

CHAPTER 1 INTRODUCTION

1.1 Introduction

Mushroom produces a variety of valuable molecules with attractive biological characteristics such as anticoagulant, antifungal protein, anti-tumour and lignin degradative enzymes such as lignin peroxidase, manganese peroxidase and laccase. Lignin peroxidase was first studied in 1983 from *Phanerochaete chrysosporium* studies on the mechanism of the lignin biodegradation (Glenn *et al.*, 1983; Tien and Kirk, 1983).

Lignin peroxidase able to degrade the rigid lignocellulos, lignin related aromatic compounds and other non-lignin related compounds (Zanirun, 2008). This make it gaining huge interest in biotechnological application and there are several applications of lignin peroxidase as it is being utilized in biopulping, biobleaching and bioremediation (Roushdy *et al.*, 2011). Besides, lignin peroxidase (LiP) is gaining huge interest in synthetic chemistry to apply in for cosmetic production as well as dermatological preparations for skin (Belinky *et al.*, 2005) where it can promote skin whitening by breaking the melanin in the skin.

According to Adinarayana *et al.* (2007), this enzyme is found abundantly in Ascomycetes, Deuteromycetes and Basidiomycetes; especially in many white-rot fungi. *Amauroderma rugosum* is a Basidiomycetes and is classed under the order Polyporales which is the largest pore fungi under Basidiomycetes. According to Chang and Lee (2004), *Amauroderma sp.* is used by indigeneous community to prevent fit, such as epileptic fit and therefore known as 'cendawan budak sawan'. This indigenous species is believed to have some valuable medicinal value which is still under discovered.

However, the major barrier restricted the wide application of the lignolytic enzyme in industrial sector is the lack of the easily available, low cost and robust lignolytic enzyme (Jing *et al.*, 2007; Jing 2010). Thus, submerged fermentation of the fungi appears to be the cost effective process for the production of the mycelia biomass and allow for mass production of bioactive compounds (Xu *et al.*, 2011). Moreover, the use of submerged fermentation of mushroom for the production of the extracellular enzymes is a cost effective method as the agricultural by-products can be used as substrate.

The extraction and purification of mushroom lignin peroxidase can be performed using the aqueous two-phase system (ATPs). This method is especially useful in extraction and purification of biomolecule such as enzyme and had been applied on extraction of various enzymes such as papase, lipase and laccase from different sources. According to Wood (1980), there are several methods for purification of lignin peroxidase such as exclusion chromatography, ion exchange chromatography and ultrafiltration. However, the use of these methods are labourious which involve multi-step processes include the lengthy extraction and purification process which will affect the conformation and biological activities of the biomolecules and result in low yield and high operation cost (Mayolo-Deloisa *et al.*, 2009). Thus, aqueous two-phase system (ATPs) appears to be the most suitable method for extraction and purification of lignin peroxidase. The simplicity and rapidness of aqueous two-phase system (ATPs) compared to other methods available to date accounts for its popularity for extraction of biomolecules (Hatti-Kaul, 2000).

The aqueous two-phase system (ATPs) was applied in this study by forming a mixture of polyethylene glycol (PEG) polymer and salts such as potassium phosphate and resulting in two immiscible phases for the extraction and purification of biomolecules (Mayolo-Deloisa *et al.*, 2009). The use of aqueous two-phase system (ATPs) create a

selectively partition environment and force the separation of biomolecules to particular phase without amending the conformation of the biomolecule and retaining the native structure hence allow the protection of the function since both phases in aqueous two-phase system (ATPs) are predominantly water based (80-85 %) (Ooi *et al.*, 2009). Apart from simplicity and biocompatibility, this method poses some advantages over the conventional method in term of low cost and ease of scale-up for large scale industries (Benavides and Rito-Palomares, 2008).

1.2 Objective

The objective of the study were:

1. to investigate the optimum condition of lignin peroxidase recovery using aqueous two- phase system (ATPs)
2. to characterise the purified lignin peroxidase produced by *Amauroderma rugosum*.

CHAPTER 2 LITERATURE REVIEW

2.1 Basidiomycota

Basidiomycota is one of the major divisions of fungi with 30,000 described species (Kirk *et al.*, 2001) and also well-known as the “club-fungi”. Basidiomycota makes up to 30 % of the known species of fungi (Hawksworth *et al.*, 1995).

According to Deacon (2005), the basidiomycota is well known for the ability to produce large fruiting bodies such as mushrooms, puffballs, brackets, gill fungi, stinkhorns, coral fungi, bird’s nest fungi, jelly fungi; it also includes yeast and small microscopic fungi, the rust fungi and smut fungi that parasites the plant.

The basidiomycota can be unicellular and multicellular and is capable of both sexual and asexual reproductions; however asexual reproduction is not common. The sexual reproduction may consist of basidiopores and the asexual types can either be in simple thallus, budding form for yeast and also spores (Alexopoulos *et al.*, 1996). Basidiomycota plays an important role in ecosystem as the dead organic matters decomposer by decomposing the dead wood and leaves (Swann and Hibbett, 2007). They are found both in terrestrial and aquatic and can be either freshwater or marine habitats for aquatic environment (Kohlmeyer and Kohlmeyer, 1979; Hibbett and Binder, 2001).

According to Matheny *et al.* (2007), the most familiar types of the basidiomycota to the public are those mushroom producing types or known as macrofungi. The very famous edible mushroom species, *Agaricus bisporus* or commonly known as button mushroom are classified under basidiomycota. Nevertheless, the mushroom produced under this division can be equally fatal and harmful for human consumption. For example, the alpha amanitin and phalloidin, a type of powerful hepatotoxins isolated from the deadly

poisonous mushroom *Amanita phalloides* (Zheleva *et al.*, 2004) can lead to fatal liver necrosis. Besides toxins, the basidiomycota is also reported for producing the hallucinogens. However, basidiomycota plays an important role in industrial biotechnology by producing the lignolytic enzymes such as the lignin peroxidase (LiP), laccases (Lac) and manganese peroxidase (MnP). Both LiP and MnP were reported to have high decolourizing effect on the kraft pulp mill effluents (Ferrer *et al.*, 1991; Michel *et al.*, 1991; Moreira *et al.*, 2003).

2.1.1 Polyporales

Polyporales known as the larger group of the macrofungi and are widely distributed all over the world. To-date, the species of macrofungi in tropical Asia alone is estimated to be in the range of 10,000 to 25,000 species according to Mueller *et al.* (2007). Many researches had been done in order to discover the fungi, however, 70-80 % of the fungi are yet to be discovered in Malaysia (Corner 1996; Lee *et al.*, 1995)

Donk (1965) further divided the poroid mushrooms into five families, i.e: Polyporaceae, Hymenochaetaceae, Ganodermataceae, Bondarzewiaceae and Fistulinaceae. According to Hibbette and Justo (2012), polyporales do exist in non-poroid form and the examples are such as gilled mushrooms (genus *Lentinus*, *Panus*) and “cauliflower fungi” (genus *Sparassis*) and they are mostly the corticoid fungi with resupinate and crust-like forms.

According to Salmiah and Jones (2001), amounts of rainfalls, substrates quantities and suitability, consistency of high air humidity in damp forest and also forest types will influence the distribution of the polyporales in various locations.

According to Ryvardeen (1991), the polyporales play crucial roles in ecosystems as comprising huge group of wood-decaying fungi and they are able to degrade different components of wood. However, some of the polyporales are the timber pathogens that cause harm to the timber such as the mine fungus *Antrodia vaillantii* which is also the enzyme producer in ecosystem.

Besides, wide application also found in medical field where intensive research had been carried out on the cancer treating ability of the *Ganoderma lucidum*, which is also one of the most famous Polyporales. The ability of this macrofungi to produce the lignolytic and cellulolytic enzymes enables the application in the “green” technologies such as biofuel production and also in bioremediation (Hibbette and Justo, 2012).

2.1.2 *Amauroderma rugosum* (Blume and Nees) Torrend 1920

Amauroderma rugosum (Figure 2.1) is first being discussed by Blume and Nees on 1920. It belongs to the order Polyporales and family Ganodermataceae. The obligate synonyms of *Amauroderma rugosum* are *Polyporus rugosus* and *Fomes rugosus*. Blume and Nees on 1920 discuss the growing properties of *Amauroderma rugosum* on the lowland and montane and were widely distributed in tropics, especially South East Asia. This species also grow in humid and humus soil, attach to the buried roots in hardwood forest and usually does not grow high on trunk expose to sunlight. However, this species can also be found in GuangDong, Hainan, Guangxi, Guizhou, Yunnan, Taiwan, Africa, South America and Oceania (Bi *et al.*, 1993). Bi *et al.* (1993) further explain the physical appearance of the *Amauroderma rugosum* with round to round pileus, 6-9cm broad, and 0.7-1.3 cm thick, up to 1.7cm thick near stipe, ravenous to black in colour with white to pale

brown context. The exact medical properties of this species are under discovered but it was believed by the locals to treat the epileptic fit. The recent journal written by Chan *et al*, 2013 revealed the nutritional value as well as the anti-oxidant and anti-inflammatory properties of this indigeneous species and have attract much interest on the species's medicinal values.



Figure 2.1: The picture of *Amauroderma rugosum*.

2.2 Lignin

The word lignin was first derived from the Latin word “lignum” with the meaning of wood (Sarkanen and Ludwig, 1971). According to Sjöström, (1993), lignin is comprised of many small units of phenylpropane that linked together to form three-dimensional network polymer. This complex and unique aromatic compound is an important part of the plant cell walls and is the second most abundant biopolymer after cellulose, make up to about 30 % of the non-fossil organic carbon and can reach up to 30 to 40 % of the wood's dry mass (Boerjan *et al.*, 2003).

Lignin plays crucial function as the mechanical support to plant and the outer impermeable layer that accounts for rigidity and enables protection to the plant. Lignin is the important part in plant, it protect the easily degradable cellulose from pathogens attack and impart rigidity of the cell wall (Prasongsuk *et al.*, 2009). Moreover, lignin also plays major role in protecting the plants against the impeding penetration of the destructive enzymes from the cell wall (Sarkanen and Ludwig, 1971; Sjöström, 1993).

Lignin is the most rigid compounds in plant compared to cellulose and hemicelluloses as it comprise of the non-hydrolysable bonds and the high molecular weight of lignin of about 100 kDa restricts the uptake and utilization inside the microbial cell, thus the degradation of the lignin can only be carried out using extracellular enzymes (Argyropoulos and Menachem, 1997).

Moreover, the rapid development in the agricultural industries led to accumulation of the agricultural by-products, especially the lignocellulosic residues (Villas-Boas *et al.*,

2002) whereby about 40 to 50 million tons of lignocellulosic residues are being produced per annum worldwide.

Roushdy *et al.* (2011) state that in order to minimize environmental damage from weathering and coal burning, it is important for biotechnology to convert lignin to clean, and cost-effective energy sources for the welfare of human beings. Thus, Kareem and Akpan (2004) proposed that the use of the agricultural by-products especially the lignocellulosic residues which are also the renewable carbon sources can be a good source as enzyme substrate which can lower down the production cost and hence enables the large scale productions of industrial enzymes in the tropics.

However, in the natural environment, the ability of utilizing the rigid lignin compound can only be done by a few groups of organisms and they are best exemplified by the white rot fungi and other species such as *Phanerochaete chrysosporium*, *Streptomyces viridosporus*, *Pleurotus eryngii*, *Trametes trogii*, *Fusarium proliferatam*, *Agaricus*, *Erwenia*, *Copricus* and *Mycema*, *Sterium* (Martin, 1977). Since *Amauroderma rugosum* is a member of the polyporales as well as white rot fungi, thus this species would have lignin degrading ability.

