LACCASE IMMOBILIZATION ON POLYETHYLENE TEREPHTHALATE BASED NANOFIBER MAT

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DEDICATION

This thesis is dedicated to my whole family, the MClan, especially my parents, who always believed in myself and support every decisions that I made for myself. Thank you for everything, I always love you <3.

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ABSTRACT

Enzymes as biocatalysts in industries are more advantageous compared to chemical catalysts because of their low operating conditions, less complexity, and environmentally-friendly property. Laccase, an oxidoreductase enzyme, is foreseen to be used in various industries as a biocatalyst. However, the use of free laccase in industries often suffers some limitations, such as high production cost, instability issues, low recovery, and reusability problems. These problems can be overcome by enzyme immobilization technology, which offers enhancements to free enzyme's stability. The advent of nanotechnology has introduced electrospun nanofiber mat as a carrier for enzyme immobilization. The mat is foreseen to be implemented in the enzymatic membrane bioreactors of continuous flow processes. In this study, polyethylene terephthalate (PET) was grafted with maleic anhydride (MAH) (PET-g-MAH) and spun through electrospinning into nanofiber mats with an average fiber diameter of 844 ± 149 nm before it was used as the carrier of immobilized laccase. Three different immobilization methods were used: physical adsorption on PET nanofiber mats, covalent bonding, and covalent bonding of cross-linked laccase aggregate with glutaraldehyde as the cross-linker. The combination of 0.28 mg/ml laccase concentration, pH 3 citrate buffer, 0.45 % (v/v) glutaraldehyde concentration, 1.5 hr of covalent bonding time at 22.7 ^{o}C, and 1 hr of cross-linking time at 20 ^{o}C contributed to the optimum immobilization yield (87.64 %). The adsorption of laccase on PET-g-MAH nanofiber mats obeyed the pseudo-first-order, and the biosorption isotherms correlated well with the Freundlich isotherm model. The optimized immobilized laccase was able to withstand high temperature (60 ^{o}C) and also oxidized 2, 2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid (ABTS) at a broad range of pH (pH 3 to pH 6) and temperature (20 ^{o}C to 70 ^{o}C). It also managed to retain 77.55 % of its initial activity after 10 repeated cycles of ABTS oxidation and 29.22 % after 30 days storage at 4 ^{o}C in pH 3 buffer. In conclusion, the results showed that the laccase immobilized on the PET-g-MAH nanofiber mat might have great potential to be used in industries.

ABSTRAK

Enzim sebagai biomangkin mempunyai kelebihan berbanding mangkin kimia untuk digunakan dalam industri kerana keadaan pengoperasiannya yang rendah, mudah dan bersifat mesra alam. Lakase, sejenis enzim oxidoreduktase, adalah berpotensi digunakan dalam pelbagai industri sebagai biomangkin. Walau bagaimanapun, terdapat beberapa kekangan yang dihadapi dalam penggunaan enzim tersebut, antaranya adalah kos penghasilan enzim yang tinggi, risiko ketidakstabilan enzim, dan masalah kebolehgunaan semula enzim yang rendah. Kekangan yang dihadapi dalam pengunaan enzim tersebut boleh diatasi dengan teknologi imobilisasi enzim iaitu satu kaedah yang boleh meningkatkan kestabilan enzim. Kemajuan teknologi nano, telah memperkenalkan tikar gentian nano elektroputar sebagai bahan kepada imobilisasi enzim. Tikar gentian nano berpotensi untuk dilaksanakan dalam bioreaktor membran berenzim proses aliran berterusan. Dalam kajian ini, polietilena tereftalat (PET) dicantumkan dengan maleat anhidrida (MAH) (PET-g-MAH) melalui proses putaran elektro menjadi tikar gentian nano dengan diameter 844 ± 149 nm sebelum digunakan sebagai bahan sokongan untuk lakase imobilisasi. Terdapat tiga kaedah imobilisasi yang digunakan: penjerapan fizikal pada tikar gentian nano PET, ikatan kovalen dan ikatan kovalen paut silang agregat lakase dengan menggunakan glutaraldehida sebagai pemaut silang. Hasil gabungan lakase pada kepekatan 0.28 mg/ml, larutan penimbal pada pH 3, kepekatan glutaraldehida 0.45 % (v/v), 1.5 jam masa ikatan kovalen pada suhu 22.7 ^{o}C dan 1 jam masa penyambungan silang pada suhu 20 ^{o}C menyumbang kepada hasil imobilisasi yang optimum (87.64 %). Penjerapan lakase pada tikar gentian nano PET-g-MAH mematuhi model tertib pertama pseudo dan isoterm bioerapan mempuyai korelasi yang baik dengan model isoterm Freundlich. Lakase yang telah melalui proses imobilisasi pada keadaan optimum dapat menahan suhu yang tinggi (60 ^{o}C) dan juga mengoksidasikan 2, 2-azino-bis 3-etilbenzotiazolina-6-asidsulfonik (ABTS) pada julat pH (pH 3 hingga pH 6) dan suhu (20 ^{o}C hingga 70 ^{o}C) yang besar. Di samping itu, proses imoblisasi enzim mampu mengekalkan aktiviti enzim sekitar 77.55 % setelah 10 kitaran pengoksidaan ABTS secara berulang dan 29.22 % setelah disimpan selama 30 hari pada suhu 4 ^{o}C dalam larutan penimbal pH 3. Kesimpulannya, lakase yang diimobilisasikan pada tikar gentian nano PET-g-MAH mempunyai potensi yang besar untuk digunakan dalam industri.

