w/v) of chitosan was used to form dry beads. The SEM result showed that the particle formed was not uniform in size and shape.

From the presumption that the density of chitosan was too low, 3% and 5% (w/v) of chitosan was then used to increase the density of chitosan solution. From SEM analysis, at both concentration of chitosan used, the sphere particle was found to be regular and uniform. Their sizes were around 500 μm of dry beads. As 3% and 5% (w/v) of chitosan solution had the same characteristics, chitosan solution at 3% (w/v) was then chosen. Next, dry and wet chitosan beads prepared from 3% (w/v) of chitosan solution was further used as support for HRP immobilization. Optimum conditions were investigated.

4.2 Optimization of the immobilization procedure

The covalent attachment of HRP seems to be the preferred method according to the most published papers. In previous study, Sakuragawa (1998) used GA which reacted with amino group of chitosan to form a Schiff base. Usually, the reaction process between an aldehyde group and amino group is modified by the effect of electrophilicity of the carbonyl group in the aldehyde. The functional group of chitosan, which is amino group, bonds with the carbonyl group of GA (step 1) resulting in a double bond. And then, another carbonyl group of GA bonds with the amino group of enzyme, horseradish peroxidase, resulting in Schiff base formation which is double bond (step 2). An advantage of this method is that strong covalent

bonds are formed that do not lose enzyme into the surrounding solution (Kenedy and Cabral, 1987).

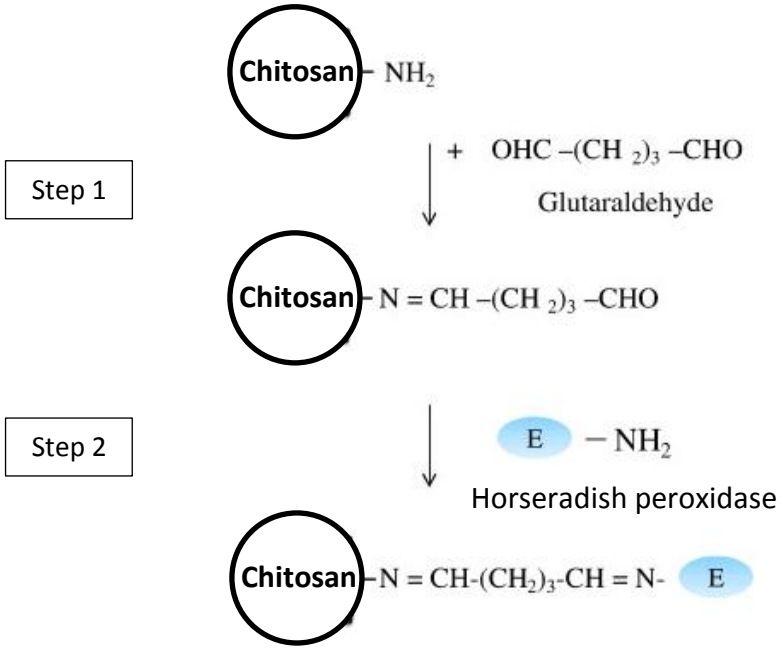

Figure 28. Chitosan activation and covalent coupling to HRP: (1) activation of chitosan carrier; (2) covalent immobilization (Sassolas *et al.*, 2012)

In this study, HRP was immobilized by covalent bond on dry and wet chitosan beads. Initially, same surface area of both types of beads was used. From surface area measurement, it was to use 50 mg of dry beads and 150 mg of wet beads. However, when HRP was immobilized and further assayed for its activity, it was found that the reaction proceeded too fast. The amount of wet chitosan beads was then reduced 5 times. Therefore, the amount of dry and wet chitosan beads in this experiment was 50 and 30 mg, respectively.

GA was used as a crosslinking agent to attach the enzyme molecule onto the surface of dry and wet chitosan beads. The optimum concentration of GA in HRP immobilization on dry and wet chitosan beads were found to be 1.5% (v/v) and 1.0% (v/v), respectively. The GA concentration that has been reported is normally in the range of 0.25-5% (Yang and Su, 1989).