2.2.1 Lignin peroxidase (LiP)

Ligninase or more commonly known as lignin peroxidase (LiP) is one of the enzymes in the group of lignolytic enzymes. Lignin peroxidases (EC 1.11.1.14) is one of the members of oxidoreductases (Higuchi, 2004; Martínez *et al.*, 2005; Hammel and Cullen, 2008). This lignolytic enzyme is firstly being discovered in 1983 from *Phanerochaete chrysosporium* during the studies on the mechanism of the lignin biodegradation (Glenn *et al.*, 1983; Tien and Kirk, 1983). Lignin peroxidase (LiP) is gaining huge interest among the scientists in the biotechnology field as it is capable of degrading the lignin or lignin related aromatic compounds make it a potential tool in biopulping, biobleaching and bioremediation (Roushdy *et al.*, 2011).

Laccase although is the most commonly used lignolytic enzyme, however it can only oxidize phenolic lignin units (Bourbonnais and Paice, 1990). Unlike laccase, lignin peroxidase is capable of oxidizing phenolic and non-phenolic compounds such as aromatic amines, aromatic ethers, and polycyclic aromatic hydrocarbons and this made lignin peroxidase the most powerful ligninase (Breen and Singleton, 1999; Martínez *et al.*, 2009). Lignin peroxidase can interact with a vast range of substrate even the unrelated molecules (Barr and Aust, 1994). According to Oyadomari *et al.* (2003), the high redox potential enables lignin peroxidase to oxidize a huge range of reducing substrates.

Besides, Haemmerli *et al.* (1986) also report the findings that lignin peroxidase is able to oxidize lignin monomers, dimers and trimers as well as polycyclic aromatic compounds such as benzopyrene. Gold and Alic (1993) further discussed on the properties of lignin peroxidase and state that this enzyme is also a hemeprotein with an unusual high

redox potential and low optimum temperature with the degradative ability took place only in the presence of hydrogen peroxide (H₂O₂). Fakoussa and Hofrichter (1999) also reported that the pH range of fungi lignin peroxidase falls between pH 2.0 to pH 5.0 with the optimum pH approximately at pH 2.5 to pH 3.0. According to Fakoussa and Hofrichter (1999), the range of subunit molecular mass range of white rot-fungiLiP is at 38-47 kDa and lies between 38-50 kDa for the peroxidases. In addition, lignin peroxidase exists as a series of isozymes with isoelectric point (pI) of 3.2-4.0 (Renganathan *et al.*, 1985; Leisola *et al.*, 1987).

2.2.2 Veratryl alcohol and Hydrogen Peroxide

Veratryl alcohol plays vital role in the study of lignin biodegradation process (Chia, 2008). Since the veratryl alcohol can be oxidized to veratrylaldehyde in the presence of lignin peroxidase with the absorbance at 310 nm (Arora and Gill, 2001), it is being used as enzyme assay in detection of lignin peroxidase.

Lignin peroxidase catalyzes the hydrogen peroxide (H₂O₂) dependent oxidation of the substrates in a multi-step reaction sequences and this catalytic mechanism is a common mechanism sharing by all the peroxidases (Edwards *et al.*, 1993). First, the ground state ferric peroxidase (Fe³⁺) reacts with hydrogen peroxide to yield two electron oxidized intermediate (compound I); secondly, the compound I is reduced by substrates to intermediate called compound II in a one electron reaction; lastly the compound II is being subsequently reduced back to the ground state by another substrate in a one electron reaction (Verdin *et al.*, 2006).

Table 2.1: Lignin peroxidase application on different sectors.

Sectors	Lignin Peroxidase (LiP) Applications	References
Food Industry	Source of natural aromatics Production of vanillin	Lesage-Meessen <i>et al.</i> , 1996 ;Lomascolo <i>et al.</i> , 1999; Barbosa <i>et al.</i> , 2008
Pulp and paper industry	Decolouriment of kraft pulp Mill effluents	Ferrer <i>et al.</i> , 1991; Bajpai, 2004; Sigoillot <i>et al.</i> , 2005
Textile Industry	Textile dye degradation and bleaching	Mc Kay, 1979; Cripps <i>et al.</i> , 1990; Cooper, 1993; Riu <i>et al.</i> , 1998; Wong and Yu, 1999; Abadulla <i>et al.</i> , 2000; Pointing, 2001; Kasinath <i>et al.</i> , 2003; Blanquez <i>et al.</i> , 2004; Hou <i>et al.</i> , 2004; Shin, 2004; Champagne and Ramsay,2005;Rodríguez <i>et al.</i> , 2005; Rodríguez andToca,2006;Kunamne ni <i>et al.</i> , 2008; Robles-Hernández <i>et al.</i> , 2008; Gomes <i>et al.</i> , 2009
Bioremediation	Degradation of azo, heterocyclic, reactive and polymeric dyes Mineralizationof environmental contaminants Xenobiotic and pesticides degradation	Bumpus and Aust, 1987; Abraham <i>et al.</i> , 2002; Ohtsubo <i>et al.</i> , 2004; Robles-Hernández <i>et al.</i> , 2008; Gomes et al. 2009; Wen et al. 2009
Organic synthesis, Medical, Pharmaceutical, Cosmetics and Nanotechnology Applications	Functional compounds synthesis Cosmetics and dermatological for skin Bioelectro-catalytic activity at atomic resolution	Christenson <i>et al.</i> , 2004; Higuchi, 2004; Belinky <i>et al.</i> , 2005; Barbosa <i>et al.</i> , 2008

(Modified from Maciel *et al.*, 2010)

2.3 Aqueous two-phase system (ATPS)

Aqueous two-phase system (ATPs) is first discovered by Beijerinck (1896) when he observed the mutual immiscibility of aqueous starch and aqueous gelatin solution. However, the popularity of an aqueous two-phase system (ATPs) system only starts after one decade.

Aqueous two-phase system (ATPs) is formed when mixing two mutually incompatible aqueous solutions either water-soluble polymers or one polymer and salt in high concentration. The basis of molecular separation in aqueous two-phase system (ATPs) is the selective partitioning of substances between two phases controlled by several parameters; the properties of the phase system, the substance and their interaction (Albertsson, 1986). Salabat *et al.* (2010) also mention that aqueous two-phase system (ATPs) can be formed between an alcohol and a salt or an ionic liquid and a low molecular salt in aqueous solution.

Among all of the compounds use for forming the aqueous two-phase system (ATPs), the most common system is made up of polymer/polymer system of dextran and polyethylene glycol (PEG). Both of these compounds which are non-toxic in chemicals, high biocompatible and environmental friendly made them a perfect option in human drug formulations; however in such systems, the molecular weight of polymers might affect the partition of the desired substances (Kula, 1979). Thus, PEG/salt systems appears to be a better choice as they have wide range of hydrophobicity between two different phases and the low cost application make them a popular method for bioseparation of proteins (Li *et al.*, 2001).

Unlike other conventional downstream processing method for biomolecules separation and purification such as chromatography, electrophoresis, and precipitation methods that are expensive, low yields, and difficulty to scale-up, aqueous two phase system (ATPS) is a more attractive technique for separation and purification of biomolecules (Ratanapongleka, 2012).

Antov *et al.* (2006) stated that the easily scalable and the ability to hold high biomass load also account for the popularity of aqueous two-phase system (ATPs) in comparison to other conventional separation methods. This makes their application for enzymes recovery from submerge fermentation attracts most interest recently.

The other advantages of aqueous two phase system (ATPS) are high biocompatibility, high water content (70-80 %), low interfacial tension (Veide *et al.*, 1989), high yield, less labour intensive, easily scale-up and enable polymer recycling (Naganagouda and Mulimani, 2008).

2.3.1 Factors affecting partition behavior

In an aqueous two-phase system (ATPs), the interactions such as hydrogen bond, van der Waals' interaction, electrostatic bonds, steric effects, hydrophobicity, biospecific affinity interactions and conformational effects between the phase components and the substance (Albertsson *et al.*, 1990; Albertsson, 1986); surface charge, existing of specific binding site and molecular weight of biomolecules also affecting the partitioning of the particular substance (Oliveira *et al.*, 2003).

Moreover, the type of the polymer and salt that form the aqueous two-phase system (ATPs) will have effect on the partitioning of the bio-molecules. Besides, other factors such as times for phase separation and temperature will influence the partition behavior of the particular biomolecules and it is necessary to maintain a constant phase separation times and temperature while carry out the experiments.

However, by optimizing the concentration of phase components, volume ratio (V_R), pH and even the addition of sodium chloride, aqueous two-phase system (ATPs) with better purification and yield can be achieved (Rito-Palomares, 2004)

2.3.2 Application of aqueous two-phase system (ATPS)

Apart from the large-scale purification of biomolecules from fungi or microbial cell, this method also being widely applied in. For example, the aqueous two phase systems (ATPs) are also being applied in the separation of membrane proteins such as cholesterol oxidase and bacteriorhodopsin (Sivars and Tjerneld, 1997); thylakoid membrane (Albertsson, 1997); viruses (Albertsson, 1986); bioremediation (Rogers, 1997), recovery of viral or plasmid gene therapy vector (Garca-Perez *et al.*, 1998).

Besides, there are wide applications of aqueous two-phase system (ATPs) mainly focused on the PEG/phosphate system. For example, the recovery of viral coat proteins from recombinant *E. coli* (Rito-Palomares and Middelberg, 2002); preparation of highly purified fractions of small inclusion bodies (Walker and Lyddiatt, 1998) and also the recovery of aroma compounds under product inhibition conditions (Rito-Palomares *et al.*, 2000).

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental Flow Chart

The experimental design of the study was summarized in Figure 3.1.

