REUSABILITY AND LEAKAGE OF IMMOBILIZED LACCASE ENZYME

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REUSABILITY AND LEAKAGE OF IMMOBILIZED LACCASE ENZYME

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Engineering (Hons.) Chemical Engineering (Biotechnology)

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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Engineering (Hons.) Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedication

To those who made this project fun

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ABSTRACT

Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase), a copper containing enzyme which can catalyse the oxidation of various organic and inorganic substrates, is usually used to decolorize the wastewater effluent and render phenolic compounds to less toxic component. The objective of this research is to compare the reusability and leakage between entrapped and covalently bonded laccase enzymes. The reusability of immobilized laccase enzyme was studied by reacting a batch of immobilized laccase enzymes with ABTS repeatedly for 15 cycles. The study of the leakage of immobilized laccase enzyme was carried out by storing the immobilized laccase enzymes in acetate buffer solution for 32 days. The acetate buffer solution samples were taken and reacted with ABTS. The samples reacted with ABTS were analysed using a UV-Vis spectrophotometer at 415 nm. The absorbance readings were recorded and enzyme activities were calculated. The data collected showed that the retained enzyme activities of entrapped and covalently bonded enzyme after being reused for 15 cycles are 33.50% and 48.19% respectively. On the other hand, the leakages of entrapped and covalently bonded laccase enzyme after 32 days are 13.9 % and 14.46 % respectively. In conclusion, the covalently bonded laccase enzymes are more stable in terms of reusability and storage stability compared to entrapped laccase enzymes.

ABSTRAK

Laccase (EC 1.10.3.2 , benzenediol : oxidoreductase oksigen) , sejenis enzim yang mengandungi tembaga yang boleh menjadi pemangkin kepada pengoksidaan pelbagai substrat organik dan bukan organik , biasanya digunakan untuk menjernihkan efluen yang berwarna dan menukarkan sebatian fenolik kepada komponen yang kurang toksik . Objektif kajian ini adalah untuk membandingkan kebolehan enzim lumpuh untuk diguna semula dan kebocorannya dari bahan imobilisasi. Perbandingan dibuat antara enzim laccase yang dilumpuhkan dengan teknik terperangkap dan ikatan kovalen. Kebolehgunasemulaan enzim laccase lumpuh telah dikaji dengan bertindak balas kelompok enzim lacccase lumpuh dengan ABTS sebanyak 15 kitaran. Kajian tentang kebocoran enzim laccase lumpuh dilakukan dengan menyimpan enzim laccase lumpuh dalam buffer asetat selama 32 hari. Sampel buffer asetat telah diambil telah bertindak balas dengan ABTS dan dianalis dengan mengunakan UV-Vis spektrofotometer pada 415 nm untuk mendapatkan absorbansi. Bacaan absorbansi itu telah digunakan dalam perhitungan aktiviti enzim. Data yang dikumpul menunjukkan bahawa aktiviti-aktiviti enzim terperangkap dan ikatan kovalen adalah adalah 33.50 % dan 48,19 % masing masing selepas digunakan semula selama 15 kitaran. Sebaliknya , kebocoran enzim terperangkap dan ikatan kovalen selepas 32 hari adalah 13.90 % and 14.46 % masing-masing . Kesimpulannya , enzim laccase ikatan kovalen adalah lebih stabil dari segi kebolehan digunasemula dan kestabilan sepanjang tempoh simpanan berbanding dengan enzim laccase terperangkap.

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LIST OF ABBREVIATIONS

Resonance Raman	RR
Surface-enhanced Raman scattering spectroscopy	SERS
Layer-by-layer	LbL
Manganese carbonate	$MnCO_3$
Polyethylene Glycol	PEG
Calcium chloride	$CaCl_2$
Alumina pellets	Al_2O_3

1 INTRODUCTION

1.1 Motivation and Statement of Problem

Wastewater treatment is an essential step in producing water that can be released into the environment. However, the common wastewater treatment technologies such as coagulation, oxidation, and filtration are usually inefficient in removing phenols in the effluent. Examples of phenol found in effluent are estradiol and lignin (Cardinal-Watkins & Nicell, 2011; Shankar *et al*., 2013). These substances are harmful to the human body as well as aquatic animals and thus have to be eliminated before being discharged as effluent. Therefore, searches for economic and effective ways to treat wastewater in the face of the ever increasing production activities are carried out to decrease the cost of wastewater treatment (Ibrahim *et al.*, 1996).

Research found that laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase), a copper containing enzyme, has the ability to catalyse the oxidation of various phenols (Teerapatsakul *et al.*, 2008). Even though laccase enzymes are able to remove phenols in wastewater, the stability of the free laccase enzymes in the process is very low and not profitable. Therefore, researches are carried out to optimise the production yield, activity and reusability of immobilised laccase enzymes (Mayer and Staples, 2002).

The aim of immobilizing laccase enzymes is to increase the stability and reusability. Different types of laccase enzymes have different optimal performance conditions such as pH value and temperature (Mahdhavi and Lele, 2009). Various methods of laccase enzymes immobilization are available. They can be categorized into entrapment, encapsulation, adsorption, covalent bonding, and self-immobilization. However, due to the different types of laccase enzymes, different support will be required for a particular immobilization. Hence, many researches are still discussing about the best method in immobilization of laccase enzymes from different species and for various functions.

To compare the enzymatic activity of immobilized enzymes with that of free enzymes, enzymes immobilization has to be carried out first. A suitable support is needed such as diatomaceous earth support Celite R-633 (Cabana *et al.*, 2009), modified PVDF microfiltration membrane (Jolivalt *et al.*, 2000), gold, silver and indium tin oxide (Mazur *et al.*, 2007), magnetic chitosan microspheres (Jiang *et al.*, 2005) etc. These supports are able to increase the mechanical resistance of laccase enzymes at different

pH and temperature conditions. However, due to the cost of materials, it is still very expensive for the commercial industry in Malaysia. In addition, some of these techniques require long preparation procedure which means that there might have some losses or inaccuracy during the preparation. Most importantly, some of the support substances might be hazardous to human, for example, polyamine 6, 6 fibers (Silva *et al.*, 2007) and Eupergit C. benzoate (Hublik and Schinner, 2000). These chemical may cause irritating effect on skin after contact.

Laccase enzyme is produced with different properties and optima conditions. These different properties will affect the method of immobilization and the support used. Therefore, choosing a suitable immobilization method for a specific enzyme species and storing it in suitable medium is very crucial as each enzyme has a very narrow range of optimum pH and temperature.

1.2 Objective

The objective of this research is to compare the reusability and storage stability between entrapped and covalently bonded laccase enzymes from the species of *Trametes Versicolor*.

1.3 Scope of This Research

The scope of this research includes the immobilization of laccase enzymes by entrapment, comparing the leakage between entrapped laccase enzymes and covalently bonded laccase enzymes for a period of 32 days and comparing the reusability between entrapped laccase enzymes and covalently bonded laccase enzymes for 15 cycles.

1.4 Main Contribution of This Work

This work can be the reference for future researches and application as the effectiveness of the immobilization technique on laccase enzymes is determined by comparing the enzymatic activities of free laccase enzymes and immobilized laccase enzymes. Next, the reusability between entrapped and covalently bonded laccase enzymes is studied to determine the optimum number of cycle that each batch of immobilized laccase enzymes can be used while the storage stability is studied to determine the optimum storage period for entrapped and covalently bonded laccase enzymes.

1.5 Organisation of this Thesis

The structure of the remainder of the thesis is outlined as follow:

Chapter 2 provides a description of the applications of laccase enzyme and its optimum condition to obtain high enzymatic activity. Different types of immobilization methods are also discussed. Comparisons were made and an immobilization method was chosen. A general description on the types of enzyme assay was discussed and the most suitable assay was chosen. This chapter also provides a discussion of the reusability test as well as leakage test.

Chapter 3 gives a description of chemicals used in this experiment. The procedures of immobilizing laccase enzyme by entrapment and covalent bonding were also mentioned. The compositions of enzyme assay as well as the procedures for reusability test and leakage test were discussed.

