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ACTIVITY AND STABILITY OF LACCASE ENZYMES IN BIOCOMPATIBLE IONIC LIQUIDS

PREPARED BY:

NUR ATIKAH BTE RAZALE

14283

SUPERVISOR:

DR. MUHAMMAD MONIRUZZAMAN

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CERTIFICATION OF APPROVAL

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Approved by,

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(Dr Muhammad Moniruzzaman)

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH, PERAK

September 2014

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

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NUR ATIKAH BTE RAZALE

ABSTRACT

The usage of ionic liquids (ILs) as a solvent together with laccase enzyme can increase the activity & stability of the enzyme (Moniruzzaman, 2013). Unfortunately, most of the ILs deactivate the enzyme. Many researches had been geared up to find the suitable ionic liquids that promotes most stable and high activity of enzymatic reaction. Therefore, this proposal will conduct a study on the activity and stability of the laccase enzyme reaction in various type ILs. The study will focus in analysis the trends of enzymatic performances in ionic liquids (ILs), physical & chemical properties of ILs and the factors & conditions that favours enzymatic reactions.

The enzyme activity and stability will be analysed by using Trametes sp. laccase together with 0.01 M of sodium acetate as a buffer solution and ILs and 2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) or (ABTS) (50mM) as the substrate for the enzymatic reaction. There are 4 types of ionic liquids used in this experiment which are 1-ethyl-3-methylimidazolium acetate [EMIM] (OAC), 1-ethyl-3-methylimidazolium octyl sulphate [EMIM] (OSO_4)**,** 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] (DEP) and 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] (MESO4). The mixture was stirred at 400rpm at 50^oC for about 3 minutes. Acetic acid was added in order to stop the reaction. Then, the sample was put into the Ultraviolet-visible spectroscopy UV-Vis to get the absorbance value. The value of specific activity and residual activity (RA %) were calculated.

The result obtained show that the specific activity of laccase enzyme decreases with the increase of IL (wt %). The higher the amount of ILs, the higher the enzyme activity. [EMIM] [OSO4] recorded the highest specific activity followed by [EMIM] [DEP] and [EMIM] [OAC]. On the other hand, residual activity of all ILs decreases with Incubation time. The longer the incubation time, the lower the residual activity. [EMIM] (DEP) recorded the highest stability followed by [BMIM] (MESO4) and [EMIM] (OAC). Besides, for the temperature variation, the result indicate that the activity of laccase enzyme can be achieved at T=50^oC.

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Nomenclatures

ILs- Ionic Liquids

[EMIM] (OAC)- 1-ethyl-3-methylimidazolium acetate

[EMIM] (OSO4)- 1-ethyl-3-methylimidazolium octyl sulfate

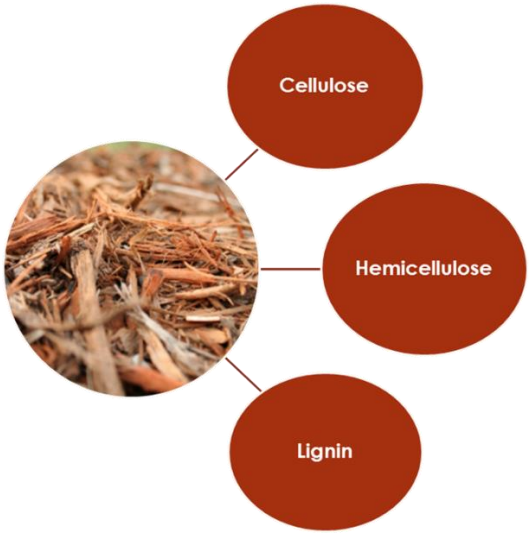
[EMIM] (DEP)- 1-ethyl-3-methylimidazolium diethyl phosphate

[BMIM] (MESO4)- 1-butyl-3-methylimidazolium methyl sulphate

CHAPTER 1: INTRODUCTION

1.1 Background

Wood is a carbon-neutral renewable resource of bioenergy and biomaterial production. Amorphous multicomponent polysaccharide hemicellulose, rigid semi-crystalline polysaccharide cellulose and the amorphous aromatic polymer lignin are three composition consist in a wood. (Muhammad Moniruzzaman, 2013)



Picture 1: Composition of wood

Cellulose that present in the wood or biomass are widely used as a raw materials for production in many industries such as pulp paper, textile, construction and biofuels. There are many methods that can be employed in order to extract cellulose and it can be classified into two classes. The first one is physical and chemical treatment while the second one is biological treatment. Both of the methods have their own advantages and disadvantages.

For example, department for environment, food and rural affairs in United Kingdom had reported the about the method that they had been using to extract cellulose from wood which sulphate method or Kraft pulping method. But unfortunately the, this method requires high temperature, pressure and pH that

will then contributes to high cost of the treatment and causing a very serious environmental hazard. The Kraft pulping method is the most popular and is responsible for around 80% of world cellulose production.

In other perspective, biological method by using enzyme treatment are more environmental friendly but time consuming and low production of cellulose. The researchers had tried to find the suitable biological method for higher cellulose production. On 2012, researches had found that usage of ionic liquids as a solvent together with laccase enzyme can increase the activity & stability and result in a higher cellulose production. (Moniruzzaman, et.al, 2013).

The presence of enzyme as a catalyst will increase the yield and speed up the reaction time of the process. Thus, produce more desired products within a short time. Laccase enzyme is among the famous type of enzymes used nowadays. Laccases can be found in fungi, microorganism and plants. It has captured the attention of many researches since the studies of enzymatic degradation of wood which is first started in Japan (Baldrian.P, 2006). The most interesting part of using laccases are it promotes green chemistry which is environmental-friendly, highly efficient and sustainable.

In a meanwhile, ionic liquids is a salt consists of organic anions and inorganic cations. Ionic liquid had been called as designer solvent where people can choose different types combination of anion and cation in order to get their desired ionic liquid characteristics. Ionic liquid had also been labelled as 'green solvent' due to their properties which are low volatility, high stability and good ionic conductivity (Xinxin Yu, et.al, 2013). Based on several investigation that had been conducted in recent years, it has been found that the usage of ionic liquids as a solvent together with laccase enzyme had result in higher cellulose percentage extraction which is 73.1% compared to the wood treated in the absence of ionic liquids (Moniruzzaman, et.al, 2013).

Many researches had been geared up to find the most suitable ionic liquids that promotes the most stable and high activity of enzymatic reaction for the cellulose extraction in industries. Therefore, in this study, laccase enzyme had been chosen to be tested for the enzymatic activity and stability analysis in biocompatible ionic liquids.

1.2 Problem Statement

The extraction of cellulose from wood or biomass can done by using many types of methods. As mentioned in the introduction above, the method for cellulose extraction can be classified into 2 classes which are physical& chemical method and biological method. Both of the processes have their own advantages and disadvantages. Most of the physical and chemical methods require high temperature, pressure as well as chemical concentration for the cooking process (Muhammad Moniruzzaman et.al, 2013). It will result in high cost since more energy needed to meet the parameters that had been set. Besides, sulfates and sulfite pulping process is among a famous methods employed by industries to extract cellulose. Unfortunately, this method cause a very serious environmental hazard.

For biological treatment, it had been conducted with the presence of enzymes. This process is a very slow approach due to the difficulties in enzyme accessibility to the solid substrate and poor solubility of lignin (Sousa et. al., 2009). Therefore, researchers had geared up their research on how to extract more cellulose by using a safe biological treatment. Based on several investigation that had been conducted in recent years, it has been found that the usage of ionic liquids as a solvent together with laccase enzyme had result in higher cellulose percentage extraction which is 73.1% compared to the wood treated in the absence of ionic liquids (Moniruzzaman, et.al, 2013). Since then, many studies had been conducted to investigate the mechanism of the interaction between enzymes and ionic liquids.

There are many types of ionic liquids such as sodium chloride, sodium nitrate and 1, butyl-3, methylimidazolium trifluoromethanesulfonate ([Bmim]TfO). Erbeidinger et. al. in the early 2000, had discovered that ILs as a solvent can increase the activity and stability of enzymatic reactions. Until now, more researches are still ongoing to determine which ionic liquids is the suitable. Some of the ionic liquids favours the enzyme reactions and stabilizes it while some of it degrades the enzyme activity (Xinxin Yu, et.al, 2013).

Therefore, this proposal will conduct a study on the activity and stability of the enzymatic reaction in various type ionic liquids. It will present the results of enzyme activity and stability in ionic liquids. There are 4 types of ionic liquids used in this experiment which are 1-ethyl-3-methylimidazolium acetate [EMIM] (OAC), 1-ethyl-3-methylimidazolium octyl sulphate [EMIM] (OSO_4)**,** 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] (DEP) and 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] (MESO4).The activity and stability of the laccase enzyme will be represented by using the specific activity and residual activity (RA%).

1.3 **Objectives of the study**

Three objectives had been set up to be achieved by the end of the study:

i. To study the laccase enzymes activity and stability in various ionic liquids.
ii. To investigate the reaction temperature for optimum activity of laccase enzymes.