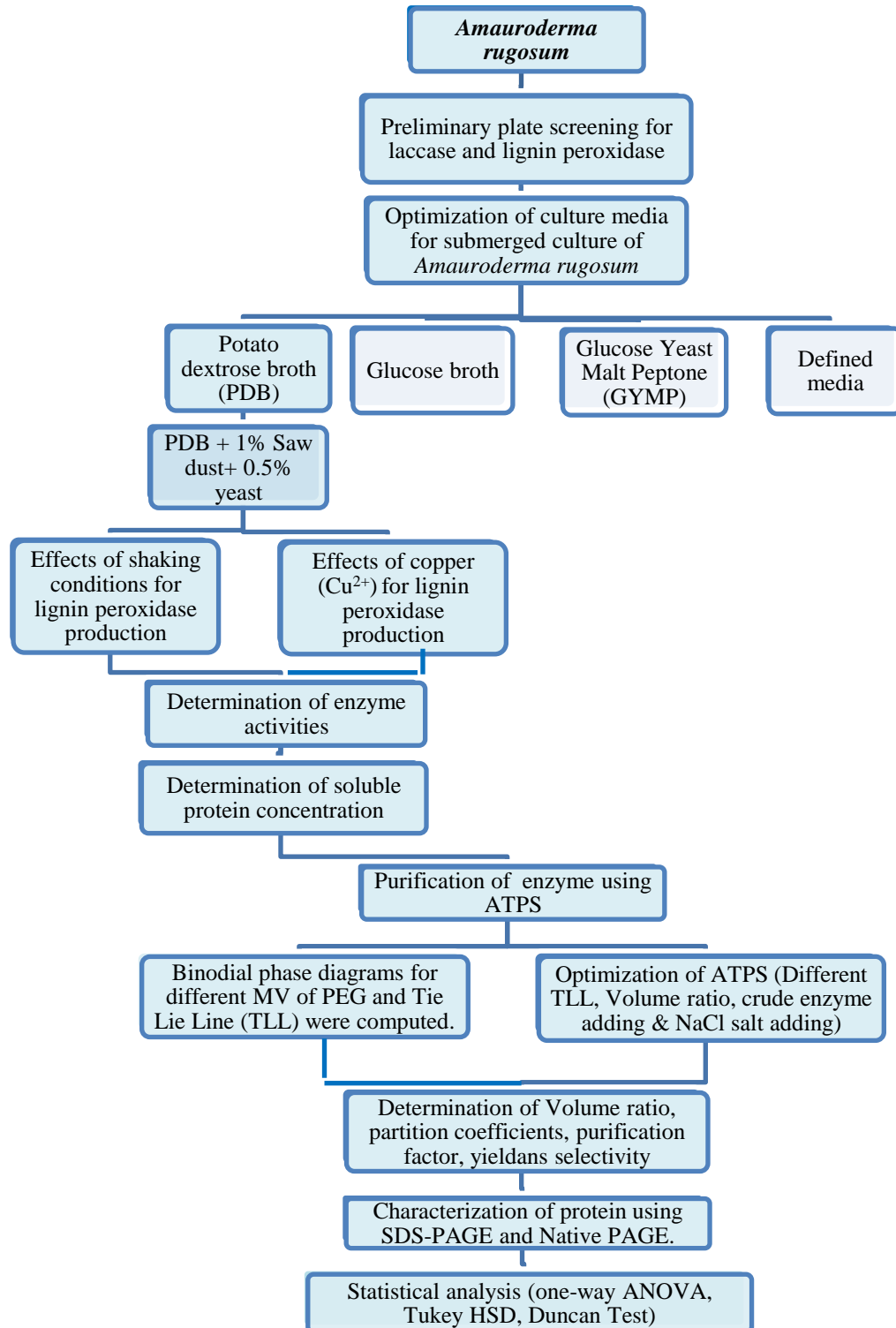


Figure 3.1: Experimental design of the project.

3.2 Chemicals and media

Polyethylene glycol (PEG) with different molecular weights (600, 1000, 1500, 3350 and 8000 g/mol) were obtained from Sigma-Aldrich (USA). Di-potassium hydrogen phosphate (K_2HPO_4) and potassium di-hydrogen phosphate (KH_2PO_4) were purchased from Merck (Darmstadt, Germany). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were both purchased from Difco. The bovine serum albumin was purchased from Sigma-Aldrich (USA). All chemicals used were of analytical grade.

3.3 Fungus strain

The mycelia of *Amauroderma rugosum* (KUM 61131) obtained from Mushroom Research Centre, University of Malaya. The fungi culture was maintained in the potato dextrose agar (PDA) by periodical subculture on every 14th day.

3.4 Preliminary plate screening

Three wells were made in the 10 days old fungus plate. Wells were labeled with A, B, C and 95 % (v/v) of ethanol was used as control. The syringaldazine (0.1 %) (v/v) was added into well B to test for laccase activity. A mixture of 1% (v/v) pyrogalllic acid and 0.4 % (v/v) hydrogen peroxide was added in 1:1 ratio into well C to test for the lignin peroxidase activity (Figure 3.1).

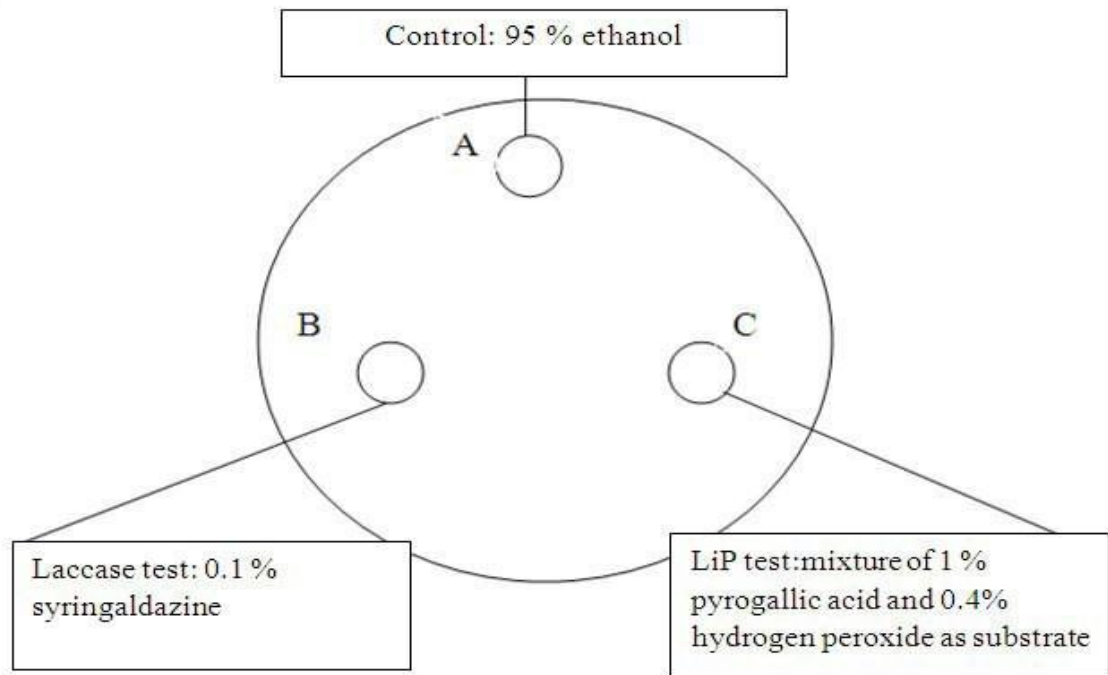


Figure 3.2: Preliminary plate screening for laccase and lignin peroxidase.

3.5 Preparation of mushroom mycelia for submerge fermentation

The extraction of lignin peroxidase (LiP) from *Amauroderma rugosum* was carried out using the mushroom mycelia instead of the mushroom fruiting body as large quantity of fruiting body was hardly available due to the specificity and scarcity of this indigenous species. The submerged culture of *Amauroderma rugosum* was carried out by inoculating 10 pieces of agar blocks from the *Amauroderma rugosum* subculture plate into a flask contained of 100 ml of culture broth.

3.5.1 Optimization of culture media for submerge fermentation

The agar blocks were inoculated into different culture media such as potato dextrose broth (PDB), glucose broth, glucose yeast malt peptone (GYMP) and defined media. Different type of substrates or inducer such as saw dust and bamboo leaves were added in proper composition for optimum lignin peroxidase (LiP) production. The submerged culture

was carried out in an orbital shaker with shaking speed of 120 rpm for 14 days at 25 °C. After 14 days of fungi incubation, the mycelia were filtered with Whatman filter paper (No.1) and were stored in refrigerator in 4 °C for future usage.

Note: The formulation of defined media used: glucose 10 (g/L); ammonium nitrate (NH₄NO₃) 2 (g/L); potassium di-hydrogen phosphate (KH₂PO₄) 0.8 (g/L); di-sodium hydrogen phosphate (Na₂HPO₄) 2(g/L); magnesium sulphate (MgSO₄.7H₂O) 0.5 (g/L) and yeast extract 2 (g/L). (Formula as described by Ding *et al*, 2012)

3.5.2 Effects of shaking conditions for lignin peroxidase (LiP) production

The effects of different shaking conditions (static, 50 rpm and 120 rpm) on the lignin peroxidase (LiP) production were tested for media consisted of potato dextrose broth (PDB), 0.5 % (w/v) of yeast and 1 % (w/v) of saw dust for 14 days at 25 °C as this media produced the highest amount of enzyme lignin peroxidase (LiP). The potato dextrose broth (PDB), 0.5 % (w/v) of yeast and 1 % (w/v) of saw dust provided the culture with sufficient carbon, nitrogen and all major and minor vitamin and minerals sources and were ideal for LiP production.

3.5.3 Effects of copper (Cu²⁺) for lignin peroxidase production

Different amount of coppers (Cu²⁺) such as 0 μM, 50 μM, 100 μM, 150 μM, 200 μM and 300 μM (w/v) were tested on the submerge fermentation at incubation length of 14 days, 120 rpm and 25°C.

3.6 Determination of enzyme activities

The laccase activity was tested by measuring the tetramethoxy-azo-bis-mtehylenequinone which was pinkish in colour resulted from the reaction of laccase with the substrate, the 0.1 mM (v/v) syringaldazine. The laccase activity was measured at absorbance of 525 nm on a spectrophotometer by referring to the protocol of Harkin and Obst (1973) and Leonowicz and Gryzwnowicz (1981) (Appendix A). The substrate blank consisted of 3.2 ml of 50 mM (w/v) sodium citrate and 0.2 ml of 0.1 mM (v/v) syringaldazine. The enzyme blank consisted of 3.2 ml of 50 mM (w/v) sodium citrate and 0.2 ml of enzyme. While the reaction mixture consisted of 3.0 ml of 50 mM (w/v) sodium citrate, 0.2 ml of enzyme and 0.2 ml of 0.1 mM (v/v) syringaldazine. The spectrophotometer was read 1 minute after the adding of syringaldazine substrate in the reaction mixture.

Calculation of laccase activity

One unit was defined as the enzyme producing one unit of absorbance change per minute.

Laccase activity on the sample was calculated as followed:

$$\text{Laccase activity (U/ml)} = (\text{Final absorbance}) \left(\frac{\text{Dilution factor}}{0.2\text{ml}} \right) \left(\frac{1}{1\text{ minute}} \right)$$

The lignin peroxidase activity was tested by determined the oxidation of veratryl alcohol to veratryldehyde in the presence of hydrogen peroxide (H₂O₂) measured at 310 nm wavelength (Have *at al.*, 1998) (Appendix A). The test was initiated by adding 0.2 ml of 0.5 mM hydrogen peroxide (H₂O₂) into a reaction mixture that consists of 2.4 ml of 100mM (v/v) sodium tartrate buffer (pH 3.0), 0.2 ml of 30 mM (v/v) veratryl alcohol and 0.2 ml of enzyme sample. The reagent blank used was without enzyme which contained 2.6 ml of 100 mM (v/v) sodium tartrate buffer, 0.2 ml of 30 mM(v/v) veratryl alcohol and 0.2 ml of 0.5 mM (v/v) hydrogen peroxide, H₂O₂. The enzyme blank used was

without hydrogen peroxide which contained 2.4 ml of 100 mM (v/v) sodium tartrate buffer and 0.2 ml of tested enzyme and the enzyme activity was measured at $\lambda=310$ after 5 minutes.