Chapter 4 is the discussion of results obtained from the analysis of this work. Standard calibration curves for free laccase enzyme were prepared and discussed. The immobilization yields for both immobilization methods were discussed. The reusability and leakage of both immobilized laccase enzymes were compared and the best immobilization method was decided.

Chapter 5 draws together a summary of the thesis and outlines the future work which might be expanded from the findings in this work.

2 LITERATURE REVIEW

2.1 Laccase Enzymes

Laccase (p-diphenol:oxygen oxidoreductases, EC 1.10.3.2), an extracellular multicopper enzyme that uses molecular oxygen as a co-substrate to oxidize various compounds by a radical-catalyzed reaction mechanism (Stoilova *et al.*, 2010), is applied in the detoxification of various aquatic and terrestrial pollutants, and in the treatment of industrial wastewater (Shuttleworth and Bollag, 1986). Laccase enzymes and their applications in several industrial sectors have been studied since the nineteenth century due to their ability to oxidize phenolic compounds (Madhavi and Lele, 2009). Laccase enzyme is better compared to peroxidise and tyrosinase as it does not require the addition of hydrogen peroxide like peroxidase, and it has broader substrate specificity than tyrosinase (Leonowicz *et al.*, 1988).

The applications of laccase enzymes are mostly in the cleaning of the industrial effluent in paper or textile industries, decolourization of dye in printing industry and elimination of pollutant in waste water treatment plant (Madhavi and Lele, 2009). Their ability to degrade phenolic compounds makes them an excellent catalyst for dye decolourization and wastewater treatment (Fernández-Fernández *et al.*, 2013). With the growing of industrial biotechnology recently, most of the food industries and medical applications have chosen laccase enzymes as their alternative catalyst for the production. Other applications of laccase enzymes include bio-oxidation, biotransformation and biosensor development (Imran *et al.*, 2012). In the field of electrobiochemistry, laccase enzymes are used in the design of biosensors to detect phenols in wastewater besides the development of biofuel cells (Fernández-Fernández *et al.*, 2013).

Enzymatic activity of laccase enzymes is influenced by pH, temperature and inhibitors. Laccase enzymes are very sensitive to pH value. The optimal pH range of laccase enzymes is from 3 to 7 for fungal laccase enzymes (Moya *et al.*, 2011) and up to 9 for plant laccase enzymes (Madhavi and Lele, 2009). The laccase enzymatic activity has a bell shaped profile with optimal pH affected by substrate, oxygen, and enzyme itself. Different types of laccase enzymes have different optimum temperature range. For example, the optimal temperature of *Trametes modesta* laccase is at 50 ℃ (Imran *et al.*,

2012), while laccase from *Ganoderma lucidum* has an optimum temperature range from 20 to 25 ℃ (Madhavi and Lele, 2009).

2.2 Immobilization of Laccase Enzymes

Lacccase enzymes are immobilized to counter the high production cost. Immobilized laccase enzymes can be reused and thus reducing the production cost. The major advantages of laccase enzymes immobilization are the increase in the thermostability of the enzyme and its resistance to extreme conditions and chemical reagents. In addition, immobilized laccase enzymes can be easily separated from the reaction products, allowing the enzymes to be employed in continuous bioreactor operations. However, the immobilization processes could result in conformational changes of the enzyme, promoting a loss of activity. In most cases, glutaraldehyde is used to stabilize the immobilized enzyme, permitting greater enzyme flexibility for conformational changes required for activity. Moreover, charged groups on the surface of a support may lead to electrostatic interaction between individual ionic amino acids on the enzyme surface. Both types of charges as well as charge density on the material surface can alter the enzymatic activity upon immobilization (Spinelli *et al.*, 2013).

To select a suitable laccase enzymes immobilization method, there are two factors that need to be considered: support and method. The chosen support must be insoluble and compatible with laccase enzymes. In addition, it needs to be able to maintain the enzyme stability in the process. The attachment of the enzymes into or onto the support should be easily accomplished during immobilization. Besides that, an immobilization technique should be easy to operate and with lower cost (Brena and Batista-Viera, 2000). There are many methods for enzymes immobilization such as adsorption, covalent bonding and entrapment.

2.2.1 Immobilization by Adsorption

Adsorption is the attachment of enzymes on a surface by weak forces, such as van der Waals force, electrostatic force, and hydrophobic interaction (Jegannathan *et al.*, 2008). This method requires a minimum of activation steps and tends to be less disruptive to the enzymatic protein as it is linked by weak forces and reduces large loss of enzymatic activity. However, the adsorbed laccase enzymes have poor operation stability and the adsorbed enzymes are more sensitive to the immobilization parameters such as

temperature and pH. In addition, desorption of adsorbed enzymes may be high due to the weak forces between them (Zhang *et al.*, 2013). Immobilization by adsorption is the hydrophobic interaction and salt linkage between the support and enzyme. Adsorbed enzymes are protected from aggregation, proteolysis and interaction with hydrophobic interfaces (Spahn and Minteer**,** 2008). Researches on this immobilization have been done with different support materials such as coconut fibres which have high water-holding capacity and good cation exchange ability; soil iron which is environmental friendly for soil remediation; and mesoporous materials which have large surface area that is ideal for the adsorption of enzymes (Zhang *et al.* 2013). In literature, laccase adsorbed on soil iron such as amorphous aluminum hydroxide, gibbsite, lepidocrocite and goethite gave immobilization yield of 26%, 31%, 36% and 64% respectively (Wang *et al.*, 2008). On the other hand, adsorption of laccase enzymes on coconut fibres gave an immobilization yield of 50%. Immobilisation by adsorption on alumina pellets and coating with polyelectrolyte layers gave an immobilization yield of 89% (Crestini *et al.*, 2010). This method of immobilization is simple and can be eco-friendly but the force of attraction is weak. In addition, the enzyme can be detached from the support if the immobilized enzyme is placed in a solution which is extreme for the enzyme-support linkage as it is exposed to the environment.

2.2.2 Immobilization by Covalent Bonding

Immobilization by covalent bonding has limited binding reaction as it must be carried out under mild conditions to avoid the active site of the enzymes being destroyed. Covalent bonding provides stable and high stability immobilized enzymes (Zhang *et al.*, 2013). In this technique, chemical groups on the support surface are activated before being reacted with nucleophilic groups on the protein (Datta *et al.*, 2013). Thus, this immobilization method requires knowledge about the effective coupling of nonessential pendant group active site of the enzymes with the functional group of carrier. Besides, some of the carriers cannot couple with the enzymes as they do not have the functional group. The best support for this immobilization should contain short spacer arms and a high density of reactive groups. These characteristics are required for the multipoint attachment of the laccase enzymes. The support employed in this immobilization method includes silica, carbon, chitosan, alumina, glass, gold, silver, graphite and most nanoparticles. Silver and gold surfaces are used as supports after the modification of the surface by thiol monolayers with carboxylic and amino groups. Immobilization has

also been studied using resonance Raman (RR) and surface-enhanced Raman scattering spectroscopy (SERS), both of which are surface sensitive techniques that can detect single molecules adsorbed or covalently bonded onto rough metal surfaces (Pointing, 1999). According to Chen *et al.* (2006), laccase enzyme covalently bonded to chitosan gave an immobilization yield of 52.2%. On the other hand, immobilization by covalent bonding on porous glass beads that were activated with 3-aminopropyltriethoxysilane and glutaraldehyde gave an immobilization yield of 90%. Immobilization by covalent bond allows easy manipulation and offering higher resistance to denaturing agents. However, this method can cause low activity recovery as a result of the modification or destruction of enzyme active sites (Zhang *et al.*, 2013).