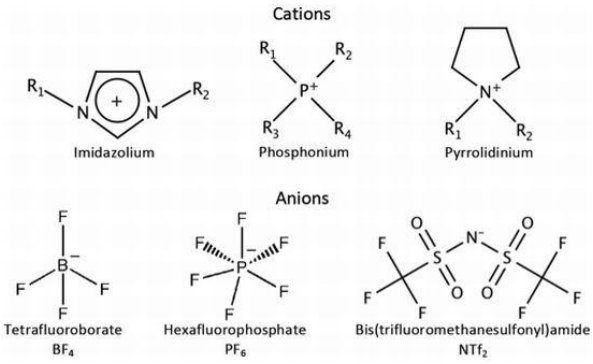
1.4 Scope of Study

The study will focus in analysis the trends of enzymatic performances in ionic liquids (ILs). Therefore, more study need to be done on the ILs itself such as the physical and chemical properties of ILs. Besides, the factors and conditions that favours enzymatic reactions will be involved as one of the scope of study. On the other hand, the other scope of study that are involved in this project are Reaction Kinetic Engineering and Biochemical Engineering. Reaction Kinetic Engineering will be used to calculate the specific activity and stability of the enzymatic activity. As this study, open up a chance of transformation for ionic liquids as the new solvent for enzyme reactions in industries, therefore it falls under biochemical engineering and bio technology field of studies. Last but not least, the method to analyse the absorbance of the sample by using Ultraviolet-visible spectroscopy (UV-Vis) falls inside analytical chemistry.

CHAPTER 2: LITERATURE REVIEW AND THEORY

2.1 Ionic Liquids and Its properties

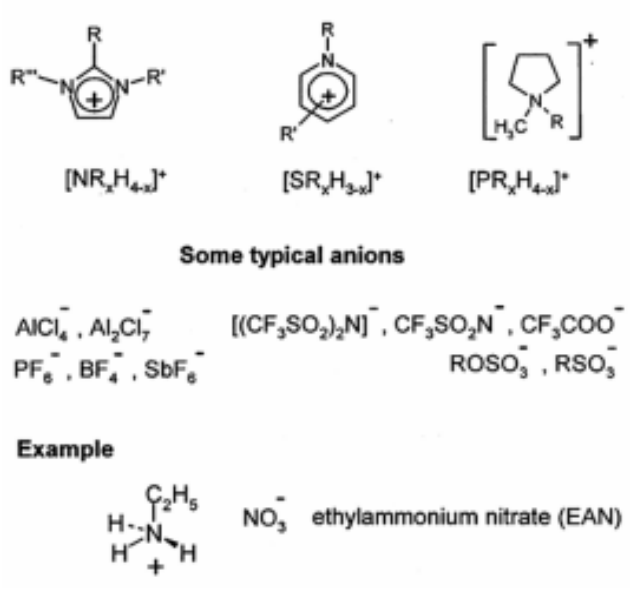
Ionic liquids are composed of organic anions and inorganic cations. Ionic liquids is labelled as ‘designer solvent’ due to its inimitable characteristic that offer a combination of different types of anion and cation that will eventually results in a wide range of solvent properties (Rogers, R.D. et.al. 2003). Besides, Ionic liquids also draw attention of many researchers with their unique volatility properties which is negligible. This indicate that almost no amount of volatile ionic liquids (VOCs) during its use. In most of ionic liquids, low or zero value of volatility will result in no meaning of vapour pressure, flash point and boiling point itself. Below is the picture of molecular structure of ionic liquids.



Picture 2: The molecular structure of Ionic Liquids

Ionic liquids had attracted many industrial personnel for the usage of ionic liquids as solvent in their processes such as catalytic reactions. Ionic liquids had also being called as ‘green’ solvent as it is eco-friendly and sustainable. However, the challenge that must be face is due to lack of information of this new solvent (Wilkes, J.S 2003). The selection of the most suitable ionic liquids for certain applications must requires knowledge on the characteristic and properties of the solvent. Until now, research on application of ionic liquids are still being carried out to boost up its involvement in the industry.

2.1.1 Structural Features of Ionic Liquids



Picture 3: Typical structure of anion and cation of ionic liquid

The figure above showing the typical structure of cation and anion that are usually used in synthesizing ionic liquids(Singh. G, et.al. 2008). Ionic liquid consists of a cation which is normally a bulk of organic structure with low symmetry. While the anion of ionic liquids may either be organic or inorganic. Table below are some examples of anion and cation of ionic liquids:

Table 1: Examples of cation and anion of ILs

Cation	Anion
1. Ammonium 2. Sulfonium 3. Phosphonium 4. Imidazolium 5. Pyridinium 6. Picolinium 7. Pyrrolidinium	1. $[BF_4]^-$ 2. $[SbF_6]^-$ 3. $[PF_6]^-$ 4. $[CF_3SO_3]^-$ 5. $[(CF_3SO_2)_2N]^-$ 6. $[Tf_2N]^-$ 7. Alkyl sulfates

Ionic liquids cation and anion were bonded through van der waals forces. Ionic liquids with $[BF4]^-$ and $[PF6]^-$ anions are air stable and neutral. Besides, Ionic liquids containing $[CF3SO3]^-$ and $[Tf2N]^-$ anion possess low melting point and stable in water and medium containing Lewis acids.

2.1.2 Physical and Chemical Properties of Ionic Liquids

The physico-chemical properties of ionic liquids are very sensitive. It can be easily altered by the presence of impurities such as organic solvents, water and chloride ions (Singh. G, et.al. 2008). Therefore, it is very crucial to be careful when handling the process involving ionic liquids due to its sensitivity towards impurities. Precautions need to be taken to prevent any presence of impurities when handling ionic liquids.

Besides, the combination of the cation and anion will determine the melting point value of the ionic liquids. The melting point of ionic liquid can be correlated with the composition and structure of ionic liquids. Combination of different anion and cation will result in different melting value (Singh. G, et.al. 2008). The symmetry of the ionic liquid cation affects the melting point value. The cation with higher symmetry will have higher melting point compared to the one with lower symmetry. This is among the uniqueness of ionic liquids where its properties can be designed through different combination of anion and cation.

Next, for the densities of ionic liquids it highly depended on the bulkiness of the organic cation and the choices of the anion. The magnitude of ionic liquids density depends on the constituent of its cation and anion. But generally ionic liquids is denser than water with the range of 1.05 to 1.36 g/cm^3 at ambient temperature. On the other hand, different ionic liquid possess different viscosity value. It is a fact that a high viscosity solvent is not suitable to be used as a solvent media. Some ionic liquids have high viscosity and it must be alert that these type of ionic liquids are not suitable to be used as a solvent. It will affect the progress of the chemical reaction.

Ionic liquids also manage to maintain its stability even at high temperatures. Most of it are still stable until 400 degree celcius. For the thermal stability of ionic

liquids, it tells a whole different story compared to other properties. It is the nature of anion itself will determine the thermal stability of an ionic liquids rather than cation. Besides, before choosing an ionic liquids as a solvent, it is very important for us to know its diffusivity and conductivity value. The rate of ionic diffusion are following this order: [EMIM][Tf_2N] > [EMIM] [BF_4] > [BP] [Tf_4N]> [BP] [BF_4]. The diffusion coefficient of ionic liquids are strongly influence the pair of cation and anion also. While the conductivity of ionic liquids are influenced by its size and pairing of ion (cation and anion).

The lack of volatility of ionic liquids may be the single most attractive property of ionic liquids for use as reaction solvents. Because the volatility is low or zero, the vapor pressure, boiling point, critical pressure, heat of vaporization and flash point have no meaning in most ionic liquids (Wilkes, J.S. 2004). Further research on ionic liquids properties are still need to be continued. The properties of ionic liquids affect the progress of chemical reaction. Therefore, the right ionic liquids with suitable properties must be chosen in order to favours and assist the chemical reaction. (Wilkes, J.S. 2004).

Welton. T, (1999) in his journal on Room-Temperature Ionic Liquids. Solvent for Synthesis and Catalysis had mentioned on the physical properties of Ionic Liquids which are unique and very fascinating solvents for synthesis. Among the properties stated are ionic liquids serve as a good solvents for many types of organic and inorganic materials even reagent with unusual combinations can be brought into the same phase.

Besides, Ionic liquids are also immiscible with a number of organic solvents and provide a non-aqueous polar alternatives for two phase system. Ionic liquids did not evaporate and can be used in high-vacuum systems because there are non-volatile. On the other hand, Ionic liquids also composed of poorly coordinating ions, so they have potential to be highly polar yet coordinating solvents (Welton. T, 1999).

The densities of most of the ionic liquid is greater than water except for pyrrolidinium dicyanodiamide and guanidinium with density ranging from 0.9 to 0.97 g/cm^3. For I-methylimidazolium ionic liquids, its density decrease with the increasing temperature. But it is opposite for the viscosity properties. Ionic liquids are viscous liquids compared to the conventional organic solution. (Zhang. S, et.al. 2005). Apart

from that, the price of ionic liquids in the market is expensive with the range of USD 700 and above. People might refuse to choose ionic liquids as a solvent for their chemical process due to the high cost demand in the market. However in fact, many should know that although the price is high but it can be recycled to be used again and offer comparable performance in chemical transformation. (Singh. G, et.al. 2008).

2.2 Laccase Activity and stability

There are several researches had been conducted in order to investigate the compatibility and conformity of laccase enzyme in different types of ionic liquids. Xinxin, Y. et.al. had presented the result of her study on the conformity, activity and stability of laccases in three trifluoromethanesulfonate ionic liquids which are 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([Bmim]TfO) , 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate ([Bmpyr]TfO) or tetramethylammonium trifluoromethanesulfonate ([TMA]TfO). Based on the study, laccase enzyme had been destabilized by ([Bmim]TfO) but ([TMA]TfO) stabilize it. The findings concluded that ([TMA]TfO) improve greatly the stability of enzymes but not a good activating agent.