Calculation of lignin peroxidase (LiP) Activity

The lignin peroxidase (LiP) activity was determined as the unit of activity in μmol of VAD released per minute, thus

$$\text{LiP activity (U/ml)} = \left[\frac{\text{Final absorbance} + 0.003}{0.0313} \left(\frac{\text{dilution factor}}{0.2} \right) \left(\frac{1}{5 \text{ mins}} \right) \left(\frac{1000}{1 \text{ mg}} \right) \left(\frac{1 \mu\text{mole}}{166.18 \mu\text{g}} \right) \right]$$

3.7 Determination of soluble protein concentration

The total soluble protein was determined using the methods described by Bradford (1976) as it can be performed at room temperature and suitable for all protein types. This Bradford method is the colorimetric assay using Coomassie[®] Brilliant Blue G-250 reagent that will bind to the arginine, lysine and other amino acids under acidic condition. The changing of colour from green/brown to blue indicated the present of protein. With the more protein, the more intense will the blue colour be and this make Bradford method a simple and accurate method to test for soluble protein concentration. In Bradford assay, crystalline bovine serum albumin (BSA) was being used as a standard to determine the protein concentration. About 0.2 ml of the protein sample was added with 5ml of Coomassie[®] Brilliant Blue G-250 reagent and the sample was vortex and incubated for 5 minutes at room temperature. The reaction of the protein sample and the Coomassie[®] Brilliant Blue G-250 reagent was measured spectrometrically at 595 nm. The determination of soluble protein concentration shall not exceed one hour as the dye lost its stability after one hour.

3.8 Preparation of ATPS

The purification of the enzyme from *Amauroderma rugosum* was then carried out using the aqueous two-phase system (ATPs) which offers easy steps for extraction and purification without affecting the native conformation and function of the enzyme. A binodial phase diagrams for different molecular weights of polyethylene glycol (PEG), PEG600, 1000, 1500, 3350, and 8000 and 40 % (w/v) potassium phosphate salt ($\text{KH}_2\text{PO}_4=18.3$ g/100ml; $\text{K}_2\text{HPO}_4=21.70$ g/100 ml) at pH 7.0 were obtained by referring to protocol by Albertsson (1986). The tie lie line (TLL) was computed based on the binodial phase diagrams. The aqueous two-phase system was carried out based on the protocol described by Vaidya *et al.* (2006). The predetermined quantities of dissolved PEG and potassium phosphate were prepared in a 15 ml centrifugal tube and 1 g (w/v) of crude enzyme was then added to top up to a total weight of 10 g (w/v). Gentle agitation was carried out and the centrifugal tubes are then subjected to 4000 rpm centrifugation for 10 minutes for phase separation. Both upper and bottom layers of the system were then tested for enzyme activity and soluble protein concentration.

3.8.1 Optimization of aqueous two-phase system

The effects of different parameters on lignin peroxidase partitioning were studied such as different tie-line length (TLL), volume ratio (V_R), pH, crude enzyme adding and NaCl (1-4 %, w/v) salt adding. The effects of different TLL were studied by selecting 5 systems from the selected PEG molecular weight and the TLL with the best enzyme partitioning result was selected. Along the selected TLL, five systems with a different volume ratio were evaluated for their influence on LiP partitioning. The volume ratio producing the best result was chosen for pH study. The phosphate salt in range of pH 6 to 9 were prepared by mixing different composition of potassium di-phosphate salt

(KH₂PO₄) and di-potassium phosphate salt (K₂HPO₄) as described by Lin *et al.* (2012). The system pH of phosphate salt that gave the best result of LiP partitioning was then selected for crude enzyme amount added (10-20%, w/v) in the system. Lastly, the effect of sodium chloride (NaCl) salt adding ranging from 1-4 percent was studied and the optimal parameters for ATPs was recorded.

3.9 Determination of volume ratio, partition coefficients, purification factor, yields and selectivity

The volume ratio (V_R) was defined as the ratio of volume in the top phase (V_T) to the volume of the bottom phase (V_B) (Equation 1):

$$V_R = \frac{V_T}{V_B} \quad (1)$$

The partition coefficient of protein (K_P) was defined as the ratio of concentrations of protein in top (P_T) and bottom phase (P_B) (Equation 2)

$$K_p = \frac{P_T}{P_B} \quad (2)$$

Where P_T and P_B are concentrations of protein in top and bottom phase, respectively.

The partition coefficient of enzyme (K_E) of the LiP was calculated as the ratio of the LiP concentration in top and bottom phases (Equation 3)

$$K_E = \frac{C_T}{C_B} \quad (3)$$

Where C_T and C_B were the LiP activity in U/ml in the top phase and the bottom phase, respectively. The Log K was expressed as the log of K_E .

The specific activity (SA) was the enzyme activity (U/ml) in the phase sample divided by the protein concentration ($\mu\text{g/ml}$) and was expressed U/ μg of protein (Equation 4):

$$SA = \frac{\text{Total LiP activity}}{\text{Total protein concentration}} \quad (4)$$

The purification factor (P_{FT}) was a comparison of the lignin peroxidase (LiP) purity in the purification process to the purity of lignin peroxidase (LiP) before the purification. The P_{FT} was calculated as the ratio of specific activity in the collected phase to the initial original specific activity (Equation 5):

$$P_{FT} = \frac{\text{SA in collected phase}}{\text{Initial S}} \quad (5)$$

Yield of LiP in top phase was determined using (Equation 6):

$$Y_T(\%) = \frac{100}{1 + [1/(V_R * K)]} \quad (6)$$

Where K was partition coefficient and V_R was the volume ratio.

Lastly, the selectivity (S) was determined using (Equation 7):

$$S = \frac{K_E}{K_P} \quad (7)$$

Where K_E was partition coefficient of enzyme and K_P was partition coefficient of protein.

3.10 Characterization of protein

Molecular mass of the crude enzyme and top phase enzyme of ATPs were analyzed using the Sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE). The SDS-PAGE was performed according to Laemmli and Favrel, (1973) protocol using 12 % (w/v) resolving gel and 5 % (w/v) stacking gel (Appendix A). The SDS-PAGE of lignin peroxidase (LiP) crude enzyme was carried out by adding 18 μ l of crude enzyme and 4 μ l of loading dye for crude enzyme molecular mass determination. A total of 25 μ l consisted of 20 μ l of recovered LiP enzyme on PEG phase and 5 μ l of loading was used to determine the molecular mass of recovered enzyme from top phase. Electrophoresis was carried out at 100 V for 120 minutes and the gel was stained with the Coomassie® Brilliant Blue R-250. The native-PAGE was then carried out to determine the lignin peroxidase (LiP) activity. The native-PAGE using the same protocol as described above without SDS and β -mercaptoethanol. The bands from the native-PAGE were excised and subjected to lignin peroxidase (LiP) activity test (Have *et al.*, 1998).

3.11 Statistical analysis

All experiments were conducted and analyzed in triplicates. Means and standard deviations were calculated and compared using the one way ANOVA. Multiple comparison tests were done by Tukey HSD and Duncan test. Significant of differences was defined as $p < 0.05$. Analysis was performed using SPSS package (PASW Statistics 18 for windows, SPSS Inc. Chicaga, IL).

CHAPTER4 RESULTS

4.1 Preliminary plate screening

The data was based on the result given at 30 minutes by observed towards the plate as shown in Figure 4.1. At this particular time, both the laccase and lignin peroxidase (LiP) activity reached the peak by showing colour changes. From Table 4.1, the laccase activity was strongest at 11th day and 12th day of culture and no laccase activity was observed on 13rd and 14th day;. For lignin peroxidase activity, a clear trend on increasing colour intensity was shown from 10th to 14th day; however the enzyme was lost after 14th day.

Table 4.1: The preliminary screening of the *Amauroderma rugosum* to determine laccase and lignin peroxidase activity.

	10 th Day	11 st Day	12 nd Day	13 rd Day	14 th Day
Control (95% ethanol)	-	-	-	-	-
Laccase	++	+++	+++	-	-
Lignin peroxidase	+++	+++	++++	++++	++++

*(The colour intensity was determined by the changes of purple colour from very light purple + (low), light purple ++ (intermediate), purple +++ (high), dark purple++++(very high), - indicates no colour changes).



Figure 4.1: Preliminary plate screening of *Amauroderma rugosum*. The changes of well colour to yellow indicate the presence of lignin peroxidase and the changes to pink-purple colour indicates the presence of laccase.

4.2 Submerged liquid fermentation

4.2.1 Optimization of culture medium for submerged fermentation.

From Table 4.2, none of the media successfully produce laccase although laccase activity was detected during preliminary screening. Almost all the media tested produced lignin peroxidase (LiP). As the increase in carbon and nitrogen sources, the enzyme productivities increase. Overall, the best media for lignin peroxidase (LiP) production was using defined media with 29.46 ± 0.70 U/ml followed by potato dextrose broth (PDB) with 1 % of saw dust and 0.5 % of yeast with 26.70 ± 3.70 U/ml after 14th day of culture at shaking speed of 120 rpm at 25 °C.

Table 4.2: Laccase and LiP activities of *Amauroderma rugosum* cultivated in different media.

Medium	Laccase activity (U/ml)	Lignin Peroxidase activity (U/ml)
Potato Dextrose Broth (PDB)	-	3.13 ± 0.84 ^(a)
Potato Dextrose Broth (PDB) + 1% saw dust	-	13.52 ± 0.20 ^(c)
Potato Dextrose Broth (PDB) + 1% saw dust + 1% malt	-	4.80 ± 0.29 ^(a,b)
Potato Dextrose Broth (PDB) + 1% saw dust + 0.5 % yeast	-	26.70 ± 3.7 ^(d,e)
Potato Dextrose Broth (PDB) + 1% bamboo leaf + 0.5 % yeast	-	24.61 ± 1.51 ^(d)
Glucose-Yeast-Malt-Peptone (GYMP)	-	3.26 ± 0.23 ^(a)
Glucose 1% + bamboo leaves 1% + yeast 1%	-	7.19 ± 0.19 ^(b)
Glucose 1% + saw dust 1% + yeast 1%	-	7.23 ± 0.04 ^(b)
Glucose 1% + 1% saw dust	-	4.54 ± 0.29 ^(a,b)
Glucose 2 % + 1% saw dust	-	5.44 ± 0.19 ^(a,b)
Defined media	-	29.46 ± 0.70 ^(e)

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Tukey HSD ($p < 0.05$). Uses harmonic mean sample size=3.00. The boxes in the table indicated the media with highest LiP activity (U/ml).

4.2.2 Effects of shaking conditions for lignin peroxidase production

In the static submerged culture, no lignin peroxidase (LiP) activity was detected in the 14 days of submerge fermentation. At shaking speed of 50 rpm, the enzyme production was low which was 1.60 ± 0.78 U/ml for medium containing potato dextrose broth (PDB) and 1 % of saw dust only. At the same shaking speed of 50 rpm, medium with potato dextrose broth (PDB), 1% of saw dust and 0.5% of yeast, the lignin peroxidase (LiP) activity was 4.76 ± 1.50 U/ml.

When the shaking speed increased to 120 rpm, the mycelia in both of the media showed increased of lignin peroxidase (LiP) activities. The lignin peroxidase (LiP) activity for medium containing potato dextrose broth (PDB) and 1 % of saw dust was 13.52 ± 0.20 U/ml. While potato dextrose broth (PDB) with 1 % of saw dust and 0.5 % of yeast, the lignin peroxidase (LiP) activity of 26.70 ± 3.70 U/ml was expressed by the *Amauroderm rugosum* (Figure 4.2).