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LIST OF ABBREVIATIONS

ABTS	-	2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid
BCA	-	Bicinchoninic Acid
CL	-	Covalent bonding of cross-linked laccase aggregates
CV	-	Covalent bonding
DCM	-	Dichloromethane
FTIR	-	Fourier transform infrared
MAH	-	Maleic anhydride
OFAT	-	One factor at time
PA	-	Physical adsorption
PET	-	Polyethylene terephthalate
PET-g-MAH	-	Polyethylene terephthalate grafted with maleic anhydride
RSM	-	Response surface methodology
SEM	-	Scanning electron microscope
TFA	-	Trifluoracetic acid

LIST OF SYMBOLS

nm	-	Nanometer
µg	-	Microgram
cm	-	Centimeter
hr	-	Hour
K_m	-	Michaelis-menten constant
kV	-	Kilo Volt
mg	-	Miligram
min	-	Minuter
ml	-	Mililitre
^{o}	-	Degree
^oC	-	Degree celcius
rpm	-	Rotation per minutes
U	-	Unit of enzyme activity
v/v	-	Volume per volume
V_{max}	-	Maximum velocity
w/v	-	Weight per volumer
ΔG	-	Gibbs free energy
ΔH	-	Enthalpy
ΔS	-	Entropy

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are protein macromolecules produced by living organisms and are essential for the organisms' survival; their function is to speed up chemical reactions and act as natural catalysts inside living cells to ensure organisms' survival. Enzymes are highly selective and specific because they only catalyze specific substrates in a set of chemical reactions. Enzymes consume little energy, act at low temperatures, produce few by-products, are non-toxic and degradable, compared with chemical catalysts (Osbon and Kumar, 2019; Sheldon and van Pelt, 2013). Also, they are more cost-effective, environmentally friendly and sustainable than chemical catalysts. Enzymes implementation in processes has been recognized because of the advantages offered by them.

Despite the advantages, several limitations hinder the implementation of enzymes as biocatalysts. The production cost of enzymes are high, and their purification process is rather complex (Ferreira et al., 2018; Klein-Marcuschamer et al., 2012; Zheng et al., 2017). Enzymes also suffer from low recovery and reusability problems, which are major problems in industries. The separation of enzymes from products after reactions also becomes a bottleneck that hampers the use of enzymes as biocatalysts. Moreover, free enzymes' stability is still limited, which is due to enzyme inactivation and denaturation under harsh reaction conditions. These problems have become major issues for enzyme usage in industries.