On the other hand, based on Vernekar, M. et.al, in his research project on laccase properties and application, he had analysed the effects of pH value, temperature and inhibitors in influencing the activity of laccase. The study had concluded that the optimal pH of laccase for the highest rate of activity is depending on the type of substrate used. For example, the optimum pH of ABTS substrate for laccase activity is between pH 3 and pH 5 (Henzkill et al. 1998). The same trend was observed in second parameter which is temperature. But Farnet et al. (2000) had found that at 40^{o}C and 50^{o}C with the pre-incubation of enzymes will rapidly enhance the activity of laccase.

Some anion had been identified able to inhibit the activity of laccase such as cyanide, hydroxide, azide and halides (excluding iodide). The anion will bind with the copper atom in laccase that will eventually halted the internal electron transfer of the enzyme.

Ozsolen. F. et al. (2010) in his journal on enhanced production and stability of laccase had revealed a findings on the effect of using different solid substrates. Based on the figure 2 below, it shows that, the highest laccase activity detected when Ground clover was used as the substrate and T-versicolor is a source of laccase production.

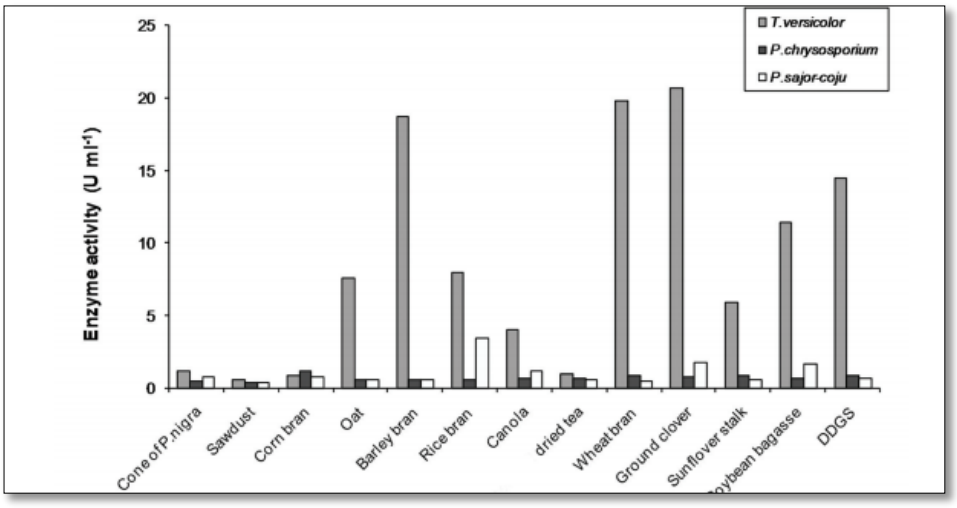


Figure 1: The effect of different solid substrate on laccase activities

Besides, Ozsolen. F. et al. (2010) had continued their research for its stability by preincubate the laccase enzyme for 30 minutes at different temperatures (20-90^{o}C). It can be observed that between 20^{o}C to 50^{o}C, optimum stability of laccase achieved.

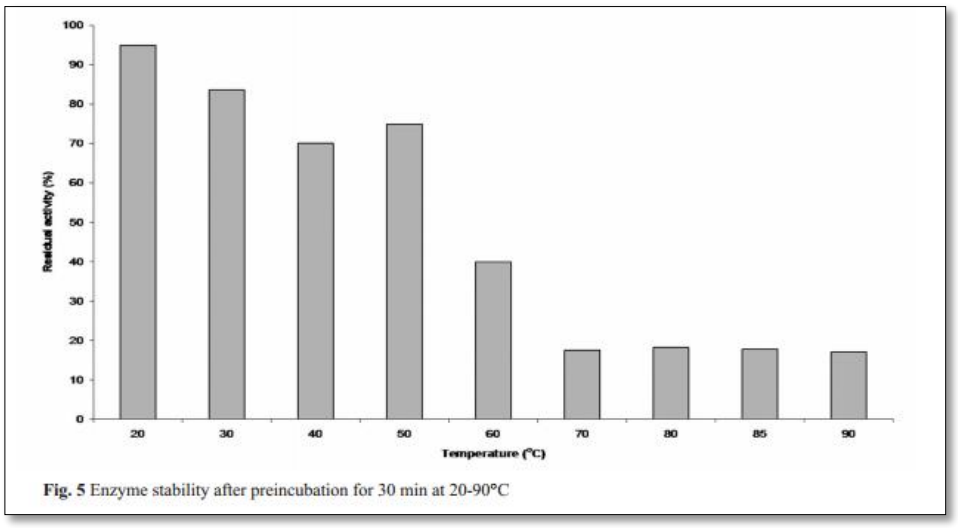


Figure 2: Enzyme stability after pre-incubation for 30 min at 20-90 degree celcius

2.3 Laccase Enzyme activity and stability in ionic liquids

Tavares A.P, et.al. (2008) had conducted a research on the alternative of using ionic liquids as the co-solvent for laccase. They had done a research on the laccase enzyme activity and stability on three different water soluble ionic liquids which are (1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulfate, [emim][[MDEGSO4], 1-ethyl-3-methylimidazolium ethylsulfate, [emim][EtSO4], and 1-ethyl-3-methylimidazolium methanesulfonate, [emim][MeSO3]). Besides, the researcher had also compared to the activity and stability of laccases enzyme in two other organic solvents.

The result shows that early enzyme condition is the same among the ILs if the same parameters or conditions were used. A high reduction on initial enzyme activity was found with acidic pH (5.0). The effect of pH and solvent concentration on enzyme stability were investigated in more detail for 1 week. The enzyme maintained a high stability at pH 9.0 for all ILs tested. [emim][MDEGSO4] was the most promising IL for laccase with an activity loss of about 10% after 7 days of incubation.

Many other researches had been conducted to find the most suitable ionic liquids and parameters that will enhance the activity and stability of laccase enzyme. Moniruzzaman, et.al, (2013) had published their findings on their research of separation and characterization of cellulose fibers from cypress wood treated with ionic liquid prior to laccase treatment. Their result shows that the usage of ILs as the solvent together with enzyme, result in higher cellulose percentage extraction which is 73%.

Xinxin. Y, et.al. (2013) had presented her research paper effect of three trifluoromethanesulfonate ionic liquids on the activity, stability and conformation of laccase. The findings concluded that ([TMA]TfO) improve greatly the stability of laccase enzymes but not a good activating agent. Besides, laccase possess catalytic activity for the degradation of the phenol in systems containing ionic liquids (Tavares, A.P.M. et al, 2012). This findings had been mentioned in the research paper entitled Biocatalyst in ionic liquid: Degradation of Phenol by Laccase. It show ILs have good performances in becoming the solvent for many enzyme activity for chemical reaction.

On the other hand, one research had also been conducted for laccase enzyme activity and stability in biocatalyst for ILs media. This experiment uses Laccase incorporated into PEG-PLA polymer to be tested with the ILs. This laccase enzyme was coated with poly (ethylene glycol). The activity and stability of PEG-PLA-laccase complex have been compared to the native laccase in an ILs. This laccase polymer retained most of its enzymatic activity and stability and record an excellent storage stability in ILs with over 70% of its initial activity retained after 12 days of storage in IL at 40^{o}C whereas it was about 20% for native laccase under the identical conditions.

2.4 Method to analyse the stability and activity of laccase enzyme

This section will discussed in several methods used for the analysis of laccase enzyme activity and stability:

2.4.1 Laccase activity and stability evaluation by using spectrophotometry (Xinxin. Yu.et. al. 2013)

For the first method in assessing laccase activity, the author had used 2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) or ABTS solution as the substrate with 20mM sodium acetate buffer at pH value of 5.0. Then, incubated laccase was mixed with sodium acetate buffer solution together with Ionic liquids. The temperature condition was maintained at 35^{o}C. After that, 20μL of ABTS plus buffer solution was added in the mixture of laccases, IL and buffer solution. The mixture then was put into the spectrophotometric and the change of absorbance at 420nm was observed and recorded.

Next, for the stability analysis method, the author using different concentration of ionic liquids. Few sample of laccase in ionic solution were incubated at 10h, 1 day, 2 days, 3 days or 5 days. After that, the step for the stability analysis are the same as mentioned in activity analysis method above.

2.4.2 Laccase activity and stability measurement method (Kubis.J.S.et. al. 2013)

The measurement of laccase activity will be conducted by preparing 2mL of buffer and 2mL of ionic liquids was mixed vigorously at the room temperature for 1 day. The buffer solution was prepared by using 0.025M of phosphate-citrate. For the reaction to take place, 1mL of the equilibrated buffer was pre-incubated at 30^{o}C followed by the addition of 5 μL of enzyme solution. The reaction will start once the ABTS solution was added into the mixture of enzyme and buffer solution. The absorbance of the sample will then being monitored at 420nm.

After that, the stability of laccase will be measured by preparing 2mL of buffer solution with 2mL of ionic liquids and mixed for 24 hours. The step will be continued with the pre-incubation of the mixture. After that, 50 $\mu L$of enzyme was added to the mixture prepared earlier and the first 50 μL sample from ionic liquid and buffer were taken off for activity measurement.

2.4.3 Measurement of Laccase activity and stability (Muhammad Moniruzzaman.et. al. 2013)

To conduct the enzymatic reaction analysis, 20 μL of laccase solution was added into 1.96 sodium acetate buffer solution (0.1 M). The solution will then stirred at 50^{o}C. Finally 20 μL of 50mM ABTS solution which is mixed with buffer solution was added to start the reaction. The change of absorbance will be recorded at 420nm once the sample being put into the UV-Vis. Catalytic activity will be determine from the slope of the kinetic curve. Besides, the stability of the enzyme was determined by mixing buffer solution together with 0, 2.5, or 5 wt% of ionic liquid containing laccase enzyme. Th solution will then incubated at 50^{o}C. After that, the sample were withdrawn at predetermined time intervals and ABTS solution was added. The stability will then being determined by the residual activity.