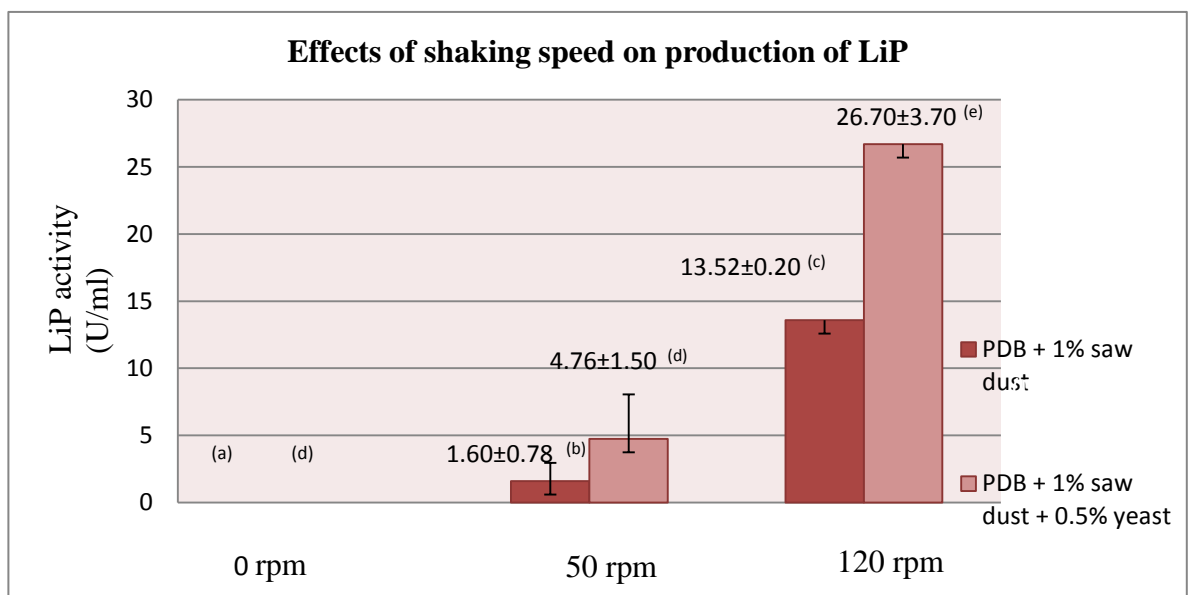


Figure 4.2: Effects of shaking conditions on the production of lignin peroxidase (LiP).

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Tukey HSD ($p < 0.05$). Uses harmonic mean sample size=3.00.

4.2.3 Effects of copper (Cu²⁺) for lignin peroxidase (LiP) production

As shown in Figure 4.3, the addition of copper into the media (PDB + 1 % saw dust + 0.5 % yeast) increased the lignin peroxidase activity in *Amauroderma rugosum*. The media without copper showed lowest lignin peroxidase activity at 3.13±0.84 U/ml. The highest activity of 105.68±5.87 U/ml of lignin peroxidase enzyme was recorded when the submerged culture was inoculated with 150 µM of copper. However, when the copper concentration increased from 200 µM to 300 µM, the lignin peroxidase activities were significantly (p<0.05) decreased.

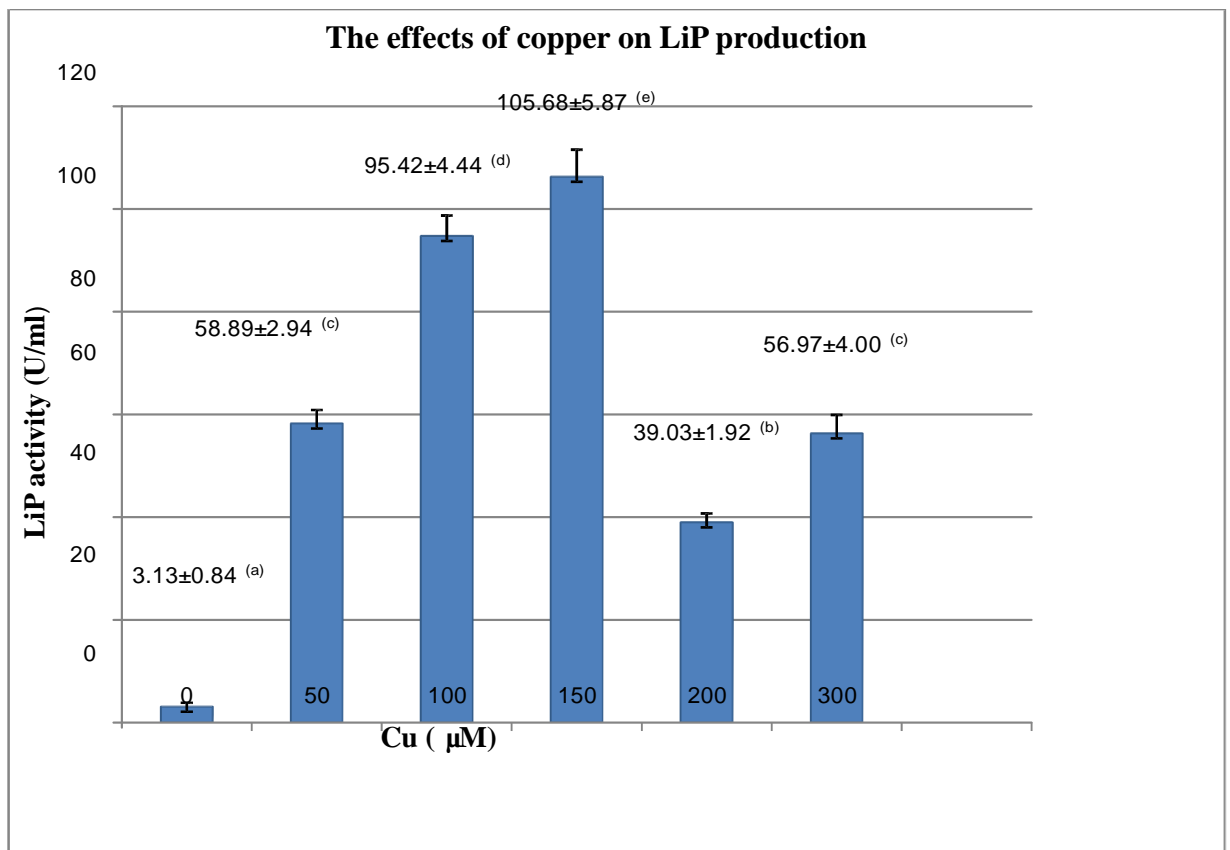


Figure 4.3: Effects of copper (Cu²⁺) for lignin peroxidase (LiP) production.

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Tukey HSD (p<0.05). Uses harmonic mean sample size=3.00.

4.3 The phase diagrams of polyethylene glycol (PEG)-phosphate system with different molecular weights.

The phase diagrams of polyethylene glycol (PEG)-phosphate system with four different PEG molecular weights (PEG 600, 1000, 1500, 3350, 8000) at constant volume ratio ($V_R=1$), pH 7 and with increasing TLL values were shown in Figure 4.4-4.9.

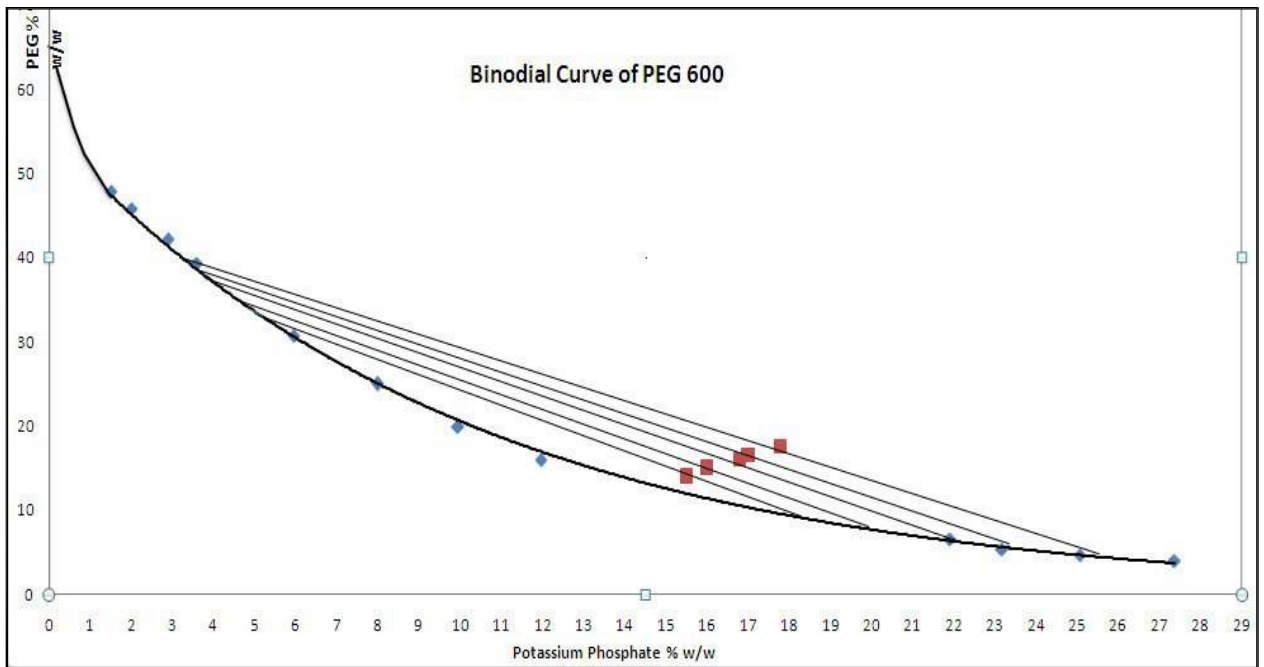


Figure 4.4: Phase diagram for PEG 600/potassium phosphate ATPS. The binodial curve for PEG 600/potassium phosphate was plotted.

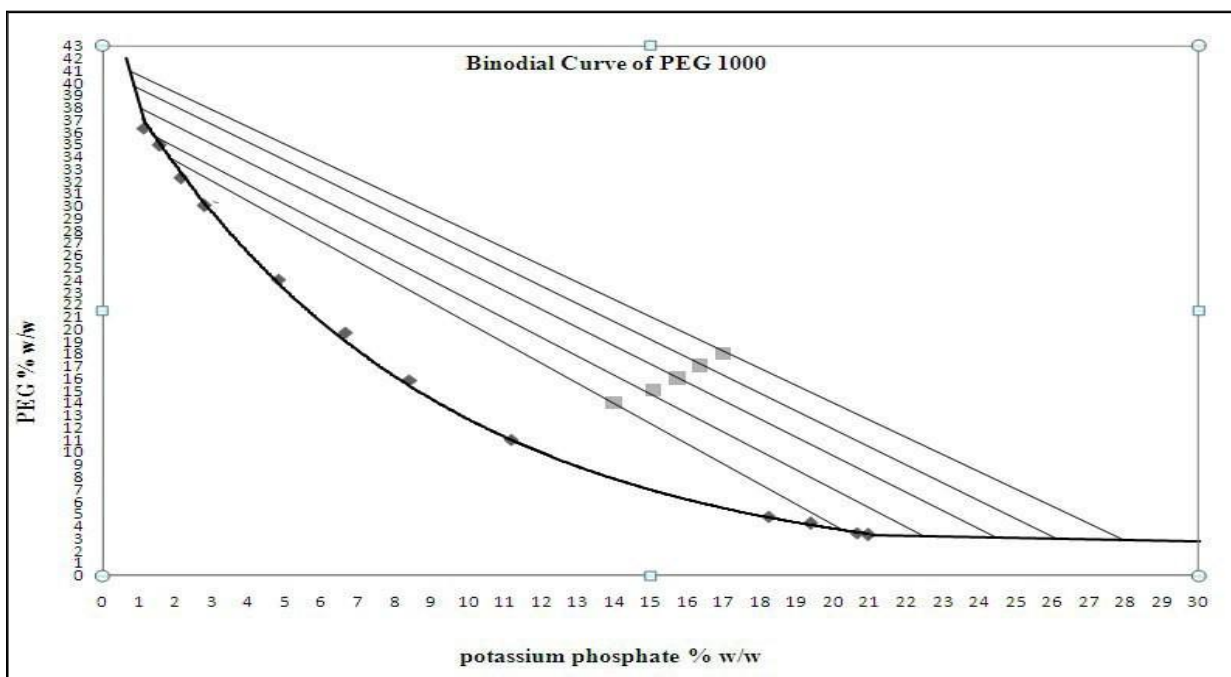


Figure 4.5: Phase diagram for PEG 1000/potassium phosphate ATPS. The binodial curve for PEG 1000/potassium phosphate was plotted.

