IMMOBILIZATION OF LACCASE ENZYME ON MAGNETICALLY-SEPARABLE HIERARCHICALLY-ORDERED MESOCELLULAR MESOPOROUS SILICA

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Philosophy

School of Chemical and Energy Engineering
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FEBRUARY 2022

DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

ACKNOWLEDGEMENT

In preparing this thesis, I was in contact with many people, researchers, academicians, and practitioners. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation to my main thesis supervisor, Associate Professor Ir. Ts. Dr. Roshanida A. Rahman for encouragement, guidance, critics and friendship. I am also very thankful to my co-supervisor Puan Noorhalieza Ali for her guidance, advices and motivation. Without their continued support and interest, this thesis would not have been the same as presented here.

I am also indebted to my parents who has supported me financially and also emotionally by not giving up upon her daughter although need to extend 10 semesters long. Laboratory technicians En Yaakop, En Hafzan and Chemad also deserve special thanks for their assistance in supplying the abundance help in solving technicalities and equipments supports.

My fellow postgraduate friends should also be recognized for their support. Thank you from my heart to Kak Jannah, Kak Ros, Kak Ayu, Syu, Aiman, Syahlan, Amir and N18 clans. My sincere appreciation also extends to my teammates Khosyi'in, Geng 12 and all beloved akhawats. Their views, tips and prayers has been accompanying me through my thick and thin journey. The most appreciation post also I bid to Dr Roket, team 2M World, and my amazing platinum members for their continuous momentum and spirit in helping the postgraduate students completing master PhD journey together. Unfortunately, it is not possible to list all of them in this limited space. May Allah grant all of you the highest level of heaven, Jannatul Firdaus.

ABSTRACT

Multicopper oxidases, known as laccase, are a sustainable biocatalyst with efficient ability to degrade a wide range of compounds, environmentally friendly properties and promise major advances in a wide range of industries. However, the use of free laccase in industries often suffers problems, such as instability, low recovery and low reusability of enzyme. Hence, laccase immobilization on a magnetically-separable hierarchically-ordered mesocellular mesoporous silica (M-HMMS) as the support material was optimized and characterized. In this study, three different immobilization methods used were enzyme adsorption, entrapped-crosslinked enzyme and entrapped-crosslinked enzyme aggregate. The optimum parameters for laccase immobilization were at 5 hr of crosslinking time, 100 mM glutaraldehyde concentration, 1 mg/ml laccase concentration, 60 min time of precipitation with pH 4.5 and temperature of 20 ℃. This optimal condition contributed to 65.03 ± 4.31 % of laccase activity recovery and enhancement by 2.6 fold. The adsorption of laccase on M-HMMS obeyed the pseudo-second-order kinetic model. The optimized immobilized laccase was able to withstand high temperature (50 ℃) and also oxidize 2, 2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid (ABTS) at a broad range of pH (pH 3.0 to pH 6.0) and temperature (20 to 70 ℃). It also retained 63.72 ± 6.59 % of its initial activity after 8 repeated cycles of ABTS oxidation and 100 % of its activity after 30 days of storage at 4 ℃ in pH 4.5 buffer. In conclusion, the optimized immobilized laccase has potential as immobilized biocatalyst for the application of bioremediation and biotransformation of contaminant molecules in water.

ABSTRAK

Oksidase pelbagai tembaga yang dikenali sebagai lakase ialah biomangkin mampan dengan keupayaan cekap untuk menguraikan pelbagai jenis sebatian, mesra alam dan menjanjikan kemajuan yang besar dalam pelbagai industri. Walau bagaimanapun, penggunaan enzim bebas di dalam industri sering menghadapi beberapa permasalahan seperti ketidakstabilan enzim, kadar penghasilan yang rendah dan kesukaran perolehan semula enzim. Oleh itu, imobilisasi lakase ke atas silika mesoporous mesoselular yang tersusun secara hierarki (M-HMMS) yang boleh dipisahkan secara magnetik sebagai bahan sokongan telah dioptimumkan dan dicirikan. Dalam kajian ini, tiga kaedah imobilisasi yang berbeza digunakan iaitu penjerapan enzim, diperangkap dan pemautsilangan enzim, dan diperangkap, pemautsilangan dan gumpalan enzim. Parameter optima bagi imobilisasi lakase adalah pada 5 jam masa pemautsilangan, 100 mM kepekatan glutaraldehida, 1 mg / ml kepekatan lakase, 60 min masa pemendakan dengan pH 4.5 pada suhu 20 ℃. Keadaan optima ini menyebabkan 65.03 ± 4.31 % kadar penghasilan lakase dan peningkatan sebanyak 2.6 kali ganda. Penjerapan lakase pada M-HMMS mematuhi model pseudo tertib kedua. Lakase yang telah melalui proses imobilisasi yang dioptimakan mampu bertahan pada (50 ℃) dan juga mengoksidakan 2, 2-azino-bis 3-etilbenzotiazolina-6-asidsulfonik (ABTS) pada pelbagai pH (pH 3.0 hingga pH 6.0) dan suhu (20 hingga 70 ℃). Di samping itu, proses imobilisasi enzim secara optima mampu mengekalkan 63.72 ± 6.59 % daripada aktiviti awal lakase setelah 8 kitaran pengoksidaan ABTS secara berulang dan 100 % aktiviti setelah disimpan selama 30 hari pada suhu 4 ℃ dalam larutan penimbal pH 4.5. Kesimpulannya, lakase yang diimobilisasi dan dioptimakan ini berpotensi sebagai biomangkin untuk digunakan dalam bidang bioremediasi dan biotransformasi molekul air yang tercemar.