CHAPTER 3: METHODOLOGY

3.1 Experiment Methodology

3.1.1 Material and Reagents

Ionic liquids was obtained from Ionic Liquids Centre of University Technology PETRONAS. There are 4 types of ionic liquids used in this experiment which are 1-ethyl-3-methylimidazolium acetate [EMIM] (OAC), 1-ethyl-3-methylimidazolium octyl sulphate [EMIM] (OSO_4)**,** 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] (DEP) and 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] (MESO4).The native laccase trametes sp. was chosen as the sample of enzyme for this project. Besides, 0.01M sodium acetate had been chosen as the buffer solution throughout the reaction. 2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) or (ABTS) is the substrate used for the experiment. Acetic acid is also needed for this experiment. On the other hand, the equipment used to measure the activity and stability of the enzyme is Ultraviolet-visible spectroscopy (UV-Vis) that will measure the absorbance of the sample. Besides, few other equipment are also needed such as stirrer, sample cell and beaker.

3.1.2 Measurement of Laccase Activity and Stability in Biocompatible Ionic Liquids

Solution Preparation

1. **Buffer Solution (Prepare sodium acetate at 0.01M at pH 4.5)**
   (a) Weigh the acetic acid, glacial at 0.038g
   (b) Weigh sodium acetate, Trihydrate at 0.049g
   (c) Mix the weighed acetic acid and sodium acetate into a 250ml conical flask and add distilled water inside the conical flask until 250ml calibration mark
   (d) Test the pH value of the solution at pH 4.5

2. **Laccases solution**
   (a) Weigh 0.002g of laccase enzyme and put into sample bottle
   (b) Measure 1 mL of buffer and add into the sample bottle
   (c) Mix the laccase solution

3. **50mM 2-2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) or (ABTS solution)**
   (a) Weigh 0.2743g of ABTS and put into sample bottle
   (b) Measure 10 mL of buffer solution and add into the sample bottle
   (c) Mix and dissolve the ABTS solution

Measurement of the laccase Activity in Ionic Liquids

1. Dissolved buffer into ionic liquids at 5 different ratio with total 2g per sample

Table 2: Ratio of Ionic Liquids (wt%) in buffer solution

Buffer solution		Ionic Liquids	
Ratio (%)	Mass (g)	Ratio (%)	Mass (g)
97.5	1.95	2.5	0.05
95	1.9	5	0.1
92.5	1.85	7.5	0.15
90	1.8	10	0.2
80	1.6	20	0.4

2. Then 20 µL of laccase solution and ABTS solution was added to the mixture of buffer and ionic liquids.
3. The mixture was then stirred at 400rpm at 50^{o}C for about 3 minutes.
4. After 3 minutes of the stirring time, acetic acid was added to stop the reaction. The presence of acetic acid will deactivate all the enzymes.
5. The mixture must be filtered if precipitation happened.
6. Then, the sample was put into the cell for UV-Vis analysis.

7. After the sample was put into the UV-Vis machine, the change in absorbance at 420nm ($\varepsilon_{420} = 3.6\ x\ 10^4\ M^{-1}cm^{-1}$) at 50°C was recorded for about 30 seconds.
8. In the end, the slope of the resulting reaction kinetic curve was determined and the specific activity was calculated.

Measurement of the laccase Stability in Ionic Liquids

1. 5 samples of 20mL of solution 2 (ABTS with buffer solution) was prepared into 5 different beakers labelled 1 until 5.
2. Solution 1(laccase with buffer solution) was mixed with the 2mL of ionic liquids in a beaker.
3. Then, 20 μL of the sample in the beaker (solution 1 with ionic liquids) were added into the beaker 1 until beaker 5.
4. All the sample in beaker 1 until beaker 5 were then incubated at different time intervals. (B1= 0 min ; B2= 1 hour ; B3= 2 hour ; B4= 5 hours ; B5= 12 hours)
5. After that, each of the sample was put into the UV-Vis machine with respect to the incubation time.
6. The change in absorbance at 420nm ($\varepsilon_{420} = 3.6\ x\ 10^4\ M^{-1}cm^{-1}$) at 50°C was recorded for about 30 seconds.
7. In the end, the slope of the resulting reaction kinetic curve was determined.

Determination of the laccase Activity in Ionic Liquids by using reaction Kinetic Analysis

1. Plot the graph of Absorbance vs Time
2. Calculate the slope of the graph Y=mX + C, m= absorbance/time ($\Delta\ A/t$)
3. Change absorbance per time into product concentration by using ΔA/min = εcl/min

   ε_{420nm} = extinction coefficient; (3.6 x $10^4\ M^{-1}cm^{-1}$)

   c = concentration of substrate in mol/L;

   l = thickness of the sample cell; 1cm

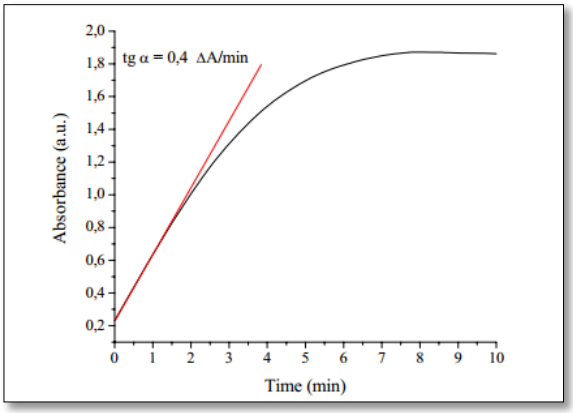


Figure 3: Graph of Absorbance vs Time

Determination of the laccase Specific Activity in Ionic Liquids

1. Calculate the specific activity of each sample by using the formula shown below

$$\text{Specific activity (mol/min.mg)} = \frac{Absorbance}{\varepsilon \ell \Delta t} \text{ x } \frac{Reaction\ volume}{Content\ of\ enzyme}$$

2. Plot a bar chart of Specific Activity vs content of ionic liquid in buffer (%).

Determination of the laccase Stability in Ionic Liquids by using reaction Kinetic Analysis

1. Take the absorbance value of each sample including Absorbance at t=0
2. Calculate the stability of the enzyme reaction by using residual activity formula as shown below.

$$\text{Residual Activity (RA) (\%)} = \frac{Activity\ at\ predetermined\ time}{Activity\ at\ t=0}$$

3. Residual activity (RA) can be calculated by substituting the absorbance value of the sample divide by the absorbance value of sample at t=0.
4. Convert the Residual activity into percentage value by taking RA at t=0 as 100% and followed by other RA percentage value for different samples.
5. Plot the graph of Residual activity vs Incubation time (hr).

3.2 Project Process Flow

This is the process flow for this research project that must be followed in order to achieve the objective of the study:

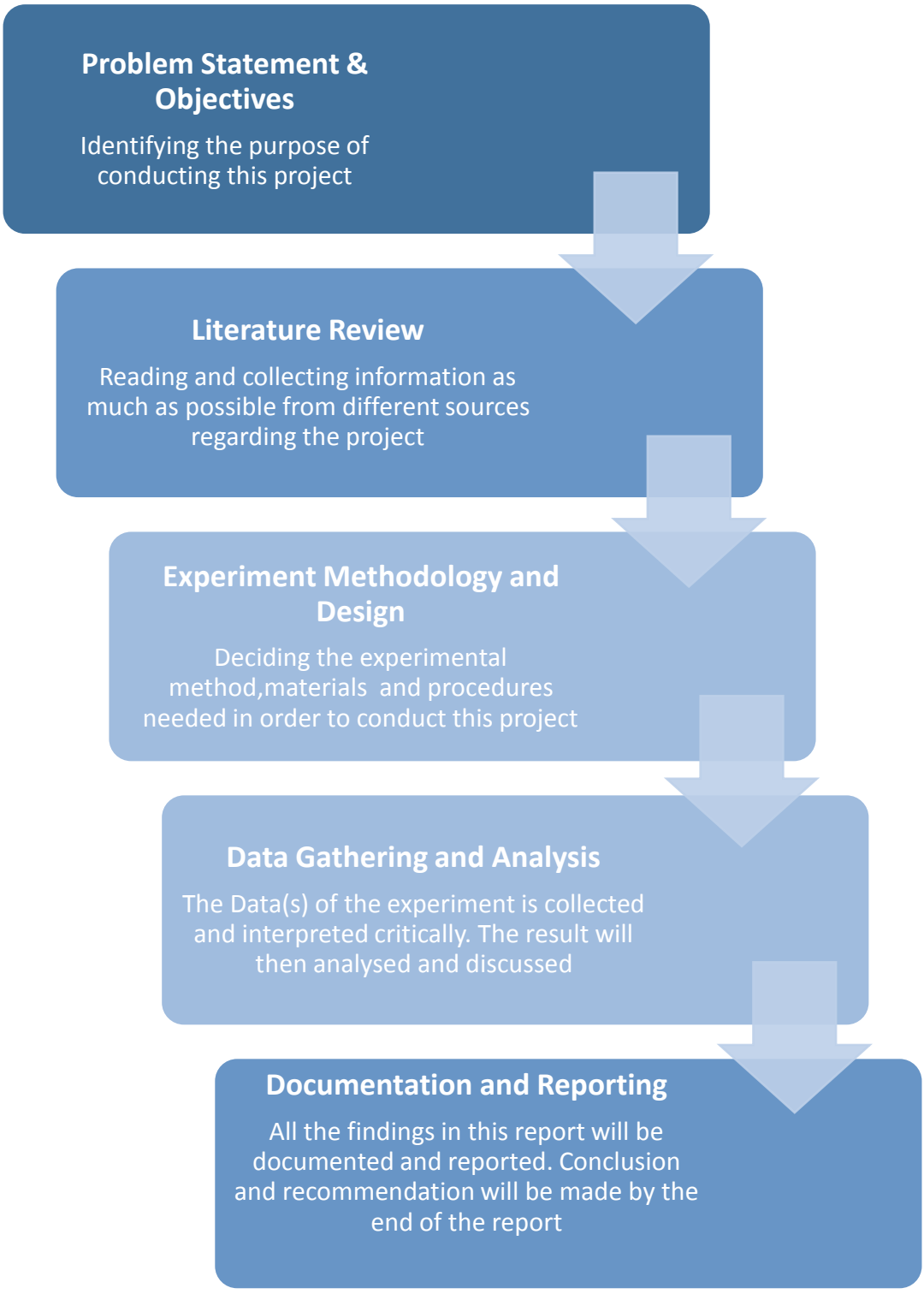


Figure 4: Process flow of the project

3.3 Gantt Chart and Key Milestone

Table 2 below show the gantt chart of the project that need to be followed during this study:

Table 3: Gantt Chart & Key Milestone

No	Details	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	Project Work continues														
2.	Submission of Progress Report														
3.	Project Work Continues														
4.	Pre-SEDEX														
5.	Submission of Draft Final Report														
6.	Submission of Dissertation (soft bound)														
7.	Submission of Technical Paper														
8.	Viva														
9.	Submission of Project Dissertation (Hard Bound)														

Gantt chart

Key Milestones

CHAPTER 4: RESULT AND DISCUSSION

In this section, the author will discuss on the result of the laccases activity and stability based on few experiment that had been conducted.

4.1 Laccase Enzyme Activity in Ionic Liquids

The result of the experiment had been analysed as shown below. This experiment for the activity of laccase enzyme had been carried out by using 3 types of ILs which are:

I. 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC]
II. 1-ethyl-3-methylimidazolium octyl sulphate [EMIM] [OSO_4]
III. 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] (DEP)

4.1.1 Result for Laccase Activity in 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC]

Table 4: The Absorbance value of the solution at different ratio of [EMIM] [OAC]

Percentage of IL (wt %)	Absorbance (IL solution w/ laccase, x)	Absorbance (IL solution w/o laccase, y)	Absorbance (x-y)
2.5	0.621	0.051	0.570
5	0.205	0.011	0.194
7.5	0.211	0.037	0.174
10	0.216	0.143	0.073
20	0.084	0.025	0.059

Table 5: The specific Activity of Laccase Enzyme in different ratio of [EMIM] [OAC]

Percentage of IL (wt %)	Specific activity ($x10^{-9}$) (mol/min.mg)
2.5	0.528
5	0.180
7.5	0.161
10	0.068
20	0.055

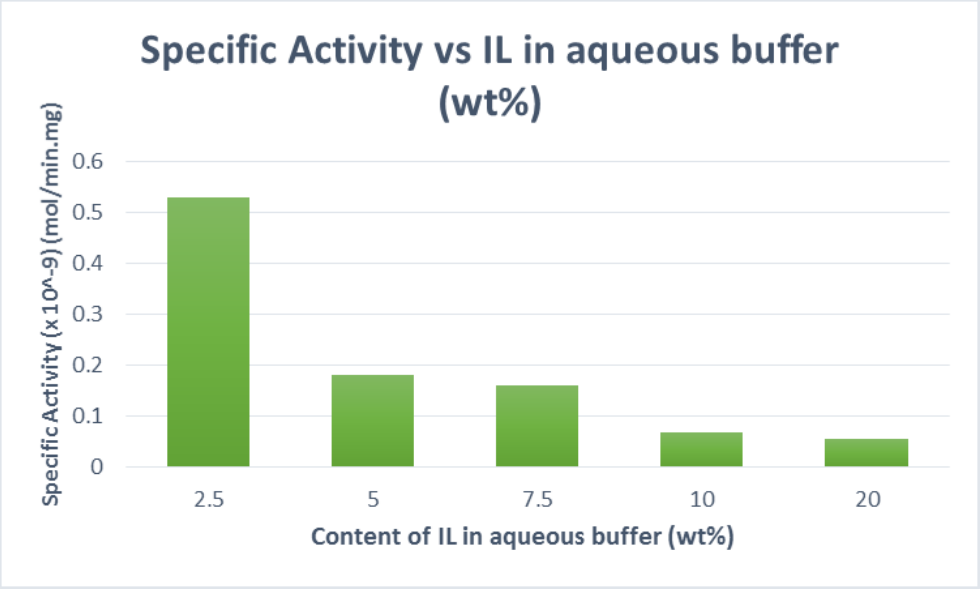


Figure 5: Specific Activity of Laccase enzyme in [EMIM] [OAC]

Figure 5 shows the specific activity of laccase enzyme in [EMIM] [OAC]. The result shows that specific activity of laccase enzyme decreases as the ILs content in the buffer solution increases. Higher specific activity gives the higher number of laccase enzyme react with the substrate inside the solution. Besides, the absorbance value in table 4 for each sample also decrease as the ILs content increases. Laccase enzyme in [EMIM] [OAC] shows a rapid decrement in its specific activity from 0.528E-9 mol/min.mg at 2.5% ILs to 0.055E-9 mol/min.mg at 20% ILs.

4.1.2 Result for Laccase Activity in 1-ethyl-3-methylimidazolium octyl sulphate [EMIM] [OSO4]

Table 6: The Absorbance value of the solution at different ratio of [EMIM][OSO4]

Percentage of IL (wt %)	Absorbance (IL solution w/ laccase, x)	Absorbance (IL solution w/o laccase, y)	Absorbance (x-y)
2.5	1.890	0.096	1.794
5	1.577	0.211	1.366
7.5	1.800	0.844	0.956
10	1.207	0.454	0.753
20	0.981	0.520	0.461

Table 7: The specific Activity of Laccase Enzyme in different ratio of [EMIM][OSO4]

Percentage of IL (wt %)	Specific activity ($x10^{-9}$) (mol/min.mg)
2.5	1.661
5	1.265
7.5	0.885
10	0.697
20	0.427

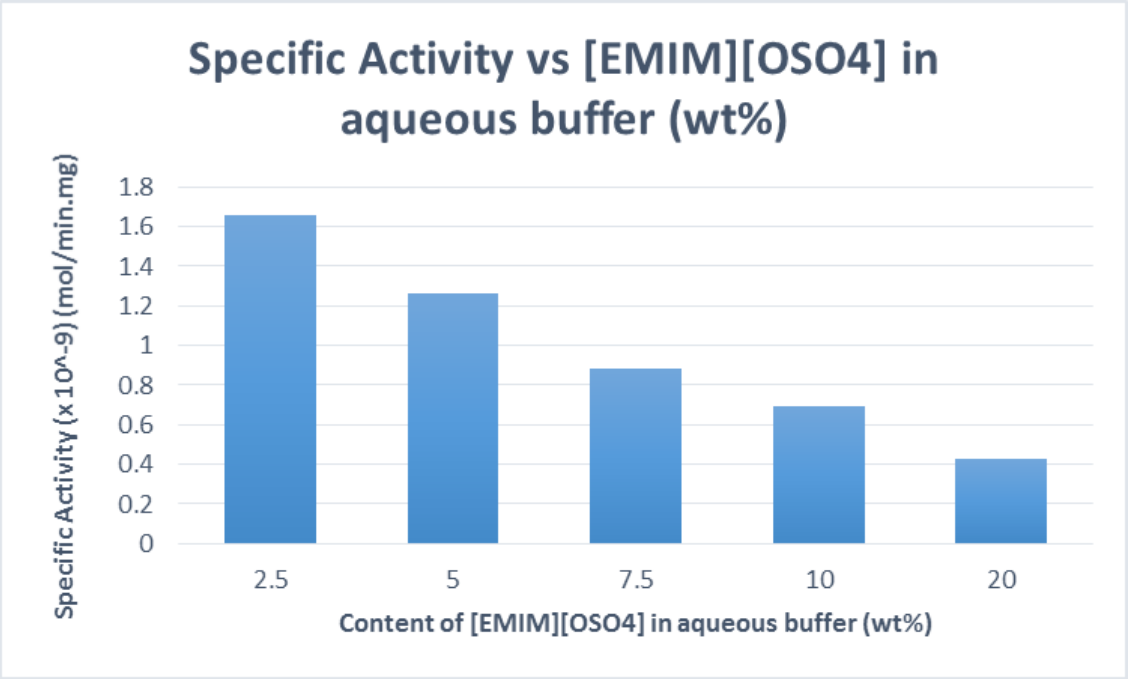


Figure 6: Specific Activity of Laccase enzyme in [EMIM] [OSO4]

Figure 6 shows the specific activity of laccase enzyme in [EMIM] [OSO4]. The result shows that specific activity decreases as the ILs content in the buffer solution increases. Besides, the absorbance value in table 6 for each sample also decrease as the ILs content increases indicating higher activity of enzyme that degrade the substrate. Laccase enzyme in [EMIM] [OSO4] shows a decrement in its specific activity from 1.661E-9 mol/min.mg at 2.5% ILs to 0.427E-9 mol/min.mg at 20% ILs.