However, according to a review by Silva *et al.* (2018), several enzyme stabilization strategies such as the screening and isolation of enzymes from extremophiles, protein engineering, enzyme functionalization, enzyme immobilization, medium engineering, enzyme storage stabilization and enzyme stabilization by silk have been studied to tackle the problems faced by enzymes. Compared with the other strategies, the immobilization technology offers enhancements to enzymes, such as the ability to operate in a broad range of temperature and pH, longer shelf life than free enzymes and high reusability, which is the most important feature for catalysts (Hong et al., 2019; Liang et al., 2020). Immobilization stabilises the enzymes by immobilizing them on or within a solid carrier using various methods such as adsorption, covalent binding, encapsulation, entrapment and cross-linking (Meryam Sardar, 2015; Sheldon and van Pelt, 2013). Since a carrier's function is to help stabilize enzyme structure (Silva et al., 2018), the selection of a carrier is one of the important factors that should be considered in immobilization technology studies.

Recently, laccase, an enzyme from the oxidoreductases class, received attention from several researchers because of its potential to be used as a biocatalyst in industries. Its potential is due to its capability to oxidize phenolic and non-phenolic lignin compounds and several environmental pollutants (Mate and Alcalde, 2015). Laccase has been immobilized using different types of carrier and evaluated in different industrial applications such as dye decolourization (Youxun Liu et al., 2016; Sathishkumar et al., 2014; Vršanská et al., 2018), phenolic compound removal (Dai et al., 2016; Qiu et al., 2019; R. Xu et al., 2017), bioremediation (Maryšková et al., 2016; Zdarta et al., 2020) and delignification (Sánchez-Ramírez et al., 2016).

However, each immobilization method still has problems with its carriers. The physical immobilization methods offer immobilization without conformational changes in the enzyme structure (Asgher *et al.*, 2017a). However, the desorption of enzymes can sometimes not be prevented because of the carriers' weak interaction. Although chemical immobilization can solve the enzyme leaching problem through

stronger covalent bonding (Chao et al., 2018; El-Aassar et al., 2019), the covalent attachment might denature, thus reduce the catalytic activity of the enzyme. As there is no universal immobilization method for every type of carrier, a suitable method should be studied for every new type of carrier.

In the present time, the advent of nanotechnology has opened up a new arena for nanomaterials to be used as a carrier for enzyme immobilization. Nanostructured materials such as nanoparticles, nanofibers, nanotubes and nanoporous materials are considered valuable carrier options because they provide high available surface areas for enzyme immobilization. Since enzyme concentration influences the reaction rate, nanomaterials would be the best choice for the carrier selections.

Among the nanomaterials, nanofibers constructed through electrospinning may be promising for enzyme immobilization because various types of polymer can produce them, and the preparation for the polymer solution is simple, depending on the desired mixture of the polymer. Electrospun nanofiber surfaces can be modified easily to meet the benefits of enzyme activity. The electrospun nanofibers have high porosity and interconnectivity, which eliminate the mass transfer limitation (Tran and Balkus, 2012; Wang et al., 2009). There have been many instances of enzyme immobilization on nanofibers; some of the nanofiber carriers are a modified nanofiber poly(acrylonitrile-co-styrene/pyrrole) nanofiber mat (El-Aassar et al., 2019), a modified polyurethane nanofiber mat (Li et al., 2019; Wu et al., 2018a), nanoparticle-incorporated nanofiber as poly(crylonitrile)/poly(vinylidene fluoride) incorporated with nano-copper (R. Xu et al., 2017), and biopolymer nanofibers such as a polyamide 6/chitosan nanofiber mat (Maryšková et al., 2016) and cellulose nanofiber (Sathishkumar et al., 2014).

Polyethylene terephthalate (PET) is an inexpensive polymer that is most widely used in the synthetic material world. Regardless of the price, the structure of PET as a nanofiber mat has been proven to have good physical and mechanical properties (Veleirinhho et al., 2008). Unfortunately, PET is also a hydrophobic and chemically inert polymer, which limits its potential to be used as a carrier for enzyme immobilization. However, polymer modification helps to improve the characteristics

of PET. Several researchers have successfully immobilized enzymes on PET fibers by modifying the fibers' surfaces. An example of those enzymes is horseradish peroxidase, which has successfully been immobilized on PET fibers grafted with acrylamide (Temoçin and Yiğitoğlu, 2009). Trypsin has also been immobilized, on a mixture of PET and polylactic acid nanofibers, and it was found that there was no enzyme leaching during the activity reaction (Silva et al., 2015).