The incubation time of GA is the additional parameter that was determined to efficiently immobilize HRP on dry and wet chitosan beads. It was found that the activities of the immobilized HRP on dry and wet beads gave the highest value of incubation time at 8 hours for dry chitosan beads and 10 hours for wet chitosan beads.

A major influence of the efficiency of HRP immobilization on dry and wet chitosan beads was HRP concentration that exposed to the support surface during immobilization process. The result shows that the increment of enzymatic concentration with the retaining activity of immobilized HRP increased linearly (Figure 11). However, the immobilization yield increased until the amount of enzyme added was 10 μg and after that the yield was constant. This could be due to the fact that the support was already saturated with the enzyme. Thus, the optimum concentration of HRP for immobilization on the support was used to 2.5 μg of HRP. Other investigators (Monier *et al.*, 2010) also explained the low immobilization yield in case of higher amount of HRP addition.

The coupling time is the supplementary parameter that was examined to efficiently immobilize HRP. The retaining activities of immobilized enzyme can be seen in Figure 12. Both results showed the highest immobilization activities at 2 hours of coupling time. This study results matched with previous research (Cheng *et al.*, 2006).

According to the experiments above, an absorbance change was detected by a spectrophotometer. In a non-stop reaction an absorbance at 650 nm was read. Some reactions were stopped and the absorbance at 450 nm was measured.

To confirm that HRP was attached on dry and wet chitosan beads, the protein on the beads were determined by silver nitrate staining method. It was found that the immobilized HRP on dry chitosan beads showed slightly higher protein than a control which was just chitosan beads without HRP. For the immobilization HRP on wet chitosan beads, it was found that they were clearly stained with silver nitrate in comparison with the non-immobilized HRP.

4.3 Properties of the immobilized HRP on dry and wet chitosan beads.

4.3.1 Effect of pH on activity and stability of free and immobilized HRP on dry and wet chitosan beads

The optimum pH of the enzyme after immobilization can be changed or remained the same as the free enzyme (Chibata, 1978). In this research, after immobilization, its optimum pH was found to be the same as that of the free enzyme. The result in Figure 16 shows the optimum pH of free and immobilized enzyme on dry and wet chitosan beads which was at 5.5. This effect largely results from the buffering properties of the bound proteins, the matrix, and the Schiff bases formed by reaction with GA, protein and carrier (Bryjak and Kolarz, 1998).

The pH stability of the immobilized HRP on dry and wet chitosan supports were examined and compared with the free enzyme. The result showed that the free and immobilized HRP on dry chitosan beads were stable over pH values of 5.0-7.0

while the free and immobilized HRP on wet chitosan beads were stable over the pH range of 5.0-11.0 (Figure 17).

4.3.2 Effect of temperature on activity and stability of free and immobilized HRP on dry and wet chitosan beads

The activity of free and immobilized HRP on dry and wet chitosan beads were determined at various temperatures. At temperature higher than 40 °C, the activity of free and immobilized enzyme on dry and wet chitosan beads decreased slowly until all activities were lost after 60 °C (Figure 14). This could probably be explained that the activity was lost at temperature above a certain limit due to the predominance of thermal denaturation of the enzyme (Chibata 1978; Parrado *et al.,* 1995).

One of the most important factors was the effect of temperature on stability. In Figure 15, the activity of free and immobilized HRP on dry and wet chitosan beads decreased when temperature was increased. The highest retained activity of all conditions of enzyme was at room temperature. Incubation at 70 °C resulted in a complete lost of enzymatic activity in all conditions of enzyme. In this study, the thermal stability was investigated at pH 7.0 according to others research (Sakuragawa *et al.,* 1998; Gomez *et al.,* 2006; Bayramoglu and Arica., 2007). Because at pH 5.5, the stability was found to be lower.