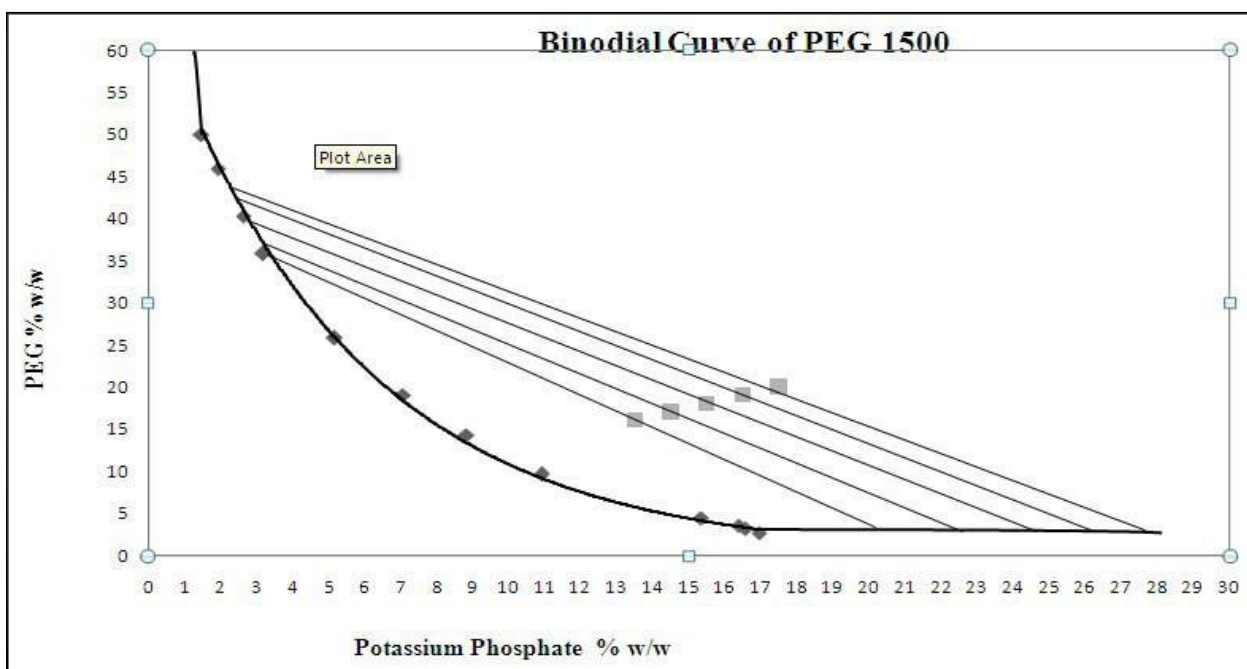


Figure 4.6: Phase diagram for PEG 1500/potassium phosphate ATPS. The binodial curve for PEG 1500/potassium phosphate was plotted.

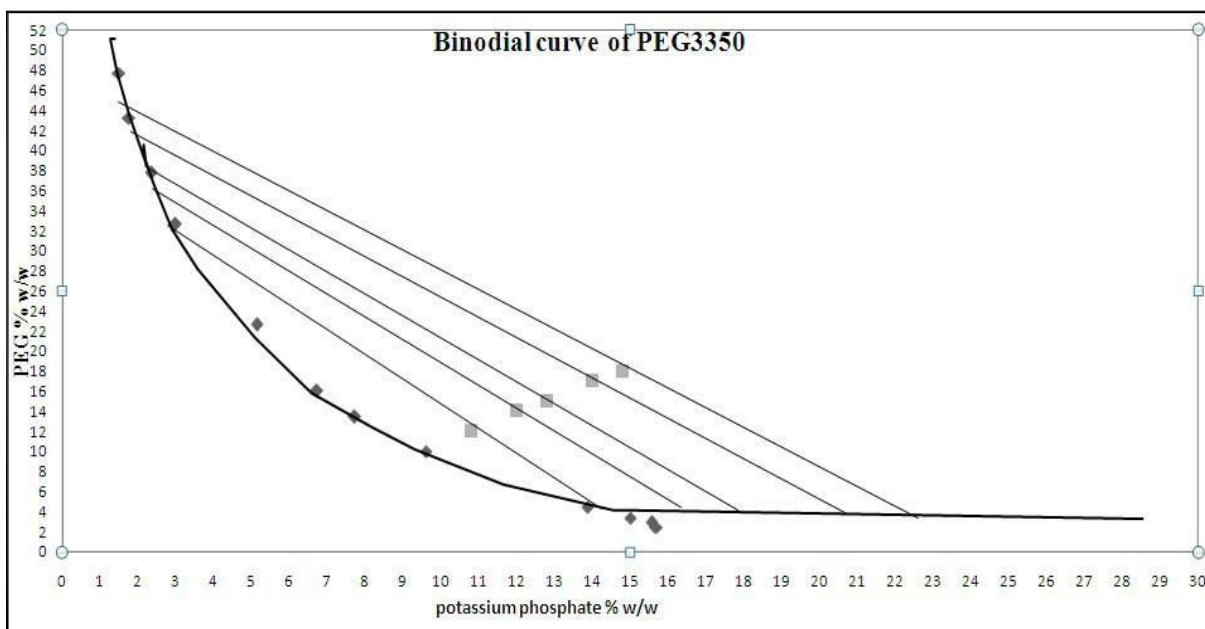


Figure 4.7: Phase diagram for PEG 3350/potassium phosphate ATPS. The binodial curve for PEG 3350/potassium phosphate was plotted.

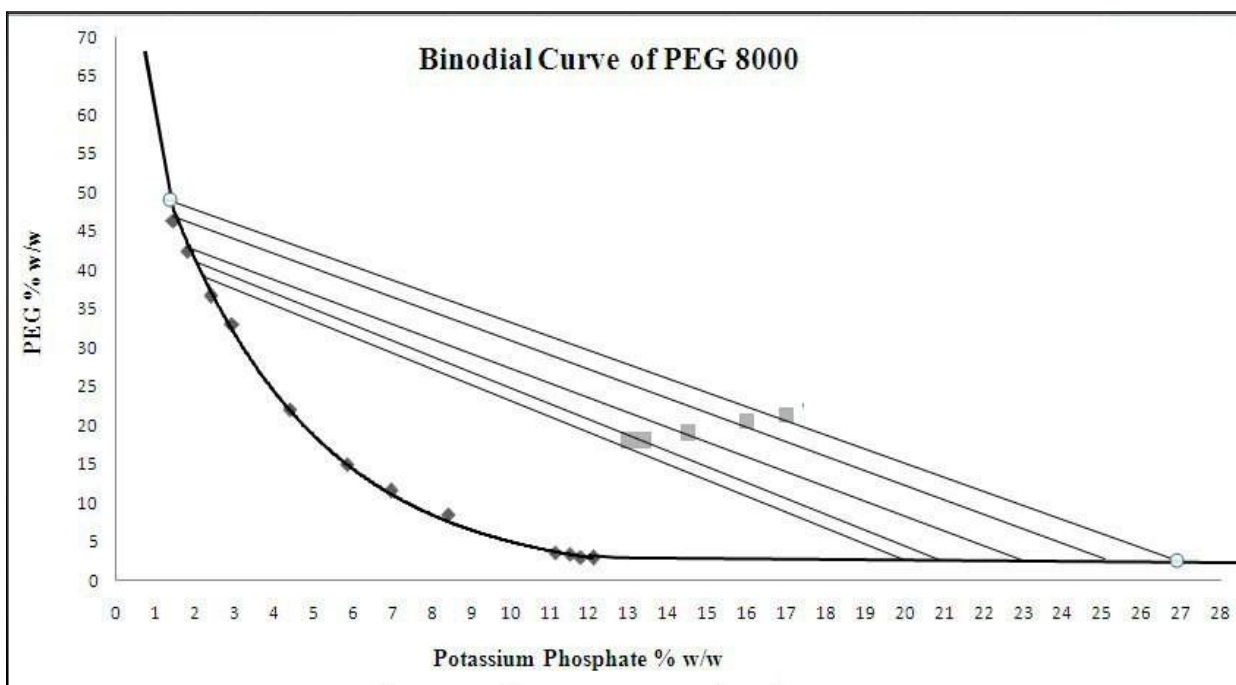


Figure 4.8: Phase diagram for PEG 8000/potassium phosphate ATPS. The binodial curve for PEG 8000/potassium phosphate was plotted.

From the phase diagram, a total of 25 systems (Table 4.3) with different tie lie line (TLL) were evaluated for the recovery of lignin peroxidase.

Table 4.3: 25 systems with 1 to 1 ratio and tie-lie length (TLL) for the evaluation of partition behavior of LiP from *Amauroderma rugosum*.

Molecular weight of PEG	System No.	% PEG (w/w)	% Phosphate (w/w)	Tie- Lie Length
PEG 600	1.	14.00	15.50	31.70
	2.	15.00	16.00	34.10
	3.	16.00	16.80	36.80
	4.	16.50	17.00	37.50
	5.	17.50	17.80	39.60
PEG 1000	6.	14.00	14.50	35.94
	7.	15.10	15.00	39.52
	8.	16.00	15.75	42.16
	9.	17.00	16.35	44.24
	10.	18.00	17.00	46.10
PEG 1500	11.	16.00	13.50	35.75
	12.	17.00	14.50	38.64
	13.	18.00	15.50	42.36
	14.	19.00	16.50	45.92
	15.	20.00	17.50	47.93
PEG 3350	16.	12.00	10.80	29.06
	17.	14.00	12.00	35.13
	18.	15.00	12.80	37.58
	19.	17.00	14.00	42.49
	20.	18.00	14.80	45.29
PEG 8000	21.	18.00	13.00	41.32
	22.	18.00	13.40	42.29
	23.	19.00	14.50	44.99
	24.	20.50	16.00	49.44
	25.	21.20	17.00	52.27

4.3.1 Influence of the different PEG molecular weight on purification factor and yield of lignin peroxidase (LiP).

The enzyme lignin peroxidase (LiP) was recovered at the top phase contained of PEG. Hence the partition coefficients (Table 4.4) for majority of the systems tested were above one which also means the enzyme was partition at the preferences of top phase. Among all the 25 systems with different PEG tested, system 2 of PEG 600 was selected. This system was selected as it showed the highest top phase lignin peroxidase (LiP) activity, 89.29 ± 19.35 U/ml, top phase purification factor of 1.33 ± 0.62 and the yield of 72.18 ± 8.50 %. The lowest top phase purification factor of 0.05 ± 0.03 and yield of 35.20 ± 5.78 was recorded for system 5 of PEG 8000. While at other systems in intermediate and higher PEG molecular weights from PEG 3350 to PEG 8000, both the top phase and bottom phase purification factors were recorded low. Thus PEG 600 was selected for further volume ratio, pH, crude enzyme adding and sodium chloride (NaCl) salt adding.