In this study, laccase was immobilized on a PET nanofiber mat that had been modified with maleic anhydride (MAH) through polymer grafting. This work marks the first time laccase was immobilized on a MAH-grafted PET nanofiber mat (PET-g-MAH). The laccase immobilized on PET-g-MAH nanofiber mat has immense potential of being implemented in industries as a biocatalyst.

1.2 Problem Statement

Laccase has received attention from researchers because of its ability to oxidize phenolic compounds, non-phenolic compounds and environmental pollutants (Fillat et al., 2017). It has been used in various industries such as paper and pulp, textile, food and bioremediation (Mate & Alcalde, 2015). However, laccase use has production cost, instability under operational conditions, and reusability limitations (Daronch et al., 2020). The immobilization technology has been proven to enhance laccase's stability (Chao et al., 2018; Li et al., 2019; Xu et al., 2017), and this technology is the most implemented strategy for enhancing the stability of enzymes as biocatalysts.

Nowadays, various carriers have been discovered to immobilize enzymes, from natural biopolymers to synthetic organic polymers. Choosing the carrier for enzyme immobilization has always been one of the crucial factors because the enzyme's properties may be affected by the structure and property of the carrier (Zhang et al., 2013) after immobilization. The material of the carrier chosen should be biocompatible with the enzyme, non-hazardous, and resistant to harsh conditions. Polyethylene terephthalate (PET) has excellent physical and mechanical properties; however, the hydrophobic and chemically inert nature of PET limits its potential to be used as an

excellent enzyme carrier. Modification of the PET surface is required to prevent these problems. Recently, sPreviously, several researchers have successfully immobilized enzyme on PET based carrier by modifying its surface (Mohamed *et al.*, 2016; Irena *et al.*, 2009; Silva *et al.*, 2015; Caramori and Fernandes, 2008). Even though the enzyme was immobilized on the carrier, the modification process of PET surface was still complex and some treatments also bring negative impact to the property of PET (Irena et al., 2009).

Another important factor to improve the immobilized enzyme is the immobilization preparation parameters because the enzyme's stability and catalytic activity should be retained throughout the immobilization process. Since enzymes are sensitive to environmental changes, unsuitable immobilization conditions may cause them to denature during the process and decrease immobilization yield. Optimization of immobilization preparation parameters should be considered in synthesizing immobilized enzymes as some previous studies reported low immobilization yield even though the enzyme was successfully immobilized on the carrier (Maryšková *et al.*, 2016; Li *et al.*, 2019).

Immobilization is the process of adsorbing an enzyme on a carrier. Knowledge on enzyme adsorption is vital as the maximum amount of enzyme adsorbed, the enzyme adsorption mechanism, and the rate-limiting step of the adsorption process need to be determined (Gilani et al., 2016). To the best of the author's knowledge, research regarding enzyme adsorption onto carriers is still limited because most of the research related to enzyme immobilization only focuses on immobilized enzymes' stability and kinetics.

1.3 Objective of Study

The research was conducted to study the immobilization of laccase on a PET-based nanofiber mat. Four sub-objectives were accomplished in this study, and they are listed as follows:

(a) To fabricate and characterize a nanofiber mat from PET-g-MAH for the immobilization of laccase.

(b) To optimize the parameters affecting the immobilization of laccase on the PET-g-MAH nanofiber mat.

(c) To determine the modelling of the laccase's adsorption on the PET-g-MAH nanofiber mat.

(d) To evaluate the laccase immobilized on the PET-g-MAH nanofiber mat's performance, structure, properties, and stability.

1.4 Scopes of Study

The scopes of work considered in achieving the objectives of this research are listed as follows:

(a) Liquid PET was grafted with MAH under different grafting temperatures (27 ^{o}C – 70 ^{o}C) before the synthesis through the electrospinning process. PET-g-MAH nanofiber mats were synthesized at different polymer concentrations (10 % (w/v), 20 % (w/v), and 30 % (w/v)) but fixed electrospinning parameters. The MAH grafting success was determined based on a hydrophobicity test and nanofiber mats' structural analysis.