4.3.3 Performance comparison between immobilized HRP on dry and wet beads

To efficiently immobilize HRP on both wet and dry chitosan beads, two experiments were performed. In the first experiment, after the chitosan beads were activated with 1.5% (v/v) of GA for 8 hours. The activated chitosan beads were than incubated for 2 hours with HRP solution. The reactions were done with and without agitation. The result in Figure 20 showed that the immobilization yield of wet beads under rotated and non-rotated condition gave higher value than that of the dry beads. The highest immobilization yield was found on wet chitosan beads when the immobilization process was done under agitation.

In another set of experiments, GA at 1.5% (v/v) and HRP solution was mixed before adding to the activated supports. The reactions were also performed with and without agitation. As shown in Figure 21, The highest immobilization efficiency occurred on wet beads under rotated condition at 4 hours of incubation time.

From the results obtained, the immobilized HRP on wet beads under rotated condition gave the highest immobilization efficiency. This could be due to the fact that the wet chitosan beads contained high water in their structure, which made chitosan more flexible than dry chitosan beads. Dry chitosan beads were packed and thus, their efficiency of reaction with GA and HRP was less than that of the wet chitosan beads.

Wet chitosan beads were further investigated under rotated and non-rotated conditions. Two set of experiments were carried out in the first set of experiment, GA concentration of 1.5% (v/v) was incubated with the support for 8 hours before HRP solution was added. Another set of experiment was performed where of GA solution

was mixed with HRP solution, prior to the addition to the supports. An incubation time of HRP with the support was performed over the range of 2-12 hours. The result in Figure 22 showed the best and appropriate conditions for immobilization of HRP on wet chitosan beads which were to incubate GA 1.5% (v/v) for 8 hours before HRP solution was added and incubated with agitation.

4.3.4 The storage stability of free and immobilized enzymes on wet beads

The storage stability is a significant factor to be considered for the applications of immobilized enzyme. The storage stability of immobilized HRP was studied and compared with the free enzyme at room temperature and at 4 °C. The result in Figure 23 showed that the free enzyme completely lost its activity after 30 days of storage at both temperature while the immobilized activity of HRP retained 7% and 19% at room temperature and 4 °C respectively. In previous study, Pueeter and Becker (1982) reported that HRP lost about 10% of its enzymatic activity daily at room temperature or at 4 °C. However, this indicated that the immobilization of HRP on wet chitosan beads increased the stability of the free enzyme.

4.4 Analysis for the optimum conditions of free and immobilized enzymes for phenol removal

The effect of phenol concentration was determined and the results are shown in Figure 24. For the phenol tested, the lowest phenol reduction efficiency was observed at the highest concentration tested (2.0 mM), the reduction efficiency was as high as 60%. Higher reduction efficiency at higher phenol concentration indicates

enzyme inhibition by the substrate or the enzyme is saturated with phenol. Therefore, the amount of enzyme should be increased proportionally to the phenol concentrations (Kennedy *et al.,* 2002). However, in this work we chose the phenol concentration at 0.5 mM for further study.

Another factor that needs to be optimized for highest phenol removal is temperature. The phenol degradation was studied with free and immobilized HRP at a temperature range of 20-70 °C and the results were shown as percentage of the phenol removal rate (Figure 25). Phenol removal efficiency of free and immobilized enzyme was found to be maximum at room temperature. The decrease in degradation rates could be due to the loss of immobilized enzyme activity at high temperature (Bayramoglu and Arica., 2008).

The effect of pH on the efficiency of immobilized HRP for phenol removal was also determined. The results were collected in Figure 26. The pH in the reaction was varied from 3 to 11. The results showed that the optimum pH for phenol removal by free and immobilized enzymes was observed at pH 10. In some cases, the optimum pH for degradation of phenolic compounds using both enzymes was observed at around pH 7 (Bayramoglu and Arica., 2007).