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LIST OF ABBREVIATIONS

ABTS	-	2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid
M-HMMS	-	Magnetically-separable Hierarchically-ordered Mesocellular Mesoporous Silica
EA	-	Enzyme Adsorption
E-CLE	-	Entrapped-crosslinked enzyme
E-CLEA	-	Entrapped-crosslinked enzyme aggregate
HMMS	-	Hierarchically-ordered Mesocellular Mesoporous
UTM	-	Universiti Teknologi Malaysia
GA	-	Glutaraldehyde
$(NH_4)_2SO_4$	-	Ammonium sulphate
OFAT	-	One factor at time
FTIR	-	Fourier transform infrared
SEM	-	Scanning electron microscope
VSM	-	Vibrating Sample Magnetometer

LIST OF SYMBOLS

hr	-	Hour
rpm	-	Rotation per minute
mg	-	Miligram
v	-	Velocity
ml	-	Mililitre
K_m	-	Michaelis-menten constat
min	-	Minute
°C	-	Degree celcius
U	-	Unit of enzyme activity
v/v	-	Volume per volume
V_{max}	-	Maximum velocity
q_e	-	Amount of enzyme adsorbed
q_t	-	Amount of enzyme adsorbed at time t
k_1	-	Pseudo-first-order rate constant
k_2	-	Pseudo-second-orer rate constant
U/g	-	Unit activity per gram minute
C_i	-	Initial concentration of enzymes
C_t	-	Final concentration of enzymes
U/ml	-	Unit activity per mililitre
mM	-	Milimolar

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are a promising tool as biocatalysts for treating numerous environmental pollutants from industrial sectors such as textile, pulp and paper, food, cosmetics, pharmaceutical, tanning and plastics industries (Ramírez-Montoya et al., 2015; Bilal et al., 2017). Unlike current treatment technologies, utilization of enzymes opens a new horizon to treat wastewater streams containing recalcitrant organic pollutants (Arica et al., 2009; Barrios-Estrada et al., 2018). This is because enzymes are easy to handle and have lower environmental and physiological toxicity (Choi et al., 2015; Madhavan et al., 2017; Torres et al., 2017).

Although enzymes are known to be environmentally friendly catalysts, they are often not ideally suited for industrial use (Krajewska, 2004). Several drawbacks to the use of soluble and native enzymes including high cost, poor operational stability, sensitivity to harsh environmental conditions and poor recovery (Bilal et al., 2018). In addition, native enzymes are not reusable after the first run and have limited shelf life (Silva et al., 2013; Ali et al., 2018).

Enzyme immobilization technology provides a practical and remarkable approach to avoid instability problems and obtain industrially desirable biocatalysts (Bilal et al., 2017). Enzyme immobilization can be defined as the binding of soluble enzymes to a carrier resulting in a reduction or complete loss of mobility of the bound enzyme using various methods such as physical methods (entrapment, adsorption, encapsulation) and chemical methods (covalent bonding and crosslinking) (Chagas et al., 2015; Meryam Sardar, 2015). Immobilization of the enzyme improved the properties, but this is strictly case by case basis. The improved enzyme properties include stability to various denaturation conditions, pH tolerance, functional stability,

easier separation of the enzyme and subsequent recovery, reusability, and increased catalytic performance (Sadighi and Faramarzi, 2013). Mechanical rigidification of enzymes through immobilization also contributes to enzyme stabilization and prevents dissociation related inactivation (Meryam Sardar, 2015).