2.2.3 Immobilization by Entrapment

Entrapment is the physical retention of enzymes in a porous solid matrix (Lu *et al.*, 2007; Phetsom *et al.*, 2009; Timur *et al.*, 2004). The enzymes are first suspended in the monomer solution, and a subsequent polymerization process keeps the enzymes trapped, preventing direct contact with the environment. Entrapment is the easiest immobilization method which induces no structural alteration of the enzymes. The solid matrixes used in this immobilization technique include polyacrylamide, collagen, alginate and gelatine (Lu *et al.*, 2007; Phetsom *et al.*, 2009; Timur *et al.*, 2004). The laccase immobilized in Cu alginate has an immobilization yield of 61% (Niladevi and Prema, 2008). According to Nor *et al.* (2010), the immobilization yield of the entrapment in PVA-alginate is 97.4% whereas according to Teerapatsakul *et al.*(2008), entrapment of laccase enzymes in alginate solution cross-linked by $CuSO_4$ gave an immobilization yield of 74.6%-146.8% depending on the concentration of alginate and $CuSO_4$ solution. The immobilization yield is higher than 100% shows that the condition inside the beads is suitable for the type of laccase enzyme used which enhances its activity (Nor *et al.*, 2010). The advantage of entrapment of enzymes immobilization is fast and economical. Besides that, the condition in the beads can be controlled by adjusting the composition of the matrix. The disadvantages of this immobilization method are mass transfer limitations (Brady and Jordaan, 2009).

2.2.4 Immobilization by Encapsulation

Immobilization by encapsulation is similar to the entrapment method. However, this immobilization method is time consuming. The disadvantage of this immobilization method is mass transfer limitation as the enzyme is protected from the environment (Brady and Jordaan, 2009). Encapsulation can also be done in micro scale which is known as microencapsulation where the enzyme is confined in the core of micron-sized spheres made from a semi-permeable material. The semipermeable membranes include polyethyleneimine, and SiO_2 (Rochefort *et al.*, 2008). The layer-by-layer (LbL) technique is another microencapsulation method that has been employed during recent years (Crestini *et al.*, 2010). For example, Crestini *et al.* (2010) microencapsulated laccase with the LbL technique which is the assembly of differently charged polyelectrolyte layers on manganese carbonate ($MnCO_3$) particles and the immobilization yield of that study is 75%. Using the LbL method, thin films can be formed and a wide variety of materials can be deposited which provides a simple way to control the thickness.

2.2.5 The Chosen Immobilization Method

Table 2-1 shows the comparison of the attributes of different immobilization methods. The immobilization technique employed in this study is entrapment. This is because, in literature, entrapment is the easiest immobilization method which induces no structural alteration of the enzymes as the enzymes are trapped, not bonded to the support. The entrapment of enzymes prevents direct contact of the enzymes with the environment. A suitable environment in the beads will enhance the enzyme activity. Besides that, immobilization by entrapment is fast and economical as the steps involve are simple and the cost of materials is low.

Table 2-1 : Comparison of the characteristics of different immobilization methods.

Characteristics	**Adsorption**	**Covalent Bonding**	**Entrapment**	**Encapsulation**
Preparation	Simple	Difficult	Simple	Difficult
Cost of immobilization	Low	High	Low	High
General applicability in industry	Yes	No	Yes	No
Enzyme activity	Intermediate	Low	Intermediate	Intermediate
Stability	Intermediate	High	High	High
Binding force	Weak	Strong	Intermediate	Intermediate
Protection of enzyme from microbial attack	No	No	Yes	Yes

In this study, entrapment is done by mixing gelatine, sodium alginate, PEG and laccase enzyme. The concentration of laccase enzyme was kept constant at 5 U/ml. According to Wang *et al.* (2008), gelatine is added into the entrapment medium as an additive as gelatine is a type of collagen which contains repeating sequences of glycine-proline and hydroxyproline-triplets (Choi *et al.*, 1999). The sequence is responsible for the formation of tertiary polypeptide of gelatine. Due to the low intensity and high brittleness which will cause high leakage, gelatine is usually used as an additive for the formation of beads (Wang *et al.*, 2003; Dong *et al.*, 2006). The mixture of sodium alginate and gelatine was used because gelatine is able to narrow the pore sizes of the beads (Wang *et al.*, 2008).

According to Nam (2014), when sodium alginate enters calcium chloride solution, ionic reaction takes place as below

$$2\ Na\ (Alginate) + CaCl_2 \text{-------}> Ca\ (Alginate)_2 + 2\ NaCl$$

Calcium chloride calcified sodium alginate to form calcium alginate. Sodium alginate was used as the material instead of calcium alginate to allow the formation of uniform beads of sodium alginate before hardening. This can ensure better beads shape and size.

Polyethylene Glycol (PEG) was added as an additive to protect the laccase enzyme from inactivation during the immobilization. This is because PEG provided more hydroxyl group to the laccase enzymes forming a stronger resistance from inactivation (Wang *et al.*, 2008). It is also proven that PEG is effective at suppressing the horseradish peroxidase inactivation (Wu *et al.*, 1998) and soybean peroxidase inactivation (Caza *et al.*, 1999). According to Bharadwaj *et al.* (2011), PEG forms a barrier and thus protecting the laccase enzyme in the beads from being inactivated by inhibitor. The higher the molecular weight of the PEG, the higher its ability to block out inhibitors and contaminants. Therefore, PEG with high molecular weight should be used to prevent the inhibitors and contaminants from entering the beads. However, in the entrapment of laccase enzyme, if the PEG chosen has a very high molecular weight (that is, have high ability to block out foreign material), the substrate may not be able to enter the beads to react with the laccase enzyme inside. Hence, PEG 4000 was chosen as it gave optimum protection to the entrapped laccase enzyme while allowing the reaction between laccase enzyme and ABTS to take place in the beads (Wang *et al.*, 2008).

Since gelatine contains free amino, hydroxyl, and carboxyl in the molecule chains (Emel *et al.*, 2006), glutaraldehyde was added to react with the free amino in gelatine which makes water discharged easily from the beads during the cross-linking and prevents the leakage of immobilized enzyme from beads (Naganagouda and Mulimani, 2006). Cross-linking with low concentration of glutaraldehyde will give poor mechanical strength as the gelatine is not stable; high concentration of glutaraldehyde will stabilise the gelatine by interacting with the free amino functions in gelatine, and therefore provide a stronger bead. However, high concentration of glutaraldehyde will inactivate the laccase enzyme. Therefore, an optimum concentration of glutaradehyde is determined as 0.6 % (v/v) to ensure the beads is stable without inhibiting the laccase enzyme (Wang *et al.*, 2008).

The covalently bonded laccase enzymes taken from a previous study are compared with the entrapped laccase enzymes in terms of immobilization yield, reusability and storage stability. Reusability of the immobilized enzymes is important as it shows the optimum number of cycle each batch of immobilized enzymes should be used. This data is important to industries as it affects the production cost. If a batch of enzyme is used less than its optimum number of cycles, the production cost will increase as that requires

frequent change of new batch enzymes. On the other hand, the quality of the product will decrease if a batch of immobilized enzymes is used more than its optimum number of cycle as the enzymes will be lost in each cycle. Storage stability is important to provide information about the maximum storage period for a batch of immobilized enzymes. Enzymes that are kept over the maximum storage period are no longer suitable to be used as the rate of reaction will be very low. This is because enzymes normally may leak into the buffer solution over the storage period.

2.3 Enzyme Assay

After immobilization, the efficiency of laccase enzymes immobilization will be tested. In general, the laccase enzymatic activity and retention percentage will be tested to determine the stability of enzymes. The assays available to determine the enzymatic activity of laccase enzyme are Bavendamm test, Poly-R agar test, azure-B agar test, ABTS test and syringaldazine test (Pointing *et al.*, 1999). Among these assays, the ABTS agar test and syringaldazine well test have the highest sensitivity of laccase enzymatic activity detection and give the result in a short time (Li *et al.*, 2012). These two methods used the oxidation reaction of laccase to the substrate to determine the activity and retention percentage. ABTS has been chosen to be used in this study as it has the highest sensitivity of laccase enzymatic activity detection, simple preparation steps and the result can be obtained in a shorter time (Li *et al.*, 2012).