Table 4.4: The ATPs results of 5 selected PEG molecular weight.

MW of PEG	LiP Crude Activity (U/ml)	LiP Top Activity (U/ml)	LiP Bottom Activity (U/ml)	Top Purification Factor	Bottom Purification Factor	Partition coefficient (Enzyme)	Selectivity	Top Recovery Yield (%)	Bottom Recovery Yield (%)
PEG600									
1	85.37±2.22	82.11±8.17	25.00±8.67	0.63±0.13	0.20±0.10	3.50±0.98	3.76±1.59	77.10±4.64	22.90±4.64
2	85.37±2.22	89.29±19.35	33.46±7.28	1.33±0.62	0.19±0.10	2.85±1.28	8.40±6.64	72.18±8.50	27.82±8.50
3	85.37±2.22	85.38±7.03	26.47±3.33	1.20±0.20	0.40±0.03	3.27±0.57	3.02±0.40	76.28±3.21	23.72±3.21
4	85.37±2.22	83.03±17.35	24.36±3.33	1.07±0.25	0.18±0.02	3.51±1.18	6.02±1.42	76.80±5.90	23.20±5.90
5	85.37±2.22	84.53±4.00	21.54±4.00	0.90±0.18	0.14±0.05	4.01±0.71	6.66±0.97	79.78±2.80	20.22±2.80
PEG1000									
1	26.70±3.70	16.60±2.94	9.36±0.98	0.37±0.12	0.13±0.01	1.78±0.25	2.86±0.96	63.75±3.23	36.25±3.23
2	26.70±3.70	43.51±10.59	7.37±6.21	0.87±0.16	0.07±0.08	13.04±14.28	33.57±38.65	84.75±14.2	15.25±14.2
3	26.70±3.70	73.21±27.89	5.06±3.90	0.91±0.50	0.03±0.02	20.76±17.17	37.03±28.69	93.21±4.49	6.79±4.49
4	26.70±3.70	49.92±8.67	19.16±4.84	0.46±0.13	0.12±0.03	2.64±0.24	3.87±0.45	72.49±1.77	27.51±1.77
5	26.70±3.70	24.93±4.84	35.18±0.00	0.26±0.12	0.21±0.03	0.71±0.14	1.23±0.46	41.21±4.82	58.79±4.82
PEG1500									
1	85.37±2.22	83.13±8.47	18.08±4.84	0.96±0.16	0.18±0.08	4.74±0.87	5.89±1.97	82.33±2.57	17.67±2.57
2	85.37±2.22	78.33±9.20	22.38±6.18	0.81±0.23	0.18±0.07	3.63±0.70	4.76±1.10	78.09±3.03	21.91±3.03
3	85.37±2.22	78.12±8.67	26.66±3.85	0.81±0.13	0.16±0.05	2.94±0.11	5.09±0.68	74.61±0.67	25.39±0.67
4	85.37±2.22	75.13±12.58	22.50±2.94	0.83±0.24	0.16±0.09	3.33±0.21	5.85±1.81	76.89±1.10	23.11±1.10
5	85.37±2.22	81.96±7.77	16.54±6.18	0.74±0.10	0.08±0.04	5.40±1.93	10.35±3.21	83.35±5.12	16.65±5.12

‘Table 4.4, continued’

MW of PEG	LiP Crude Activity (U/ml)	LiP Top Activity (U/ml)	LiP Bottom Activity (U/ml)	Top Purification Factor	Bottom Purification Factor	Partition coefficient (Enzyme)	Selectivity	Top Recovery Yield (%)	Bottom Recovery Yield (%)
PEG3350									
1	23.43±1.96	16.60±2.22	11.47±2.22	0.26±0.04	0.21±0.07	1.47±0.25	1.28±0.25	59.22±3.99	40.78±3.99
2	23.43±1.96	21.08±1.11	6.34±0.00	0.24±0.01	0.06±0.01	3.33±0.18	4.22±0.67	76.86±0.96	23.14±0.96
3	23.43±1.96	13.39±2.22	10.19±0.00	0.15±0.05	0.06±0.01	1.32±0.22	2.29±0.53	56.54±3.89	43.46±3.89
4	23.43±1.96	15.31±2.22	9.55±1.11	0.18±0.05	0.07±0.01	1.64±0.45	2.41±0.58	61.41±6.03	38.59±6.03
5	23.43±1.96	24.29±1.11	4.42±1.92	0.37±0.05	0.03±0.01	6.28±2.83	13.90±5.84	84.94±5.19	15.06±5.19
PEG8000									
1	26.70±3.70	57.76±10.48	11.03±3.33	0.79±0.34	0.14±0.06	5.35±0.59	5.65±0.45	84.16±1.53	15.84±1.53
2	26.70±3.70	48.64±8.38	15.70±5.11	0.46±0.11	0.16±0.09	3.26±0.91	3.44±1.15	75.90±4.61	24.10±4.60
3	26.70±3.70	54.41±6.66	15.83±0.99	0.48±0.13	0.14±0.02	3.46±0.60	3.47±0.70	77.31±2.91	22.70±2.90
4	26.70±3.70	69.15±14.43	15.71±3.19	0.61±0.27	0.10±0.03	4.42±0.58	6.01±0.94	81.41±1.86	18.60±1.86
5	26.70±3.70	6.34±1.93	11.48±1.92	0.05±0.03	0.06±0.01	0.55±0.15	0.74±0.28	35.20±5.78	64.80±5.78

Note: The boxes indicated the selected system for PEG600.

Table 4.5: The SPSS analysis for top phase purification factor of PEG 600-8000.

Molecular weight of PEG	System No.	% PEG (w/w)	% Phosphate (w/w)	P_{FT}
PEG 600	1.	14.00	15.50	0.63±0.13 ^(a,b,c,d,e,f,g,h)
	2.	15.00	16.00	1.33±0.62 ^(h)
	3.	16.00	16.80	1.20±0.20 ^(g,h)
	4.	16.50	17.00	1.07±0.25 ^(f,g,h)
	5.	17.50	17.80	0.90±0.18 ^(d,e,f,g,h)
	6.	14.00	14.50	0.37±0.12 ^(a,b,c,d,e,f)
PEG 1000	7.	15.10	15.00	0.87±0.16 ^(c,d,e,f,g,h)
	8.	16.00	15.75	0.91±0.50 ^(d,e,f,g,h)
	9.	17.00	16.35	0.46±0.13 ^(a,b,c,d,e,f)
	10.	18.00	17.00	0.26±0.12 ^(a,b,c,d,e)
	11.	16.00	13.50	0.96±0.16 ^(e,f,g,h)
PEG 1500	12.	17.00	14.50	0.81±0.23 ^(b,c,d,e,f,g,h)
	13.	18.00	15.50	0.81±0.13 ^(b,c,d,e,f,g,h)
	14.	19.00	16.50	0.83±0.24 ^(b,c,d,e,f,g,h)
	15.	20.00	17.50	0.74±0.10 ^(a,b,c,d,e,f,g,h)
PEG 3350	16.	12.00	10.80	0.26±0.04 ^(a,b,c,d,e)
	17.	14.00	12.00	0.24±0.01 ^(a,b,c,d)
	18.	15.00	12.80	0.15±0.05 ^(a,b)
	19.	17.00	14.00	0.18±0.05 ^(a,b,c)
PEG 8000	20.	18.00	14.80	0.37±0.05 ^(a,b,c,d,e,f)
	21.	18.00	13.00	0.79±0.34 ^(b,c,d,e,f,g,h)
	22.	18.00	13.40	0.46±0.11 ^(a,b,c,d,e,f)
	23.	19.00	14.50	0.48±0.13 ^(a,b,c,d,e,f)
	24.	20.50	16.00	0.61±0.27 ^(a,b,c,d,e,f,g)
	25.	21.20	17.00	0.05±0.03 ^(a)

Note: Top phase purification factors with different letter(s) were significantly different by Tukey HSD (p<0.05). Uses harmonic mean sample size=3.00.

4.3.2 Influence of the TLL on purification factor and yield of lignin peroxidase (LiP).

The effect of TLL on the purification factor was studied for all PEG molecular weight tested. Only PEG 1000 (system 6-10) showed increase in top phase purification factor (P_{FT}) with increasing TLL from TLL of 35.94 to TLL of 42.16 (Table 4.6). System 9 and 10 of PEG 1000 gave negative result on top phase purification factor (P_{FT}) with increasing TLL. The top phase purification factor (P_{FT}) was significantly increased. However, such increased showed no significant different by multi-comparison test, the Tukey HSD test in where the p-value was more than 0.05. Thus, PEG 1000 was not selected for further study (APPENDIX B).

Table 4.6: Influence of the PEG molecular weight and TLL on P_{FT}

PEG molecular weight (g/mol)	TLL (%)		P_{FT}
PEG 1000	6	35.94	$0.37 \pm 0.12^{(a)}$
	7	39.52	$0.87 \pm 0.16^{(a)}$
	8	42.16	$0.91 \pm 0.50^{(a)}$
	9	44.24	$0.46 \pm 0.13^{(a)}$
	10	46.10	$0.26 \pm 0.12^{(a)}$

Note: Since the p-value in Tukey HSD was 0.065 and was more than 0.05 ($p > 0.05$), there was no significant different into phase purification factors (P_{FT}) in different TLL of PEG 1000. Uses harmonic mean sample size=3.00.

4.3.3 Effect of volume ratio on top phase purification factor

Since the best system selected was at PEG 600 with TLL of 34.10 % (w/w), lignin peroxidase partitioning at different V_R values along the TLL were selected for further study. Among the 6 different volumes ratio tested which were 0.5, 0.62, 0.79, 1.00, 1.30 and 1.77. Volume ratio of 0.62 gave the highest top phase purification factor (P_{FT}) of 2.66 ± 0.14 , log K of 1.08 ± 0.07 and selectivity of 23.69 ± 6.49 (Figure 4.9). At lower volume ratio such as 0.52, the top phase purification factor (P_{FT}) was 1.89 ± 0.33 , the selectivity was 14.11 ± 2.67 and log k was 0.90 ± 0.09 . At higher volume ratio of 1.77, the top phase purification factor (P_{FT}) was 1.67 ± 0.53 , the selectivity was 3.78 ± 1.08 and the log k was 0.46 ± 0.11 (Figure 4.9).