The enzyme laccase, known as a multicopper oxidase, holds excellent advancements in recent years as a sustainable and green biocatalyst for biotechnological and environmental applications in industries (Barrios-Estrada et al., 2018). Laccase has the ability to degrade a wide range of compounds, including phenolic and non-phenolic compounds (Mogharabi-Manzari et al., 2019). Due to the exceptional properties such as catalytic efficiency, low toxicity, biodegradability, high specificity and mild reaction conditions, enzyme immobilization has been extensively studied (Bilal et al., 2019c).

However, certain properties of free enzymes, including their sensitivity to denaturants and non-reusability, low operational stability, and high production costs, make laccases undesirable for large-scale applications (Zhu et al., 2007). One way to overcome these limiting factors is to immobilize laccases on supports that improve the stability of the enzymes to extreme conditions and chemical agents, protect them from denaturation, maintain good catalytic efficiency, and facilitate their use in continuous processes, leading to more economical processes (Kashefi et al., 2019a).

The potential uses of laccases have been extensively studied in recent years, and other reports on these enzymes dealing with applications in the food industry (Bezerra et al., 2015) and wastewater treatment (Mate and Alcalde, 2017; Alshabib and Onaizi, 2019a). Due to their ability to reduce oxygen to water in the presence of phenolic compounds, immobilized laccases-based biosensors have been used to determine phenolic chemical compounds in food (Bagci, 2014). In addition, the most commonly used strategies for immobilization are those in which laccases are physically adsorbed and immobilized on various supports by crosslinking (Jesionowski et al., 2014). Enzyme immobilization by adsorption, unlike covalent binding, is generally ineffective in binding the enzyme to the support, especially under

industrial conditions. Therefore, covalent binding has gained popularity as it makes the enzyme more robust and attractive for industrial use (Bommarius and Paye, 2013).

In the past, laccase has been immobilized by adsorption and covalent binding using mesostructured silicon-containing cellular foams (Zhao et al., 2011; Bryjak et al., 2012; Zdarta et al., 2020a). Immobilization of Myceliophthora thermophilic laccase by covalent binding to epoxy-functionalized silica for decolorization and degradation of textile dyes was described by Salami et al., (2018). They discovered high enzyme binding (50 mg/g), high catalytic efficiency, and excellent reusability (61 percent of original activity after 8 cycles), as well as the ability to remove five different textile dyes. In addition, advances in the use of laccase in environmental applications have enabled more advanced technologies that include not only carrier-bound and carrier-free immobilization of laccase, but also the combination of the advantages of carrier-bound and carrier-free immobilized enzymes (Ba and Vinoth Kumar, 2017; Fathali et al., 2019).

Enzyme immobilization via physical and chemical approaches explains the combined strategy. Mesoporous silica materials are suitable candidates for the preparation of entrapped cross-linked enzyme aggregates (E-CLEA) according to Fathali et al., (2019). A simple fabrication procedure of amino-functionalized magnetic nanoparticles (MNPs) for *Trametes versicolor* cross-linked enzyme aggregates (CLEA) to immobilize laccase was also reported in another study (Kumar et al., 2014b). In this study, laccase was immobilized on magnetically separable hierarchically ordered mesocellular mesoporous silica (M-HMMS). This approach further developed the combination of immobilization methods on magnetic mesocellular mesoporous silica to increase enzyme stability and catalytic activity for the application in the bioremediation and biotransformation of contaminant molecules in water.

1.2 Problem Statement

The ability of laccase as a biocatalyst to oxidize phenolic and non-phenolic compounds has found wide application in the food, bioremediation, biofuel, and other industries (Bilal et al., 2017). However, problematic separation, low stability, and high processing cost limit its practical application (Bilal et al., 2019a). Therefore, immobilization of laccase is one of the advantageous approaches that enhanced laccase performance from the above limitations (Deska and Kończak, 2019a).

The combined method of enzyme immobilization has been explored as it represents a biocatalyst with an attractive proposition for industrial applications (Sheldon, 2011; Kumar et al., 2014b; Fathali et al., 2019). Physical methods such as entrapment, adsorption and encapsulation of laccase are inexpensive and straightforward. However, those methods cause more enzyme losses (Górecka and Jastrzębska, 2011). Chemical methods such as covalent bonding and cross-linking strengthen the bond between enzyme and carrier, but require a large amount of enzyme and altered the active site of the enzyme (Secundo, 2013). However, the advantages of both methods can be used to improve the catalytic performance of laccase on M-HMMS.

Nevertheless, data on laccase immobilization on mesoporous supports are limited and efficient protocols for laccase immobilization are still needed (Zdarta et al., 2020a). Lee et al., (2009, 2010) and Jannah Sulaiman et al., (2019) published some studies on the immobilization of laccase using M-HMMS as a carrier. However, these studies used different enzymes such as cellulase, xylanase, α-chymotrypsin, and glucose oxidase. Therefore, M-HMMS was investigated as potential carrier for the immobilization of laccase to determine the performance of the biocatalyst. It was found that nanosized silica is suitable carrier because it has excellent properties such as large surface area, high chemical purity, good stability, good dispersion and easy modification (Yang et al., 2014).