4.5 The reusability of the immobilized enzyme

One of the most important factors for evaluating the possibility of a practical application of the immobilized enzyme is its reusability. An advantage of immobilized enzyme is that it could be easily separated from reaction solution and reuse. The phenol removal efficiency of immobilized HRP retained 36% of its

original activity after 5 repeated use (Figure 27). This could be explained by the fact that the polymer produced during the enzymatic reaction may cover the immobilized enzyme and entrapped the active sites of enzyme. The decrease in the immobilized enzyme activity after each cycle was also observed by other researchers (Cheng *et al.,* 2006).

Immobilization of HRP for phenol removal can be an alternative mean for the removal of phenol in the wastewater treatment industry. Although the efficiency of the immobilized HRP on chitosan beads was similar to that of the free enzyme, the immobilized HRP can be reused.

CHAPTER V

CONCLUSIONS

1. Various types of supports including chitosan film, spray dried chitosan, 2%, 3% and 5% (w/v) dry chitosan beads and 3% (w/v) wet chitosan beads were investigated.
2. The appropriate chitosan supports were 3% (w/v) of dry and wet chitosan beads.
3. The covalent coupling time of dry chitosan beads with 1.5% (v/v) glutaraldehyde was 8 hours and 10 hours for activation of wet chitosan beads with 1% (v/v) glutaraldehyde. The amount of enzyme added on dry and wet chitosan beads was 4 ml of 2.5 μg/ml with coupling time of 2 hours.
4. The biochemical properties of the immobilized HRP were characterized.

    4.1 Optimum pH of HRP immobilized on dry and wet chitosan beads was 5.5 in 0.1 M citrate buffer at room temperature.

    4.2 The pH stability of HRP immobilized on dry chitosan beads was at pH range of 5.0-7.0 and was pH 5.0-10.0 for wet chitosan beads.

    4.3 The thermal stability of HRP immobilized on dry chitosan beads was 20-40°C and on wet chitosan beads was 20-50°C.

    4.4 The immobilized HRP when stored at 4 and 25°C presented higher stability than that of the soluble enzyme. After 30 days of storage, the immobilized enzyme stored at 4°C retained 19% of its original activity while the immobilized enzyme stored at 25°C retained only 7%.

5. The optimum condition for phenol removal by immobilized HRP on wet chitosan beads was highest at room temperature and at pH 10.0 when sodium bicarbonate buffer was used.
6. The reusability of the immobilized enzyme for the removal of phenol after 5 repeated use retained 36% of its original activity.

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APPENDICES

APPENDIX A: Preparation for assay enzyme solution

3,3’5,5’-tetramethylbenzidine (TMB)	1.0 mg
Dimethylsulfoxide (DMSO)	100 µl
0.1 M citrate buffer	10 ml
Incubated for 10 minutes	
Urea hydrogen	1.0 mg

APPENDIX B: Preparation for silver stain solution

Silver stain solution

Reagent A:	$AgNO_3$	0.8 g
	H_2O	2 ml
Reagent B:	30% NaOH	125 µl
	30% NH_4OH	700 µl
	H_2O	10.5 ml

Add reagent A to reagent B until the equivalence point titration and adjust volume to 50 ml with distilled water.

Developing solution

1% $C_6H_8O_7$	1 ml
3% formaldehyde	100 µl
H_2O	200 µl

APPENDIX C: Preparation for buffer solution

0.1 M Sodium citrate buffer pH 3.0

$C_6H_8O_7$	1.723 g
$Na_3C_6H_5O_7$	0.529 g

Adjusted volume to 100 ml with distilled water.

0.1 M Sodium citrate buffer pH 4.0

$C_6H_8O_7$	1.240 g
$Na_3C_6H_5O_7$	1.206 g

Adjusted volume to 100 ml with distilled water.

0.1 M Sodium citrate buffer pH 5.0

$C_6H_8O_7$	0.735 g
$Na_3C_6H_5O_7$	1.911 g

Adjusted volume to 100 ml with distilled water.