4.1.3 Result for Laccase Activity in 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] [DEP]

Table 8: The Absorbance value of the solution at different ratio of [EMIM][DEP]

Percentage of IL (wt %)	Absorbance (IL solution w/ laccase, x)	Absorbance (IL solution w/o laccase, y)	Absorbance (x-y)
2.5	1.325	0.114	1.211
5	1.079	0.017	1.062
7.5	0.841	0.107	0.734
10	0.455	0.105	0.350
20	0.167	0.009	0.158

Table 9: The specific Activity of Laccase Enzyme in different ratio of [EMIM][DEP]

Percentage of IL (wt %)	Specific activity ($x10^{-9}$) (mol/min.mg)
2.5	1.121
5	0.983
7.5	0.680
10	0.324
20	0.146

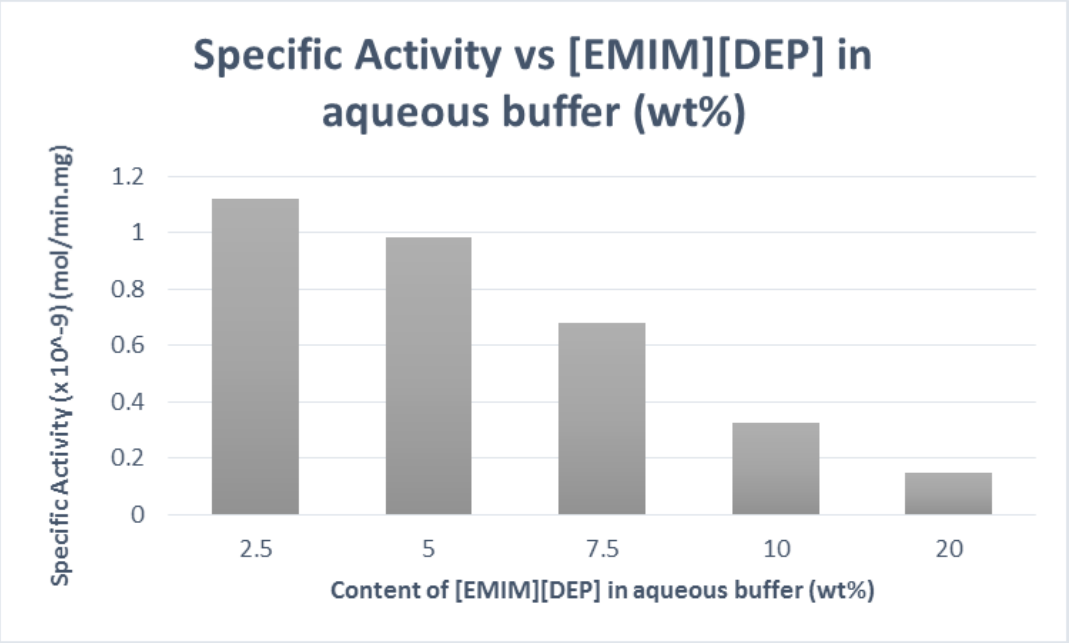


Figure 7: Specific Activity of Laccase enzyme in [EMIM] [DEP]

Figure 7 shows the specific activity of laccase enzyme in [EMIM] [DEP]. The result shows that specific activity decreases as the ILs content in the buffer solution increases. Besides, the absorbance value in table 8 for each sample also decrease as the ILs content increases indicating higher activity of enzyme that degrade the substrate. Laccase enzyme in [EMIM] [DEP] shows a decrement in its specific activity from 1.211E-9 mol/min.mg at 2.5% ILs to 0.146E-9 mol/min.mg at 20% ILs.

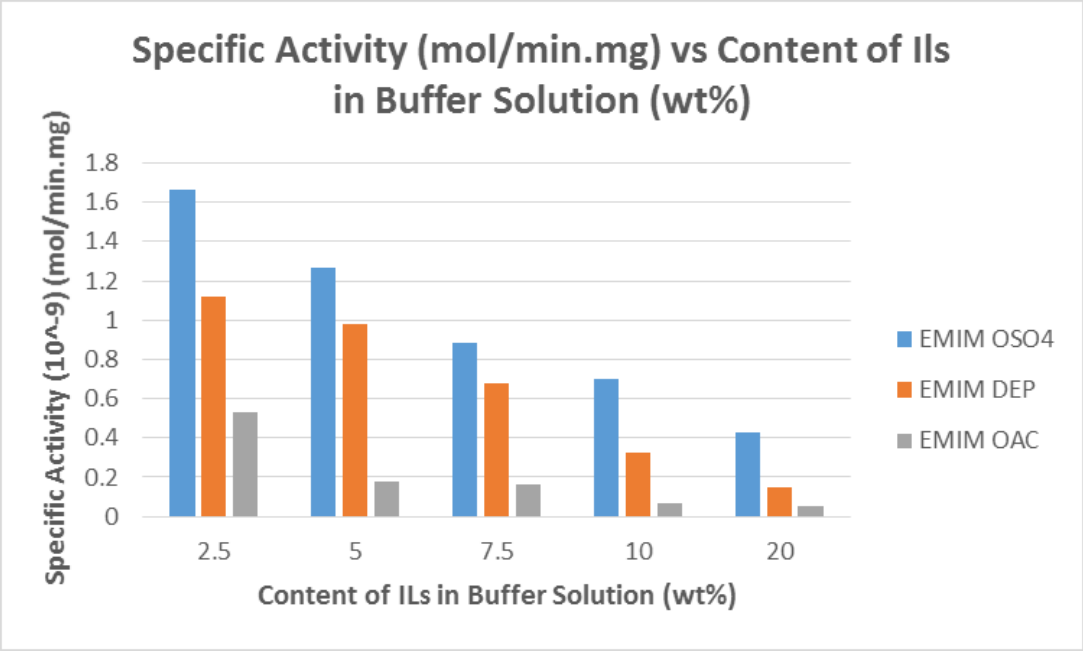


Figure 8: Comparison of the Specific Activity of Laccase Enzyme in Different Ionic Liquids

Based on the graph above, it shows that the specific activity of laccase enzyme decreases with the increment of ILs content in buffer solution (wt%). Specific activity indicate the purity of enzyme inside the solution(Somers, A.E, et.al, 2012).This term defined as the amount of substrate converted by the enzyme per mg protein in the enzyme prepared (Nelson,D. Et.al. 2000). The higher the value of specific activity, the higher the amount of enzyme react with the substrate. As the content of ILs inside the buffer solution is increase, the specific activity of enzyme decrease gradually. This proven that, the presence of ILs favours the enzyme reaction and assist in its activity with the suitable amount of ILs content in the buffer solution.

Besides, among these three ILs, **1-ethyl-3-methylimidazolium octyl sulphate [EMIM] [OSO$_4$]** recorded the highest specific activity compared to **1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] [DEP]** and **1-ethyl-3-methylimidazolium acetate [EMIM] [OAC].** This shows that, the enzyme activity favours the most with [EMIM] [OSO$_4$] as the solvent compared to [EMIM] [DEP] and [EMIM] [OAC] with the lowest enzyme specific activity.

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4.2 Laccase Enzyme Stability in Ionic Liquids

This experiment had been carried out at 5 different incubation time (0h, 1h, 2h, 5h and 12h) by using 3 types of ILs which are:

I. 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC]
II. 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] [MESO4]
III. 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] [DEP]

4.2.1 Result for Laccase Stability 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] (MESO4)

Table 10: Absorbance and Residual Activity of Laccase enzyme in [BMIM] [MESO4]

Incubation time (h)	Residual Activity (X) (%)	Ln X
0	100	4.605
1	88.1	4.478
2	97.3	4.578
5	93.6	4.539
12	54.2	3.993

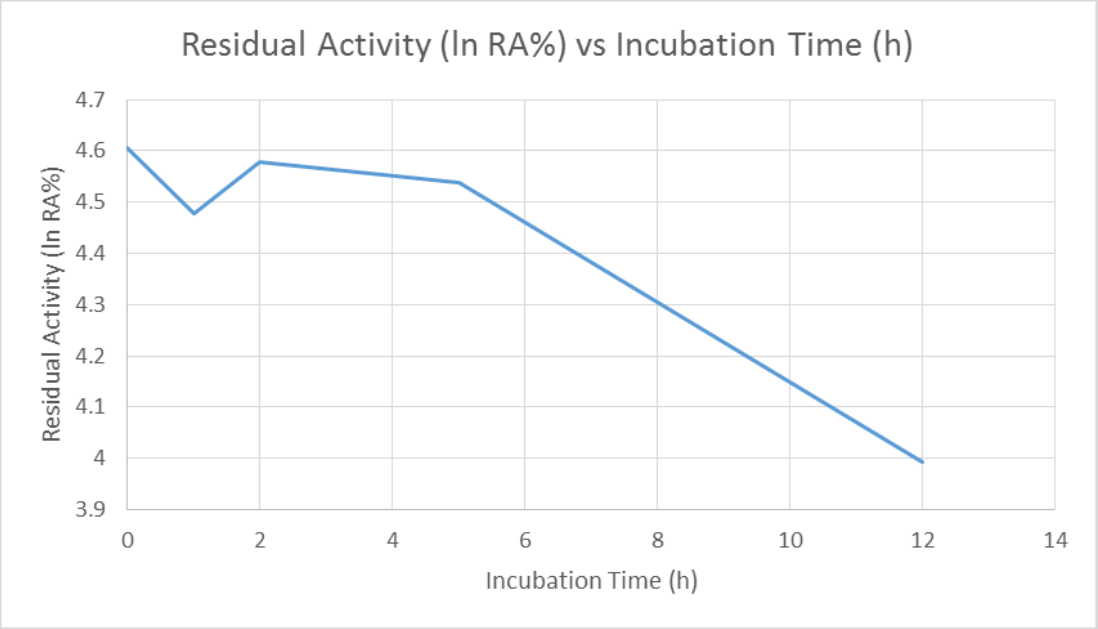

Figure 9: Residual Activity [BMI][MESO4]

Figure 9 shows the residual activity of laccase enzyme in [BMIM] [MESO4]. Residual activity can be defined as the ratio of the current activity of the enzyme to the initial activity of the enzyme. The result shows that residual activity decreases gradually as the incubation time increases. The result for enzyme stability shows the same trend for the three types of ILs tested which are the residual activity decreases as the incubation time increases.