2.4 Reusability Test

Reusability test is carried out to determine the retained activity of a batch of immobilized enzymes after each cycle of reaction. From the data, an optimum number of cycles that each batch of immobilized laccase enzymes can be used are determined.

According to Nor *et al.* (2010), immobilization of laccase enzymes by entrapment in PVA–alginate has a reusability of 14 cycles with a retained activity of 71.75%. This shows that the enzyme can be reused for more than 14 cycles as long as the retained activity is still applicable for an economical production. On the other hand, the reusability of laccase enzymes immobilized by microencapsulation has a reusability of 10 cycles with a retained activity of 68% while the retained activity of laccase enzymes adsorbed on alumina pellets and coated with polyelectrolyte layer is 84% after 10 cycles (Crestini *et al.*, 2010). In addition, immobilization of laccase enzymes by covalent

bonding on porous glass beads that were activated with 3-aminopropyltriethoxysilane and glutaraldehyde gave a retained activity of 65% after 6 cycles (Leonowicz *et al.*, 1988).

In this study, reusability test is carried out by allowing the immobilized laccase enzymes to catalyse the oxidation of ABTS for 75 minutes to ensure complete oxidation (Nor *et al.*, 2010). The immobilized laccase enzymes are then filtered and placed into a new batch of ABTS solution. The test is repeated for 15 cycles.

2.5 Leakage Test

Storage stability test is carried out to determine the optimum storage period for immobilized laccase enzymes. In literature, the leakage percentage of laccase enzymes entrapped in PVA-alginate and microencapsulated is 6.49% and 21.38%, respectively, over 30 days (Nor *et al.*, 2010; Crestini *et al.*, 2010). On the other hand, laccase enzymes adsorbed on alumina pellets and coating with polyelectrolyte layers has a leakage percentage of 15.75% over 30 days (Crestini *et al.*, 2010). Next, laccase enzymes that are immobilized by covalent bonding on porous glass beads that were activated with 3-aminopropyltriethoxysilane and glutaraldehyde gave a leakage percentage of 6% over 20 days (Leonowicz *et al.*, 1988). The highest leakage percentage is only 21.38% over a period of 30 days. This shows that immobilized laccase enzymes can be stored for at least 30 days and still have reasonable enzymatic activity.

Storage stability test is carried out by immersing the immobilized enzymes in acetate buffer solution and the buffer solution is collected and tested with ABTS solution for enzymatic activity for 32 days to determine the optimum storage period. The absorbance of the oxidized ABTS is obtained (Nor *et al.*, 2010).

The buffer used is a factor in ensuring the stability of alginate beads. Citrate and phosphate buffer are not suitable to be used as the storage medium for the alginate beads because both buffers will destabilize calcium alginate beads as they are calcium chelators (Nam, 2014). Calcium chelators tend to bind with calcium to form calcium-chelators complex such as calcium citrate and calcium phosphate (Embl.de, 2014). If the calcium alginate beads were kept in the buffer containing calcium chelators as mentioned above, the calcium content in the calcified alginate beads will decrease

causing the beads to become unstable and disintegrate. Furthermore, the formation of calcium-chelators complex will affect the buffering capacity of the buffer (Worldwide.promega.com, 2014). Therefore, acetate buffer was chosen as the storage medium in this study.

3 MATERIALS AND METHODS

3.1 Chemicals

Laccase enzymes from *Trametes versicolor* and gelatine were purchased from Sigma-Aldrich. Polyethylene Glycol (PEG) (MW = 4000), calcium chloride ($CaCl_2$), sodium acetate, glutaraldehyde and acetic acid were purchased from Merck. Sodium Alginate was purchased from R&M Chemicals and ABTS was purchased from Roche.

3.2 Laccase Immobilization

3.2.1 Entrapment with Gelatine and Sodium Alginate

100 ml of 2.0% (w/v) Na-alginate was prepared before adding 2.0 g of gelatine and 0.5 g of PEG. 0.05 g (500 U) of laccase enzymes was then added and mixed thoroughly for 10 minutes at 25℃. Next, the mixture was withdrawn using a sterile 5 ml syringe and extruded through a 21 gauge (0.51 mm inner diameter) needle into 2.0% (w/v) $CaCl_2$ solution, almost instantly forming beads of 2.0 – 3.0 mm diameter. The beads were left unstirred to harden in the $CaCl_2$ solution at 4℃ for about 2 hours, after which the $CaCl_2$ solution was removed and the beads were washed twice with distilled water. Then, the beads were subsequently incubated in 100 ml of 0.6% (w/v) glutaraldehyde solution and stirred at 4℃ for 2 hours. After the beads were washed several times with acetate buffer (pH 4.5), the immobilized laccase enzymes are stored at 4℃ in acetate buffer (pH 4.5) (Wang *et al.* 2008).

3.2.2 Covalent Bonding by Alumina Pellet Support

The immobilization of laccase enzymes by covalent bonding was not carried out in this study. The covalently bonded laccase enzymes were obtained from a previous undergraduate research project. In this immobilization method, alumina pellets (Al_2O_3) was first silanised with 2.0% (v/v) γ-aminopropyltriethoxysilane in acetone at 45℃ for 20 hours. The support was then washed with acetone one time and silanised again for 24 hours. After silanisation, the support was washed few times with deionised water and dried through air. The Al_2O_3 were then treated with 2% (v/v) aqueous glutaraldehyde 50% (v/v) for 2 hours at 25℃. The support was again washed with deionised water and dried through air. 150 g of treated supports were then immersed in 250 mL (5 U/mL) of laccase solution (obtained by dissolving the enzyme in 0.1 M citrate buffer pH 5 with 0.1 M sodium chloride) at 25℃ for 48 hours. The particles then were washed with 0.05

M phosphate buffer (pH 7) to remove the unbound enzyme and keep at 4 ℃ until further use (Silva *et al.*, 2007; Leong, 2014).

3.3 Enzyme Assays

The 0.5 mM ABTS solution was prepared by dissolving ABTS with acetate buffer. The assay mixture for test consisted of 1.9 ml of 0.1 M acetate buffer (pH 4.5), enzyme (0.1 ml enzyme in buffer; 0.09 g or 0.2 g of covalent bonded and entrapped laccase enzyme respectively with an approximate volume of 0.1 ml) and reaction was started immediately by adding 1.0 ml of 0.5 mM ABTS solution. The assay mixture for the blank consists of 1.9 ml of 0.1 M acetate buffer (pH 4.5), 0.1 ml of pure water and 1.0 ml of 0.5 mM ABTS solution. The activity of laccase enzymes was assayed spectrophotometrically at 415 nm with UV-VIS spectrophotometer (Shimadzu, UV-1800) at 25 ℃ (Vanhulle *et al.*, 2006). The absorbance of the mixture solution was recorded.