In addition, the operating conditions during the immobilization process are another important factor affecting the stability and catalytic activity of the enzyme. In

order to provide optimal conditions for the immobilized laccase, the bonds that are bound to the support must be altered in the environment (Sulaiman, 2020; Mohd Syukri, 2021). Therefore, optimizing the operating conditions in the production of immobilized laccase is crucial because of the environmental changes during the immobilization process and affect the activity (Wang et al., 2018).

Moreover, since the first step in the immobilization of laccase is adsorption, it is vital to determine the adsorption efficiency and identify the adsorption mechanism (Gilani et al., 2016). To date, adsorption studies of laccase enzymes still lack, especially on magnetic supports. It is crucial to know the adsorption mechanism as it can help determine the durability and cause of the enzyme behaviour after immobilization and improve the immobilization of the enzyme.

1.3 Research Objectives

The objectives of the research are:

(a) To determine the immobilization method for laccase on M-HMMS.

(b) To optimize the operating condition affecting the immobilization of laccase on M-HMMS.

(c) To determine laccase adsorption kinetics and mechanism on M-HMMS.

(d) To evaluate the physical properties and catalytic performance of immobilized laccase on M-HMMS.

1.4 Scope of Study

(a) Immobilization methods for laccase on M-HMMS were determined, which are enzyme adsorption (EA), entrapped cross-linked enzyme (E-CLE) and entrapped cross-linked enzyme aggregate (E-CLEA) methods.

(b) Optimization of operating conditions, i.e., cross-linking time (1 - 6 hr), glutaraldehyde concentration (4 - 500 mM), enzyme concentration (1 - 9 mg/mL), precipitation time (30 - 150 min), pH (4 - 6), and temperature (4 - 25 °C), was performed using one factor at a time (OFAT) method. Statistical analysis was performed using One-Way ANOVA from software IBM SPSS Statistics Version 26.

(c) An adsorption kinetics study involving pseudo-first-order, pseudo-second-order and intraparticle diffusion was also performed to determine the interaction between laccase and the carrier. The effects of contact time (0 - 105 min), laccase concentration (0.1, 0.5, 1.0, 1.5 mg/ml) and adsorption temperature (15, 20, 25, 30 °C) on the adsorption of laccase on M-HMMS were determined.

(d) The characterization of immobilized laccase on M-HMMS was carried out and and compared with free laccase. The analysis on functional groups, morphology and magnetization value before and after immobilization were evaluated. The immobilized laccase was characterized in terms of physical properties, optimum pH (3 - 8), optimum temperature (20 -70 °C), pH stability (3 - 6), thermal stability (30 – 70 °C), storage stability (30 days), reusability (8 cycles) and leaching. In addition, the kinetics coefficients (K_m and V_{max}) of the immobilized laccase were determined by measuring the laccase activity in different ABTS concentrations.

1.5 Significant of Study

The immobilization of laccase on M-HMMS create a new approach that combined physical and chemical method with magnetic properties. This study gives information on the adsorption mechanism between laccase enzyme and M-HMMS as a carrier. The optimum laccase operating conditions were obtained from one-factor-at-a-time (OFAT) method. Furthermore, it can work well in broader range of pH and temperature with improved reusability and stability. The immobilized laccase would benefit the industrial sector as it introduced an easy way to separate biocatalyst after

the process of degrading dyes, pharmaceutical contaminants and also in pulp and paper industries.

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LIST OF PUBLICATIONS

1. **Norsyafiqah Amalina Ahmad Jafri**, Roshanida A. Rahman, Noorhalieza Ali. (2021). A Short Review on Recent Development of Laccase Immobilization on Different Support Materials. *Journal of Sustainable and Natural Resources*. Vol. 2, 54-63. https://doi.org/10.30880/jsunr.2021.02.01.008

2. **Norsyafiqah Amalina Ahmad Jafri**, Roshanida A. Rahman, Noorhalieza Ali. (2020). Immobilization of laccase enzyme on Magnetically-Separable Hierarchically-Ordered Mesocellular Mesoporous Silica. *3rd ICA Research Symposium (ICARS) 2020 15 September 2020*. (pp. 29 – 34). https://pagoh.utm.my/icasymposium/files/2020/11/3rd-ICARS-2020-eproceeding-eISBN-978-967-17781-1-1-1.pdf