4.2.2 Result for Laccase Stability 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC]

Table 11: Absorbance and Residual Activity of Laccase enzyme in [EMIM] [OAC]

Incubation time (h)	Absorbance	Residual Activity (X) (%)	Ln X
0	0.274	100	4.605
1	0.249	90.88	4.510
2	0.245	89.42	4.493
5	0.222	81.02	4.395
12	0.164	59.85	4.092

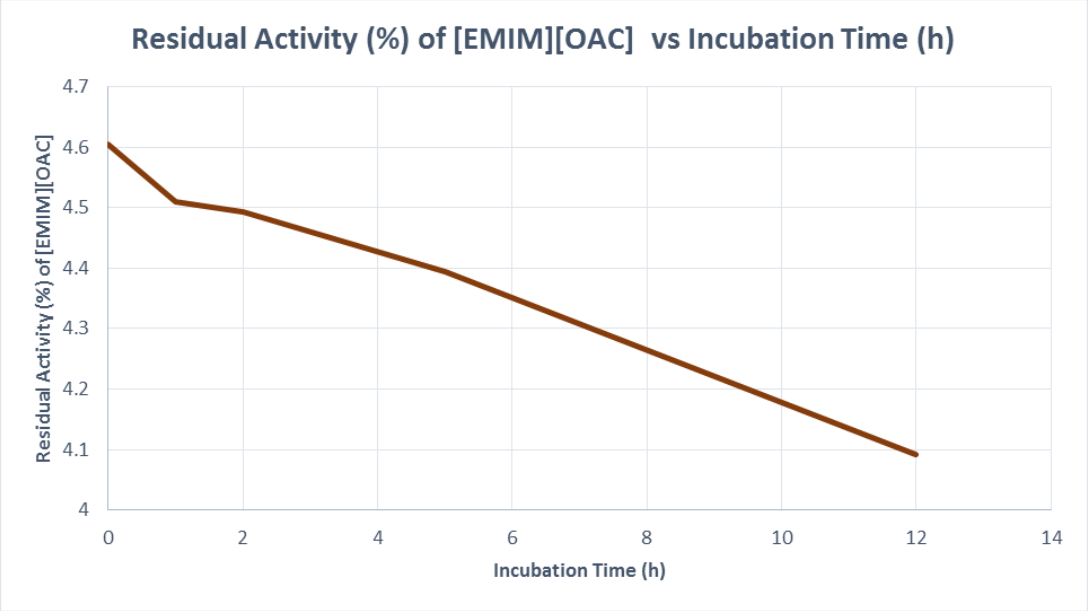

Figure 10: Residual Activity [EMIM] [OAC]

4.2.3 Result for Laccase Stability 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] [DEP]

Table 12: Absorbance and Residual Activity of Laccase enzyme in [EMIM] [DEP]

Incubation time (h)	Absorbance	Residual Activity (X) (%)	Ln X
0	1.604	100	4.605
1	1.600	99.75	4.603
2	1.525	95.07	4.555
5	1.190	74.19	4.307
12	1.143	71.26	4.266

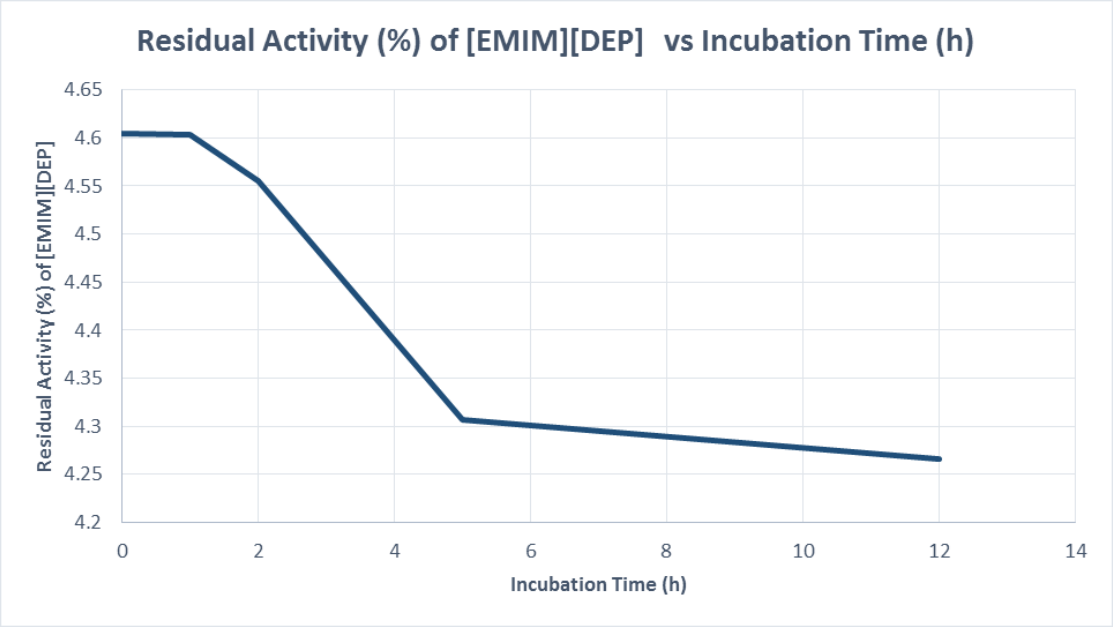

Figure 11: Residual Activity of Laccase Enzyme in [EMIM] [DEP]

4.2.4. Comparison of the stability of Laccase Enzyme in Different Ionic Liquids

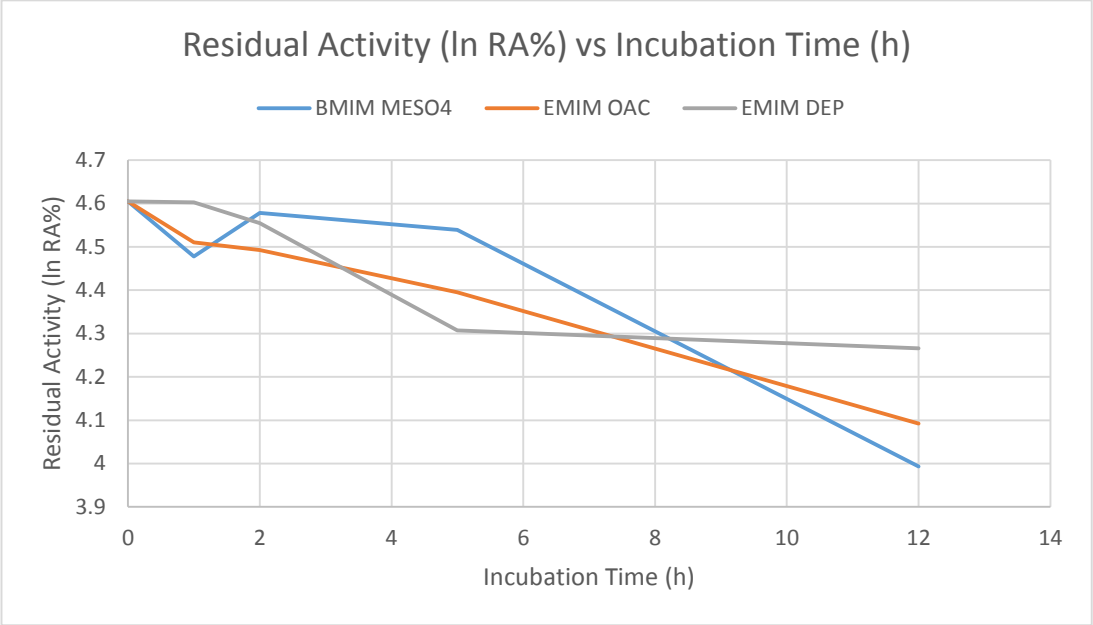

Figure 12: Comparison of the stability of Laccase Enzyme in Different Ionic Liquids

The graph above, indicate the stability of laccase enzyme in ionic liquids. Each sample are incubated at different incubation time which are at 0h, 1h, 2h, 5 h and 12h. Three types of ionic liquids that has been tested in this experiment are 1-butyl-3-methyl-imidazolium Methyl sulphate [BMIM] [MESO4], 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC], and 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM] [DEP].

Based on the result obtained, it can be observed that, at 0-2h [EMIM] [DEP] has the highest enzyme stability while at 2-8h [BMIM] [MESO4] recorded the highest enzyme stability compared to the other ILs. At 8-12 h, [EMIM] [DEP] has shown the highest enzyme stability followed by [EMIM] [OAC] and [BMIM] [MESO4]. It can be concluded that, [EMIM] [DEP] is a good solvent that promotes high laccase enzyme stability for its reaction compared to [EMIM] [OAC] and [BMIM] [MESO4].