0.1 M Sodium citrate buffer pH 5.5

$C_6H_8O_7$	0.483 g
$Na_3C_6H_5O_7$	2.265 g

Adjusted volume to 100 ml with distilled water.

0.1 M Sodium citrate buffer pH 6.0

$C_6H_8O_7$	0.242 g
$Na_3C_6H_5O_7$	2.603 g

Adjusted volume to 100 ml with distilled water.

0.1M Potassium phosphate buffer pH 6.0

KH_2PO_4	1.280 g
K_2HPO_4	0.103 g

Adjusted volume to 100 ml with distilled water.

0.1 M Potassium phosphate buffer pH 7.0

KH_2PO_4 0.834 g

K_2HPO_4 0.676 g

Adjusted volume to 100 ml with distilled water.

0.1 M Tris-HCl pH 7.0–9.0

Tris (hydroxymethy)-aminomethane 1.211 g

Adjusted to pH 7.0-9.0 with 1 M HCl and adjusted volume to 100 ml with distilled water.

0.1 M Glycine-NaOH pH 9.0–11.0

Glycine 0.751 g

Adjusted to pH 9.0-11.0 with 1 M NaOH and adjusted volume to 100 ml with distilled water.

0.1 M Sodium bicarbonate buffer pH 9.0–11.0

$NaHCO_3$ 0.848 g

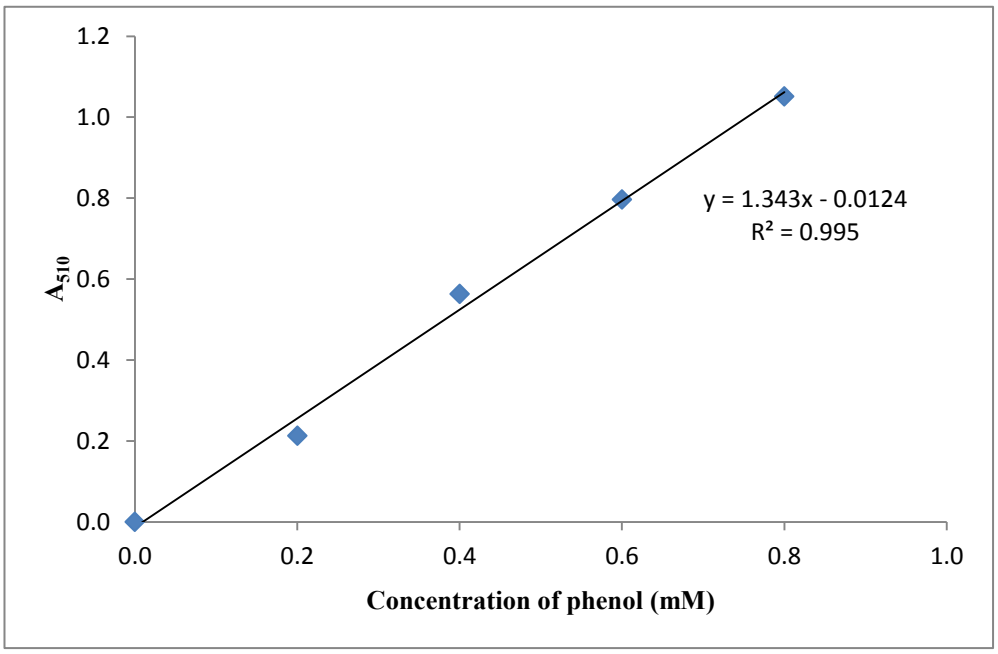
Adjusted to pH 9.0-11.0 with 1 M NaOH and adjusted volume to 100 ml with distilled water.

0.5 M Sodium bicarbonate buffer pH 10.0

$NaHCO_3$ 4.200 g

Adjusted to pH 10.0 with 1 M NaOH and adjusted volume to 100 ml with distilled water.

APPENDIX D: Standard calibration curve of phenol



VITA

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