(b) The optimization of immobilization preparation parameters was conducted in three phases. The first phase was the preliminary study of the immobilization methods (physical adsorption (PA), covalent bonding (CV), and covalent bonding of cross-linked laccase aggregates (CL)). The second phase was the screening for the best method. The immobilization preparation parameters screened were laccase concentration (0.2 – 1.0 mg/ml), pH (3.0 – 5.0), immobilization temperature (20 – 30 ^{o}C), immobilization time (1 – 6 hr), cross-linking concentration (0.05 – 0.5 % (v/v)), cross-linking temperature (4 – 20 ^{o}C), cross-linking time (1 – 12 hr), and agitation speed (150 – 250 rpm), and they were screened using a two-level factorial design. The independent variables that affected the immobilization yield significantly were further optimized using the Box-Behnken Design (BBD).

(c) The effects of contact time (0 – 105 min), laccase concentration (0.1, 0.2, 0. mg/ml), and adsorption temperature (15, 22.7, 30 ^{o}C) on the adsorption of laccase on PET-g-MAH were determined to study the adsorption kinetics of the laccase by fitting the adsorption data to adsorption isotherm models (Langmuir and Freundlich) and kinetic adsorption models (pseudo-first-order, pseudo-second-order and intraparticle diffusions) and by using thermodynamics.

(d) The laccase immobilized on the PET-g-MAH nanofiber mat was characterized and compared with free laccase in terms of 2-azino-bis 3-ethylbenzothiazoline-6- sulfonic acid (ABTS) oxidation. The immobilized laccase with optimum immobilization preparation parameters was characterized in terms of optimum pH (3.0 – 6.0), optimum temperature (25 – 70 ^{o}C), thermal stability (30 – 60 ^{o}C), and enzyme kinetics' coefficients (K_m and V_{max}) by measuring the laccase activity assay. Additionally, the immobilized laccase's storage stability (30 days), leaching, and reusability (10 cycles) were also tested to verify immobilization enhancements. Finally, analysis on structure and morphology before and after immobilization was conducted to confirm further the immobilization of laccase on the PET-g-MAH nanofiber mat.

1.5 Significance of Study

This study introduced a novel PET-based carrier for laccase immobilization. PET was grafted with MAH using a simple and effective way before the electrospinning process. The PET-g-MAH nanofiber mats fabricated in this study provide the enzyme technology field PET-based enzyme carriers ready to be used. The carriers do not require surface activation as the reactive MAH is already grafted on the surface to reduce the PET's hydrophobicity. The carrier is foreseen to be implemented as a membrane in an enzymatic bioreactor as it is physically in a mat form and can be used in several industries such as the textile industry, beverage industry, bioremediation, and paper industry. Furthermore, it can work well as a biocatalyst by looking at its performance in reusability and stability at a wide range of pH and temperature. This study also gives information on the optimum laccase preparation parameters obtained from the response surface methodology. The polynomial equation developed from the experimental data helped attain the best immobilization performance for the laccase on the PET-g-MAH nanofiber mat. Finally, the knowledge on the laccase adsorption on PET-g-MAH of this study is useful for researchers to determine the relationship between the laccase and PET-g-MAH nanofiber mat and the adsorption mechanism of the laccase.

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LIST OF PUBLICATIONS

Indexed Journal

(a) **Mohd Syukri, M. S.,** A. Rahman, R., Mohamad, Z., Md Illias, R., Nik Mahmood, N. A., & Jaafar, N. R. (2020). Optimization strategy for laccase immobilization on polyethylene terephthalate grafted with maleic anhydride electrospun nanofiber mat. International Journal of Biological Macromolecules, 166, 876–883. **(IF : 5.162)**

(b) **Mohd Syukri, M. S.,** Rahman, R. A., Mohamad, Z., Nik Mahmood, N. A., Illias, R. M., & Tokuyama, H. (2020). Laccase immobilisation on poly(ethylene) terephthalate grafted with maleic anhydride (PET-g-MAH) nanofiber mat. Chemical Engineering Transactions, 78, 37–42. **(Indexed by SCOPUS)**

Non-Indexed Conference Proceedings

(a) **Syukri, M. S. M.,** Abd Rahman, R., & Mohamad, Z (2017). Delignification of Lignocellulosic Biomass by Immobilized Laccase: A Mini Review. In *International Postgraduate Symposium In Biotechnology 2017 (IPSB 2017)* (pp. 124 – 128). https://www.utm.my/ipsb/files/2017/08/MB-11-Reviewed-Syahlan-Extended-abstract-2.pdf