4.3 The Effect of Temperature on Laccase Enzyme Activity by using 1-ethyl-3-methylimidazolium acetate [EMIM] [OAC]

Table 13: The absorbance value at different temperatures

Percentage of IL (wt %)	Temperature				
	40°C	**45°C**	**50°C**	**55°C**	**60°C**
2.5	0.723	1.967	2.087	1.919	1.758
5	0.522	1.376	1.760	1.522	0.761
7.5	0.372	1.126	1.459	1.105	0.390
10	0.277	0.508	1.285	1.099	0.400
20	0.157	0.200	0.504	0.025	0.069

Table 14: The Specific Activity of Laccase Enzyme at different temperatures

Percentage of IL (wt %)	Specific Activity ($x10^{-9}$) (mol/min.mg)				
	40°C	**45°C**	**50°C**	**55°C**	**60°C**
2.5	0.669	1.821	1.933	1.777	1.628
5	0.483	1.274	1.630	1.409	0.705
7.5	0.344	1.043	1.351	1.023	0.361
10	0.256	0.470	1.190	1.017	0.370
20	0.145	0.185	0.467	0.023	0.064

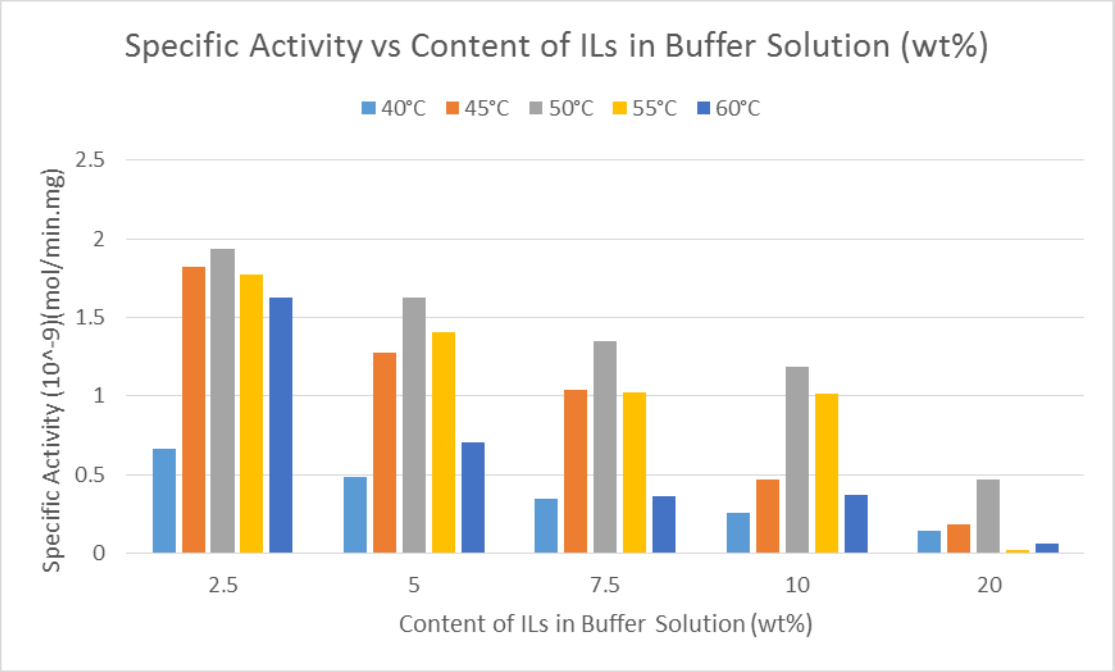

Figure 13: The effect of different temperature on Laccase Enzyme in [EMIM] [OAC]

Based on the graph above, it can be observed that the highest range of specific activity recorded at T= 50^{o}C.This shows that the enzyme experiencing the highest activity at T= 50^{o}C compared to other temperatures. As the specific activity indicating the enzyme purity in the solution, so, the higher its value the higher the number of enzyme react with the substrate (Somers, A.E, et.al, 2012). The temperature with the second highest range of specific activity is at T=45^{o}C followed by T=55^{o}C, 60^{o}C, 40^{o}C. This concluded that the optimum temperature for the enzyme activity is at T=50^{o}C at the buffer solution of pH 4.5.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

As a conclusion, hopefully this research project will give benefit to the human and many process industries. By analysing the performance of ionic liquid as a solvent that can optimize the laccase enzyme activity and stability will give a big advantage to many process industries such as pulp & paper, biofuel and manufacturing industries.

Based on the findings, it proven that ionic liquids have high potential in improving enzyme activity and stability. Different ionic liquids gives different performance on the enzyme activity and stability. For the laccase activity experiment, the author has used ILs with the same cation but different anion. The result recorded has shown that different anion gives different effect to the enzyme activity. The enzyme activity favours the most with [EMIM] [OSO_4] as the solvent compared to [EMIM] [DEP] and [EMIM] [OAC] with the lowest enzyme specific activity. Besides, for the stability study, the presence of ILs as a solvent improve the enzyme stability. [EMIM] (DEP) has been observed as a good solvent that promotes high laccase enzyme stability for its reaction compared to [EMIM] (OAC) and [BMIM] (MESO4)

Therefore, suitable ionic liquids need to be employed in order to get excellent enzyme performance. Some ionic liquids favours the enzyme reaction while some other ILs hindered the reaction. More analysis need to be done in order to classify which ionic liquids promotes a healthy medium for the enzyme reaction.

Besides, the change in temperature also affect the enzyme performance in the ILs. The optimum temperature must be maintained in order to get maximum enzyme activity for the conversion of the substrates. For this study, the author has tested the effect of temperature to the enzymatic reaction performance in 1-ethyl-3-methylimidazolium acetate [EMIM] (OAC) and the result shows that 50^{o}C is the optimum temperature for the reaction compared to other temperature tested.

5.2 Recommendations

For the recommendation, since ionic Liquids is a very sensitive solution. Any presence of impurities will disturb the physical and chemical properties of the solution and hence affect the result of enzymatic activity and stability. Therefore, the experiment must be handled in great care and following the procedures in order to avoid any impurities presence during the experiment was conducted.

Besides, more analysis and exploration need to be done in different types of ILs. As the author only has 4 months to complete the experiment, only few types of ILs can be tested for the enzyme activity and stability. The author highly encourage more researches will be conducted with different types of ILs so that we can classify which ionic liquids favours the enzyme reaction.

On the other hand, the reaction condition also need to be varied such as pH of the solution. It is important to manipulate the reaction condition, so that we can conduct the enzyme reaction at optimum pH and temperature together with the suitable ionic liquids as the solvent for the solution. Optimum condition will give the maximum performances for the enzyme reaction and thus producing more products.

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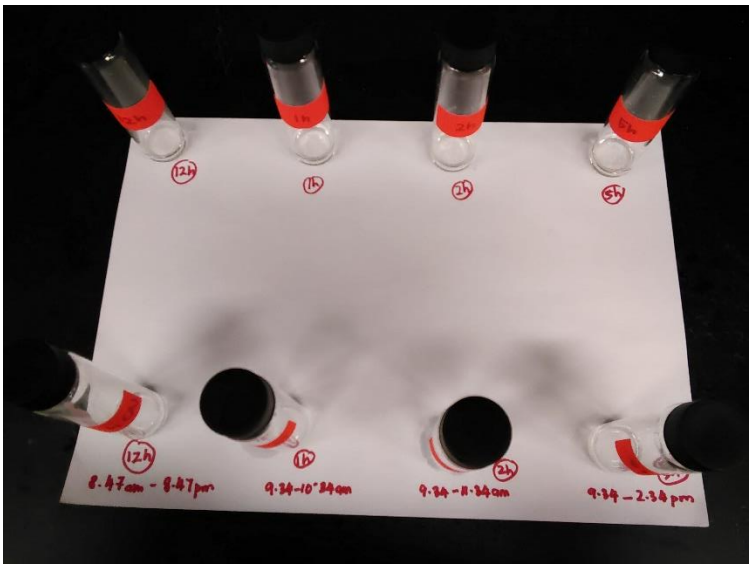
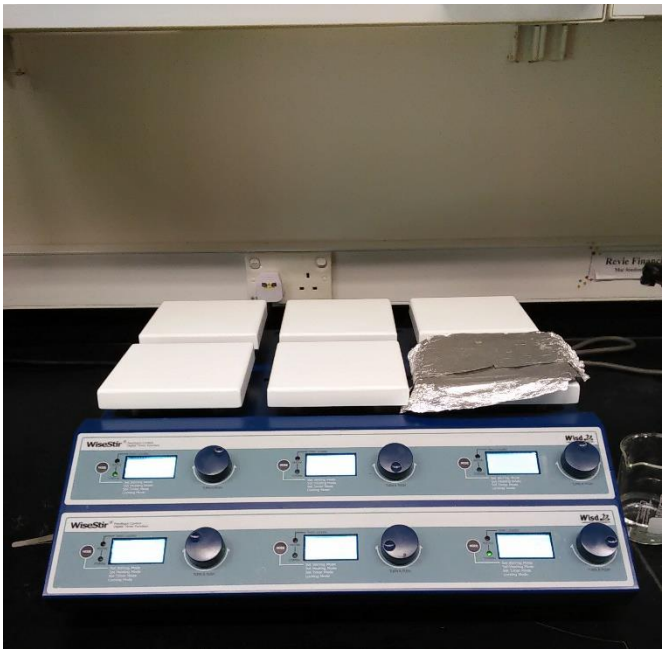
CHAPTER 7: APPENDICES

Picture 4: The picture the author conducting the laccase activity experiment



Picture 5: Laccase Enzyme incubated together with ILs and buffer solution



Picture 6: Heating Equipment

Picture 7: Ultraviolet-visible Spectrophotometry