3.4 Reusability Test

The reusability of entrapped laccase enzymes was studied for 15 cycles. 3 sample bottles each contained approximately 0.09 g covalent bonded laccase enzyme was added with 1.9 ml of buffer solution and 1.0 ml of ABTS solution. The immobilized laccase enzymes were allowed to catalyse the oxidation of ABTS for 75 minutes (Nor *et al.*, 2010). The immobilized laccase enzymes were filtered, washed and placed into a new batch of ABTS solution for the second cycle. The experiment was repeated to test the reusability of entrapped laccase enzyme by substituting the 0.09 g of covalent bonded laccase enzyme with 0.2 g of entrapped enzyme. The activity of laccase enzymes was assayed spectrophotometrically at 415 nm with UV-VIS spectrophotometer (Shimadzu, UV-1800) at 25 ℃ (Vanhulle *et al.*, 2006).The absorbance of the mixture solution was recorded. The retained activity is calculated using the equations 1 and 2.

$$Unit\ of\ enzymes_{\ (n)} = \frac{Absorbance\ of\ mixture}{\varepsilon} \qquad \text{(Eq. 1)}$$

$$Retained\ activity\ (\%) = \frac{Unit\ of\ enzyme_{\ (n)}}{unit\ of\ enzyme_{(n=1)}} \qquad \text{(Eq. 2)}$$

3.5 Leakage Test

The storage stability test was carried out to determine the leakage of the entrapped and covalently bonded laccase enzymes during the 32 days of the study period. Immobilized laccase enzymes were stored in acetate buffer (pH 4.5) and sample of the buffer solution was collected every day for the first week and then every five days thereafter. 0.1 ml of the sample buffer was added into 1.9 ml of 0.1 M acetate buffer. 1.0 ml of ABTS solution was added and the activity of laccase enzymes was assayed spectrophotometrically at 415 nm with UV-VIS spectrophotometer at 25 ℃. The assay was repeated 3 times and the average reading was determined to obtain a more accurate result. The activity of immobilized laccase enzymes was tested for 32 days. In this period the laccase enzymes were stored at 4 ℃ (Vanhulle *et al.*, 2006).

$$Unit\ of\ enzyme\ leaked\ (U) = \frac{Absorbance}{\varepsilon} \times dilution\ factor \times volume\ of\ buffer(ml)$$

$$Leakage\ (\%) = \frac{Unit\ of\ enzyme\ leaked_{(d)} - Unit\ of\ enzyme\ leaked_{(d=1)}}{Unit\ of\ enzyme\ leaked_{(d=1)}} \times 100\ \%$$

Where d = number of day

4 RESULTS AND DISCUSSIONS

4.1 Standard Calibration Curve

The free laccase enzyme activity was determined and used to calculate the yield of immobilization by entrapment and the enzyme activity for the reusability and leakage test. Free laccase enzyme standard calibration curve was done by plotting the graph of absorbance versus concentration of ABTS as shown in Figure 4.1.

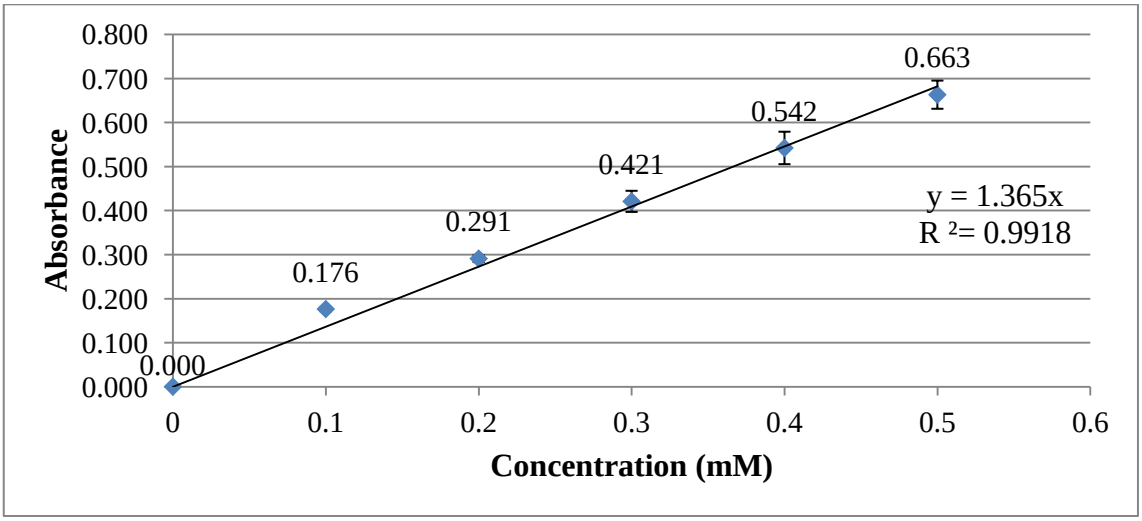

Figure 4-1 : The absorbance detected by reacting various concentration of ABTS with free laccase enzyme (5 U/ml).

From Figure 4-1, the absorbance is directly proportional to the concentration of ABTS. Laccase enzyme is able to oxidise ABTS. Oxidation of ABTS causes the mixture to change from colourless to green. Therefore, a higher concentration of ABTS will give a denser colour of oxidised ABTS and hence having a higher absorbance. According to Manole *et al.* (2008), the extinction coefficient, ε is obtained from the slope of the graph of absorbance versus concentration. From Figure 4-1, the gradient of the graph is 1.365 $mM^{-1}cm^{-1}$ which means that ε = 1.365 $mM^{-1}cm^{-1}$. According to Leong (2014), the extinction coefficient is 1.310 $mM^{-1}cm^{-1}$ which is close to the ε in this study. Therefore, it is concluded that the ε found in this study is valid and repeatable.

The reaction of free laccase enzyme with ABTS decreases over time. This can be seen from Figure 4-2 where the absorbance of ABTS increased rapidly from the beginning of the reaction until the 10^{th} minute and remain almost constant after that. This showed that the highest rate of reaction occurred in the first was almost complete by 10 minutes.

Therefore, the initial rate of reaction, ΔA/min of free laccase enzyme was determined by the first 10 minutes reaction and can be obtained from the slope of the plot in Figure 4-2. From Figure 4-2, the ΔA/min is 0.1134 Abs/min. To calculate the actual rate of reaction, the ΔA/min was multiplied with the dilution factor. The rate of reaction was determined by using the equation as follow

$$\text{Rate of reaction (U/ml)} = \frac{(\Delta A/\text{min})}{\varepsilon} \times dilution\ factor \qquad \text{(Eq. 3)}$$

The rate of reaction is

$$\text{Rate of reaction} = \frac{(0.1134)}{1.365} \times 10$$

$$= 0.83\ U/ml$$

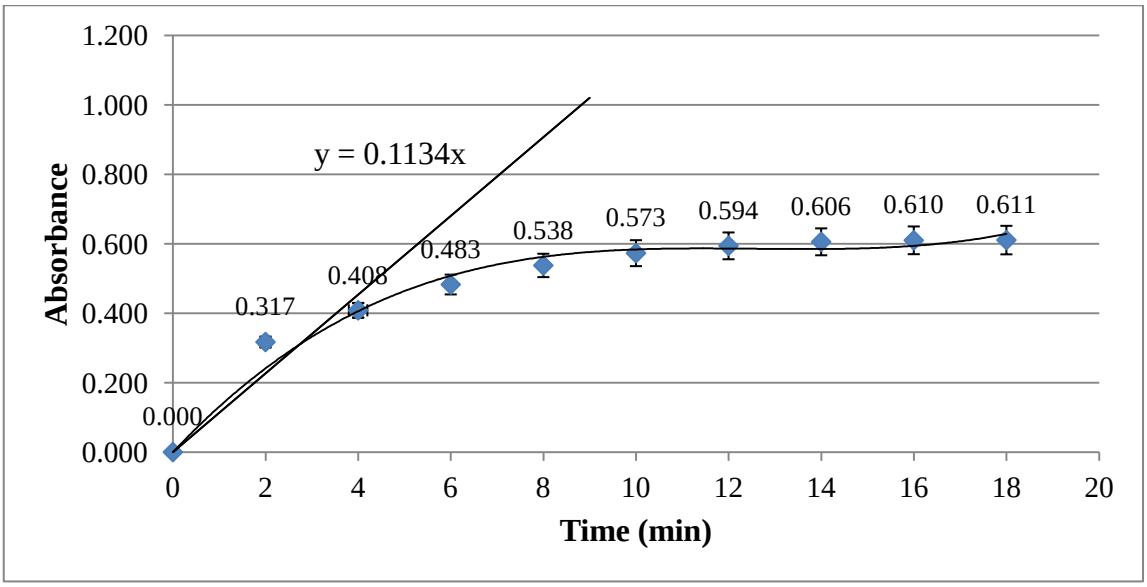

Figure 4-2 : The absorbance of ABTS (0.5 mM) versus time taken by reacting with 5 U/ml of free laccase enzyme.

4.2 Laccase Enzyme Immobilization

4.2.1 Entrapment by Sodium Alginate Cross-linked with Glutaraldehyde

After the alginate beads were soak in the $CaCl_2$ solution for hardening and glutaraldehyde for cross-linking, the $CaCl_2$ and glutaraldehyde were tested for enzymatic activity. $CaCl_2$ and glutaraldehyde reacted instantly with ABTS as there was

a different absorbance between the solution and their respective blanks. Therefore, the composition of enzyme assay for the activity of enzyme in $CaCl_2$ was 1.9 ml of 0.1 M acetate buffer (pH 4.5), 1.0 ml of ABTS and 0.1 ml of $CaCl_2$ which was used for hardening process. The blank of this enzyme assay was prepared by switching the $CaCl_2$ which was used in hardening to unused $CaCl_2$. The enzyme assay for the activity of enzyme in glutaraldehyde was prepared by substituting the $CaCl_2$ solution with glutaraldehyde.

Since the $CaCl_2$ and glutaraldehyde reacted instantly with ABTS, the increase in absorbance in Figure 4-3 and Figure 4-4 were concluded as the activity of the enzyme leaked into the $CaCl_2$ and glutaraldehyde during hardening and cross-linking process. By using the gradients of the graphs in Figure 4-3 and Figure 4-4, the enzyme activities in the respective solution were determined as 1.26 U/ml and 1.09 U/ml for $CaCl_2$ and glutaraldehyde respectively. The yield of the entrapment of laccase enzyme in this study was then calculated using the formula

$$Yield\ (\%) = \frac{500\ U - EA\ in\ CaCl2\ (U) - EA\ in\ glutaraldehyde\ (U)}{500\ U} \times 100\ \% \qquad \text{(Eq. 4)}$$

Where EA= Enzyme activity

$$Yield\ (\%) = \frac{500\ U - 125.64\ U - 109.38\ U}{500\ U} \times 100\ \%$$

$$= 53\ \%$$

According to Nam (2014), the yield of entrapment using gelatine is between 25-50 % while entrapment using sodium alginate has a yield of 80 %. However, while gelatine provides low mass transfer resistance, the leakage of enzyme to surrounding is high. In this study, a mixture of gelatine and sodium alginate was used to form stronger and more stable entrapment beads. As a result, the yield is higher than those reported using gelatine, but lower than that using sodium alginate.

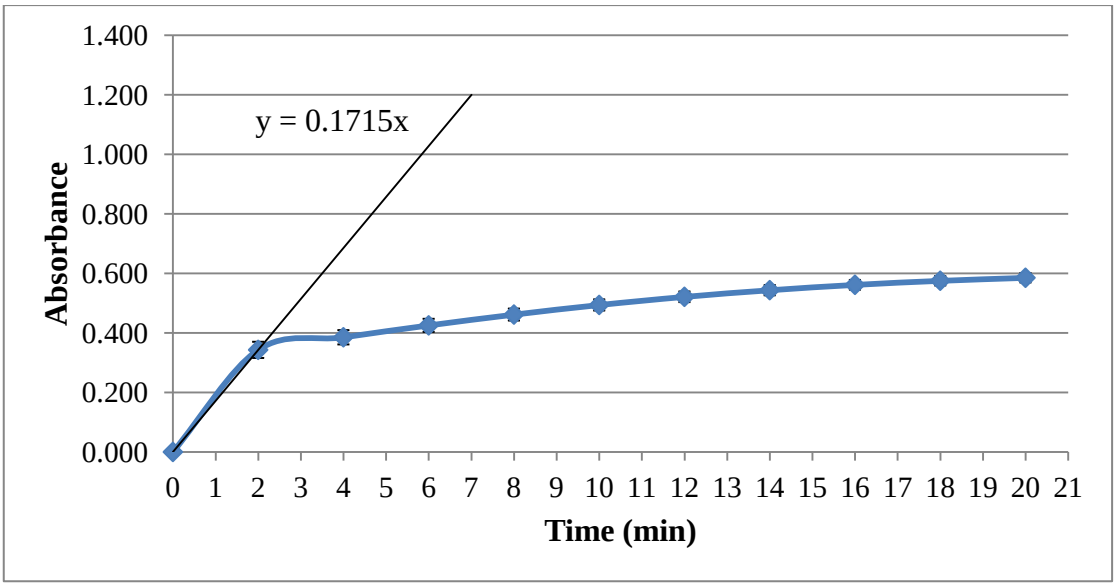

Figure 4-3 : The graph of absorbance versus time for the enzymatic activity in calcium chloride solution.

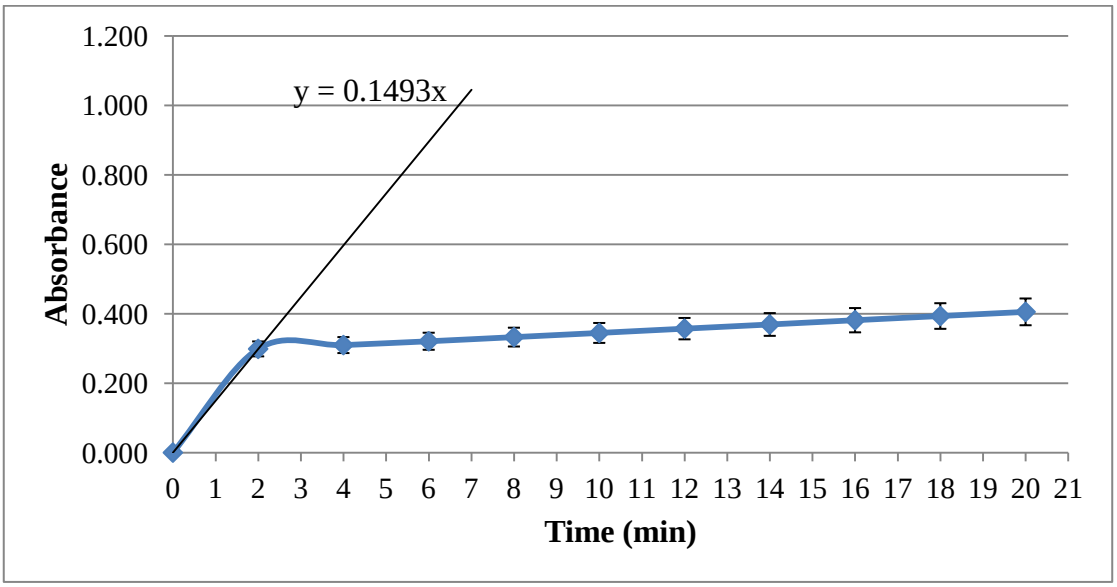

Figure 4-4 : The graph of absorbance versus time for the enzymatic activity in glutaraldehyde solution.

The yield of covalent bonded laccase enzymes from the previous study was 94 % (Leong, 2014). Therefore, immobilization by covalent bonding is a more suitable immobilization method for laccase enzyme *Tremetes versicolor*.

4.3 Reusability of Immobilized Laccase Enzyme

The immobilized laccase enzyme was reacted with ABTS for 15 successive cycles. The retained activities of covalent bonded and entrapped laccase enzymes after being reused for 15 cycles are 48.19 % and 33.50 % respectively. The retained activity is calculated by assuming the retained activity after 1st cycle is 100 %. For covalent bonded laccase enzyme the retained activity is more than 90 % for 1 cycle only. For entrapped laccase enzyme, the retained activity is less than 80 % for 1 cycle.

From Figure 4-5, it can be observed that the retained activity of entrapped laccase enzyme was higher compared to the covalent bonded laccase enzyme. However, the trend only continued until the 9th cycle where the retained activity of both immobilization methods intercepted. From the 10th cycle onwards, the retained activity of entrapped laccase enzyme was lower than the covalent bonded laccase enzyme. This happened due to the disintegration of the alginate beads during the 5th to 9th cycle. Disintegration of alginate beads caused the laccase enzyme to leaked into the ABTS and be washed off after every cycle. The disintegration occurred due to the poor immobilization handling. One example of poor immobilization handling was the presence of bubbles in the beads. Since sodium alginate dissolves slowly in water (FAO.org, 2014), sodium alginate solution was prepared by adding sodium alginate to water and stirred for 30 minutes with magnetic stirrer. During the stirring process, air tends to enter the solution causing bubbles to form in the solution. The formation of bubbles might also occur if the alginate solution was not properly withdrawn with the syringe. When bubbles were present in the beads, the chances of leakage increased as the polymerization and cross-linking bond were weaker due to the obstruction of bubbles.

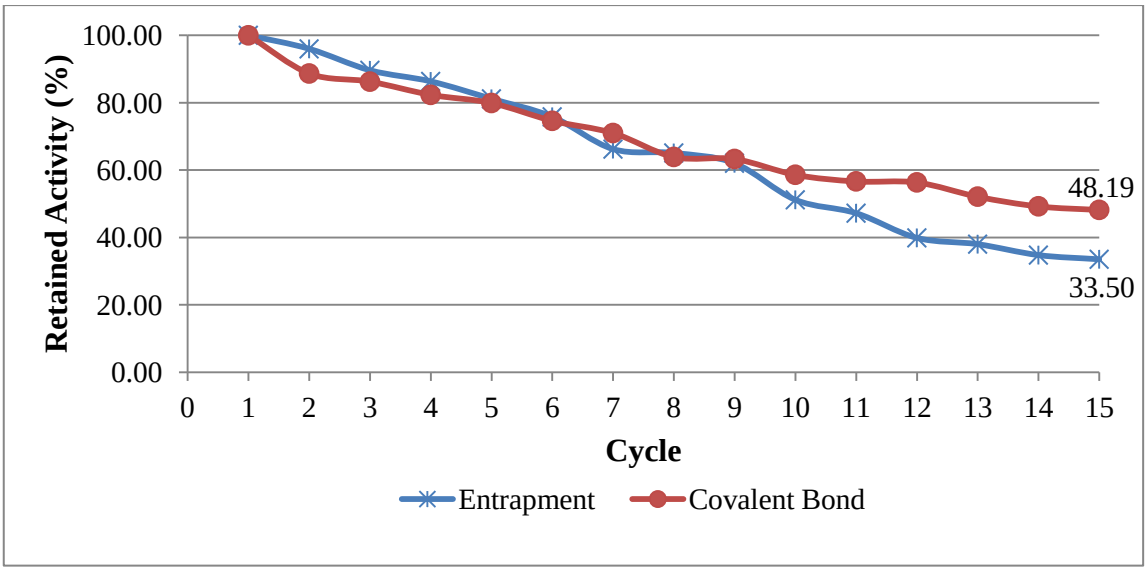

Figure 4-5 : The retained activity of entrapped and covalent bonded laccase enzymes for 15 cycles.

Secondly, the size of the syringe needles may also cause disintegration of alginate beads. The size of the needle should be small enough to produce beads with short diameter so that the ratio of contact surface area to volume will be high to give a high rate of reaction. However, if the diameter of the needle is too small which cause the ratio of contact surface area to volume to be too high, the beads might not be stable due to insufficient polymerization and cross-linking sites and hence, causing the leakage of laccase enzymes from the beads. Furthermore, according to Mahajan (2010), beads with larger diameter is more economical as the unit of enzyme can be entrapped is more compared to small beads.

Thirdly, the decrease in the retained activity may be due to the degradation of laccase enzymes from both immobilization methods. Enzymes degraded after the reaction with ABTS as they generally degrade over time (Couto *et al.*, 2007).

According to Leong (2014), the retained activity of covalent bonded laccase enzyme after 10 cycles is 56.49 %. According to Table A-2 in the appendices, the retained activity of covalent bonded laccase enzyme in this study is 58.61 %. Since the difference is only 3.6 %, it is acceptable to conclude that the procedure of this experiment is repeatable and therefore it can be used and applied in industries.

The retained enzyme activity required for a reaction to take place is 50 % (Yang *et al*., 2014). Based on Table A-1 and A-2 in the appendices, the maximum number of times that the entrapped and covalent bonded laccase enzymes can be recycled is 10 and 13 times respectively.

In conclusion, the covalent bonded laccase enzyme is better compared to the entrapped laccase enzyme in term of reusability as the retained activity and reusability of covalently bonded laccase enzyme is higher than entrapped laccase enzyme.

4.4 Leakage of Immobilized Laccase Enzyme

The immobilized laccase enzymes were kept in acetate buffer at 4^{o}C to study the leakage of laccase enzyme over a period of 32 days. Samples were taken every day for the first week and every 5 days thereafter. From Figure 4-6, the leakage of both covalent bonded and entrapped laccase enzyme increased over time. The leakages at day 32 were 14.46 % and 13.90 % for covalent bonded and entrapped laccase enzyme respectively. Since the leakages for both immobilization methods are less than 15 %, the leakage level is considered low. Low leakage level obtained due to the application of optimum storage temperature. 4^{o}C is suitable to be used to store both immobilized laccase enzymes as at this temperature, laccase enzyme is inactive and hence prolonging its shelf life.

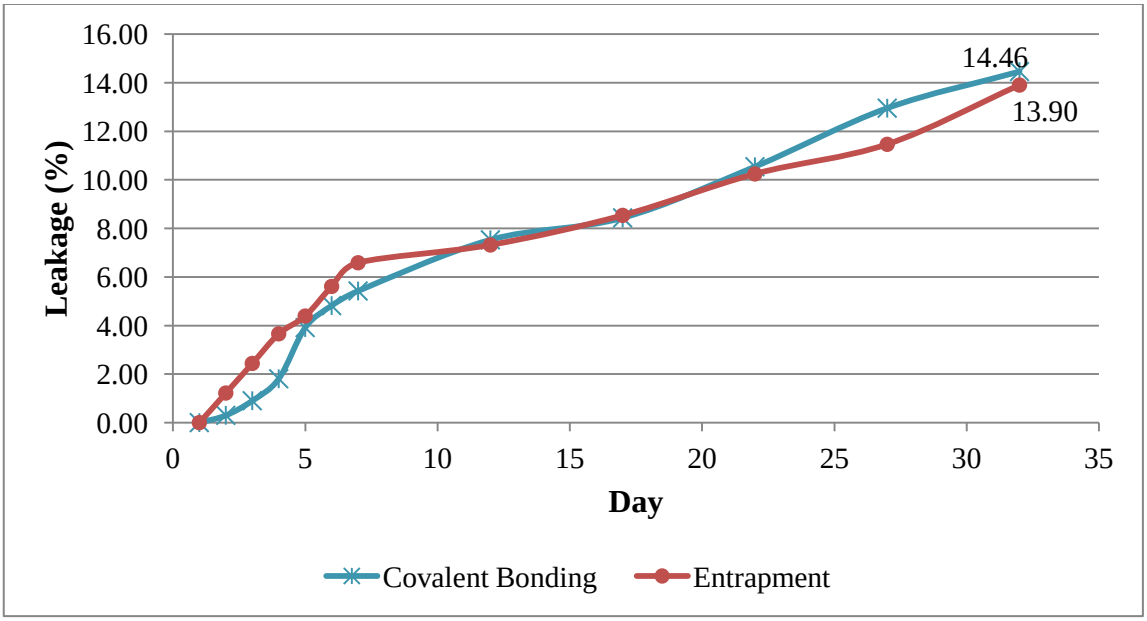

Figure 4-6 : The leakage of laccase enzyme from its respective support over a period of 32 days.

The rate of leaking increased rapidly in the first week for both immobilization method and increase slowly for the next 27 days. This happened may be due to the buffer solution that was not changed during the 32 days of study. As a consequent, the buffer solution is saturated with laccase enzyme and causing the rate of leakage to decrease.

According to Nor *et al*. (2010), laccase enzyme entrapped in PVA-alginate has a leakage of 6.49 % over 30 days. The leakage is lower as compared to the leakage of 12.68 % obtained in this study. The differences in leakage may be due to the material used. According to Wu and Wisecarver (1992), beads produced with PVA are more elastic and have high strength and durability. However, PVA is an extremely sticky material and therefore, PVA beads tend to agglomerate. When the beads clustered, the mass transfer limitation increases. Hence, laccase enzyme entrapped with PVA-alginate must be handled carefully to avoid the agglomeration of the beads. On the other hand, laccase enzyme entrapped in gelatine-alginate beads does not have the tendency to cluster. Therefore, the gelatine-alginate entrapment studied in this work is better compared to PVA-alginate entrapment as the difference in leakage is only 6.19 % but much easier to be carried out.

The low leakage for covalent bonded laccase enzyme is the result of the high affinity interaction of alumina pellet (pI = 7 to 9) with the acidic laccase enzyme and the acidic buffer solution (pH = 4.5) which enhanced the storage environment (Fernandez-Fernandez *et al.*, 2013). On the other hand, entrapped laccase enzyme has low leakage because the enzyme was protected in a matrix. The gelatine in the matrix narrowed the pore of the beads reducing the chances of leakage. Degradation of laccase enzyme was less likely to occur as the PEG was added to prevent the deactivation of laccase enzyme. Furthermore, mass transfer limitation provided by alginate made the beads more secure.

Comparing the two immobilization methods, the leakage were close to each other. This showed that the supports in these two immobilization methods were able to protect the laccase enzymes from leaking into the surrounding environment. From the observations, it is safe to assume that the support is able to protect the laccase enzyme providing the support has a low degradation rate.

4.5 Comparison of the Analysis Done on Covalent Bonded and Entrapped Laccase Enzyme

Table 4-1 shows the summary of the analysis done on covalent bonded and entrapped laccase enzyme. It can be observed that the immobilization by covalent bonding has high immobilization yield and high retained activity for reusability test as compared to immobilization by entrapment. However, the leakage of covalent bonded laccase enzyme is higher than the entrapped laccase enzyme. Since the difference of leakage between both immobilization methods is very small, it is safe to conclude that the immobilization by covalent bonding is better than entrapment.

Table 4-1 : Summary of the analysis done on covalent bonded and entrapped laccase enzyme.

Analysis	**Covalent Bonding**	**Entrapment**
Immobilization yield (%)	94.00	53.00
Retained activity for reusability test (%)	48.19	33.50
Leakage (%)	14.46	13.90

5 CONCLUSION & RECOMMENDATIONS

Laccase enzyme immobilized by entrapment in sodium alginate and gelatine was compared with the covalent bonded laccase enzyme immobilized in alumina pellets obtained from a previous study. From the results obtained, the immobilization yield of laccase enzyme that was immobilised through covalent bonding was 94 % which was higher than that of the laccase enzyme immobilised through entrapment (53 %). The reusability of laccase enzyme immobilised through entrapment and covalent bonding were 10 and 13 cycles respectively while the leakage for entrapped an covalent bonded laccase enzymes were 13.9 % and 14.46 % respectively. Both immobilization methods are considerably good as the result of analysis showed that the reusability and storage stability were generally high and the difference of the leakage between both methods of immobilization was small. Immobilization by covalent bonding was much preferred over entrapment as its immobilization yield and reusability were higher.

For future studies, it is recommended that the study on the immobilization of *Trametes versicolor* be expanded to explore more immobilization techniques that can in turn be compared to current immobilization techniques in order to determine the best method of immobilization. Aside from this, further experimental work can be carried out on the extensive application of this immobilization method. The characteristics of laccase enzymes should also be explored to expand the application of laccase enzymes in industries.

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APPENDICES

Table A-1

Data of reusability for laccase enzyme immobilized by entrapment.

Cycle	A (0.0918 g)	B (0.0980 g)	C (0.0946 g)	Average	c/min	Retained Activity (%)
1	0.596	0.578	0.587	0.5870	0.430037	100.00
2	0.575	0.560	0.555	0.5633	0.412698	95.97
3	0.540	0.521	0.517	0.5260	0.385348	89.61
4	0.453	0.523	0.544	0.5067	0.371184	86.31
5	0.422	0.500	0.507	0.4763	0.348962	81.15
6	0.398	0.455	0.482	0.4450	0.326007	75.81
7	0.360	0.384	0.422	0.3887	0.284737	66.21
8	0.395	0.374	0.377	0.3820	0.279853	65.08
9	0.354	0.370	0.368	0.3640	0.266667	62.01
10	0.296	0.306	0.299	0.3003	0.220024	51.16
11	0.268	0.273	0.290	0.2770	0.202930	47.19
12	0.241	0.228	0.233	0.2340	0.171429	39.86
13	0.215	0.224	0.230	0.2230	0.163370	37.99
14	0.219	0.172	0.221	0.2040	0.149451	34.75
15	0.203	0.195	0.192	0.1967	0.144078	33.50

Table A-2

Data of reusability for laccase enzyme immobilized by covalent bonding.

Cycle	A (0.2100 g)	B (0.2089 g)	C (0.2083 g)	Average	c/min	Retained Activity (%)
1	0.180	0.248	0.234	0.2207	0.161661	100.00
2	0.170	0.206	0.211	0.1957	0.143346	88.67
3	0.166	0.200	0.205	0.1903	0.139438	86.25
4	0.161	0.192	0.192	0.1817	0.133089	82.33
5	0.155	0.186	0.188	0.1763	0.129182	79.91
6	0.149	0.171	0.174	0.1647	0.120635	74.62
7	0.140	0.165	0.165	0.1567	0.114774	71.00
8	0.133	0.131	0.159	0.1410	0.103297	63.90
9	0.128	0.158	0.133	0.1397	0.102320	63.29
10	0.125	0.109	0.154	0.1293	0.094750	58.61
11	0.117	0.123	0.135	0.1250	0.091575	56.65
12	0.122	0.123	0.128	0.1243	0.091087	56.34
13	0.104	0.118	0.123	0.1150	0.084249	52.11
14	0.119	0.104	0.103	0.1087	0.079609	49.24
15	0.104	0.111	0.104	0.1063	0.077900	48.19

Table A-3

Data of leakage for laccase enzyme immobilized by covalent bonding and entrapment.

Day	Covalent (150ml)					Entrapment (250ml)				
	ΔA/min	ΔC/min	Enzyme activity (U/ml)	Unit of enzyme (U)	Leakage (%)	ΔA/min	ΔC/min	Enzyme activity (U/ml)	Unit of Enzyme (U)	Leakage (%)
1	0.0332	0.0243	0.2432	36.4835	0.0000	0.0410	0.0300	0.3004	75.0916	0.0000
2	0.0333	0.0244	0.2440	36.5934	0.3012	0.0415	0.0304	0.3040	76.0073	1.2195
3	0.0335	0.0245	0.2454	36.8132	0.9036	0.0420	0.0308	0.3077	76.9231	2.4390
4	0.0338	0.0248	0.2476	37.1429	1.8072	0.0425	0.0311	0.3114	77.8388	3.6585
5	0.0345	0.0253	0.2527	37.9121	3.9157	0.0428	0.0314	0.3136	78.3883	4.3902
6	0.0348	0.0255	0.2549	38.2418	4.8193	0.0433	0.0317	0.3172	79.3040	5.6098
7	0.0350	0.0256	0.2564	38.4615	5.4217	0.0437	0.0320	0.3201	80.0366	6.5854
12	0.0357	0.0262	0.2615	39.2308	7.5301	0.0440	0.0322	0.3223	80.5861	7.3171
17	0.0360	0.0264	0.2637	39.5604	8.4337	0.0445	0.0326	0.3260	81.5018	8.5366
22	0.0367	0.0269	0.2689	40.3297	10.5422	0.0452	0.0331	0.3311	82.7839	10.2439
27	0.0375	0.0275	0.2747	41.2088	12.9518	0.0457	0.0335	0.3348	83.6996	11.4634
32	0.0380	0.0278	0.2784	41.7582	14.4578	0.0467	0.0342	0.3421	85.5311	13.9024