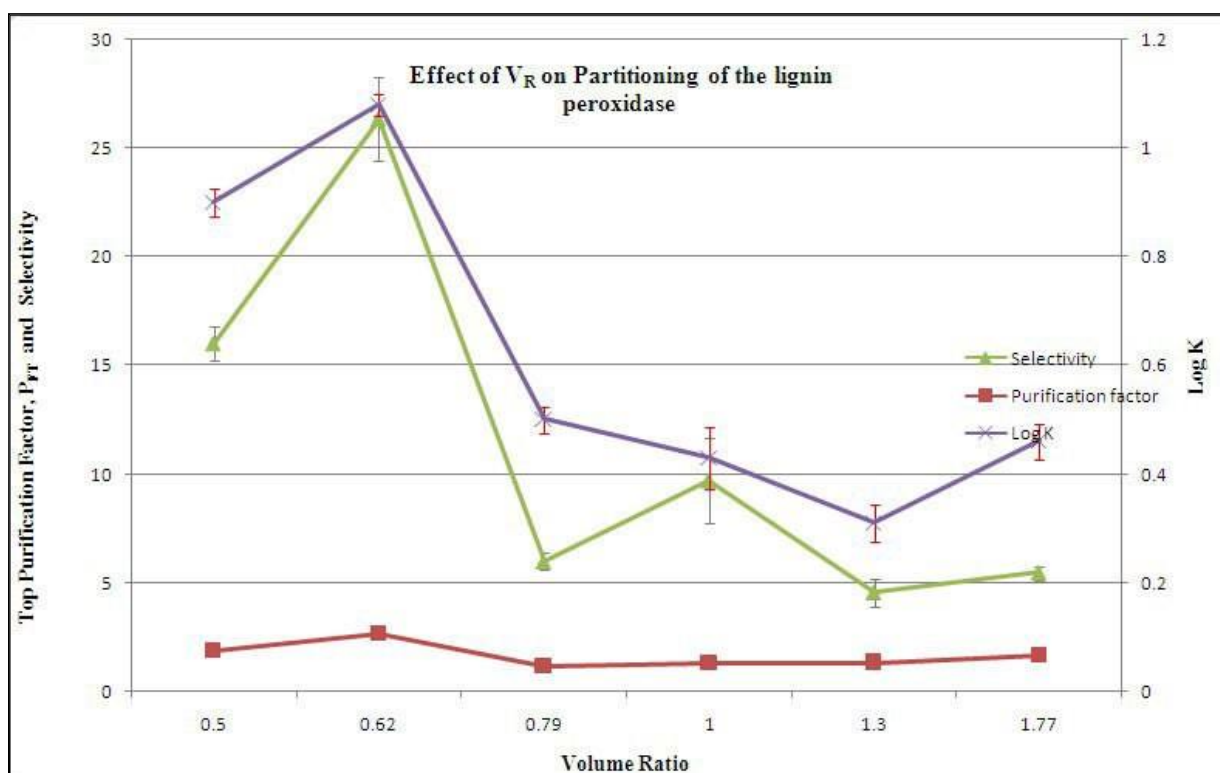


Figure 4.9: Influence of the volume ratio on partitioning of the lignin peroxidase (LiP) by showing the purification factor (P_{FT}), Selectivity and Log K.

4.3.4 Effect of pH on P_{FT}

The pH in ATPs system was measured using the method described by Lin *et al.* (2012) by mixing different composition of potassium di-phosphate salt (KH_2PO_4) and di-potassium phosphate salt (K_2HPO_4) in the pH range of 6-9.

The increased of the system pH generally induced an increased in the purification factor of lignin peroxidase (LiP) ranging from pH 6.5 to pH 8.0 followed by a sudden dropped at pH 8.5 and pH 9.0 (Figure 4.10). Although there was no significant differences for the top phase purification factor between pH 7.5 and pH 8 which was 5.71 ± 1.78 and 6.25 ± 3.21 respectively, the system tested with pH 8 was selected as it gave a better partition coefficient of 7.38 (Appendix B).

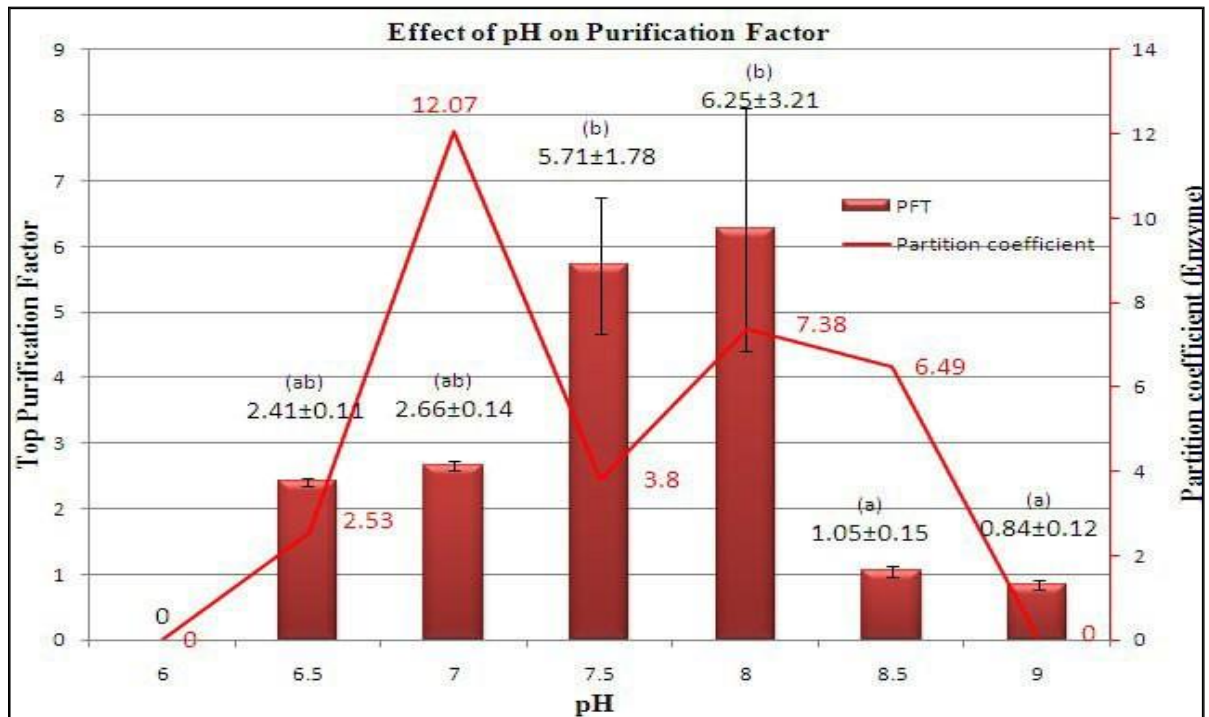


Figure 4.10: Influence of the pH on top phase purification factor (P_{FT}) of lignin peroxidase (LiP).

Note: Top purification factors with different letter(s) were significantly different by Turkey's HSD ($p < 0.05$). Uses Harmonic Mean Sample Size=3.00.

4.3.5 Effect of crude enzyme amount added in the system on P_{FT}

The maximum capacity for a 10 g ATPs system was 10 % of crude loading (w/w) with the highest purification factor of 6.25 ± 3.21 with the yield of 81.08 ± 4.92 % (Table 4.7). However, when the amount of crude enzyme increase to 20 % (w/w), there was a sudden dropped of the purity of enzyme with purification factor 0.69 ± 0.20 and the yield of 69.76 ± 2.24 %.

Table 4.7: Influence of the crude feedstock load from 10 % (w/w) to 20 % (w/w) on the P_{FT} and yield of lignin peroxidase (LiP).

Crude Load (% w/w)	Purification factor , P_{FT}	Yield (%)
10	6.25 ± 3.21	81.08 ± 4.92
20	0.69 ± 0.20	69.76 ± 2.24

4.3.6 Effect of sodium chloride (NaCl) salt on partition coefficient and PFT of lignin peroxidase (LiP).

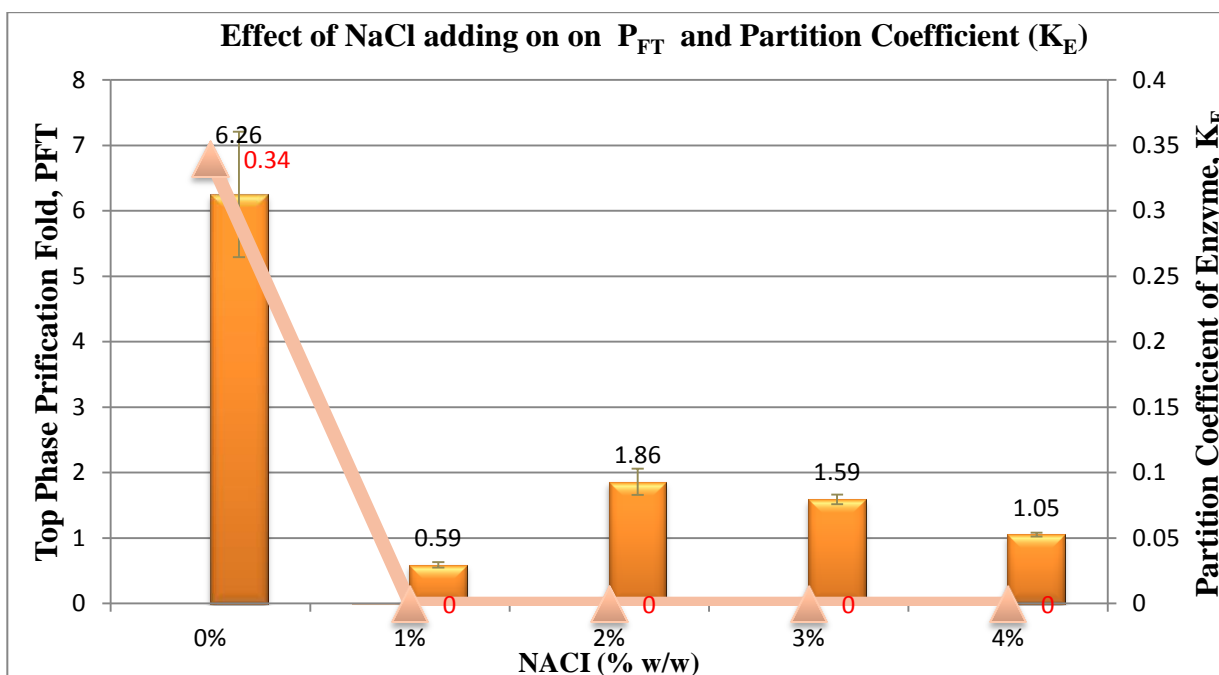


Figure 4.11: Influence of the NaCl (% w/w) on top phase purification factor (P_{FT}) and partition coefficient of enzyme (K_E) of lignin peroxidase (LiP).

The effect of sodium chloride (NaCl) salt from 1 % (w/w)-4 % (w/w) added to the system was shown in Figure 4.11. It was clearly shown that by adding the salt, majority of the top phase enzyme activities decreased (Figure 4.11), indicated the purity of enzyme on the top phase was greatly reduced. Moreover, the top phase purification factor (P_{FT}) was decreased with no enzyme partition coefficient reading. In addition, no lignin peroxidase (LiP) activity was detected at the bottom phase as well indicated the shifting of the enzymes partition not only at both top and bottom phase but possibly towards intermediate phase. Since addition of salt had no effect on LiP purification, this parameter had been excluded. Thus, the best top phase purification factor (P_{FT}) of lignin peroxidase was 6.25 ± 3.21 with yield of 81.08 ± 4.92 % at volume ratio of 0.62, pH 8.0 and crude enzyme adding of 10 %.

4.4 SDS-PAGE and Native-PAGE analysis of purified lignin peroxidase from ATPs extraction.

The characterization of the lignin peroxidase was assessed by SDS-PAGE and Native-PAGE analysis. From Figure 4.12 (lane 3), three distinctive bands were shown in both gels. These bands sizes were estimated to be 38 kDa; 45-46 kDa; and 66 kDa. Band at lane 6 with molecular size of 66 kDa showed clumps on native-PAGE. The three bands obtained (band e-g, lanes 4-6, Figure 4.12) from the Native-PAGE were then excised and subjected to lignin peroxidase (LiP) activity test as described by Have *et al.* (1998). All bands excised from native-PAGE showed positive lignin peroxidase (LiP) activities of 4.67 U/ml, 6.02 U/ml and 6.02 U/ml for bands d, e and f, respectively. The enzyme activity retained after the native-PAGE was 68.68 % (Table 4.8).

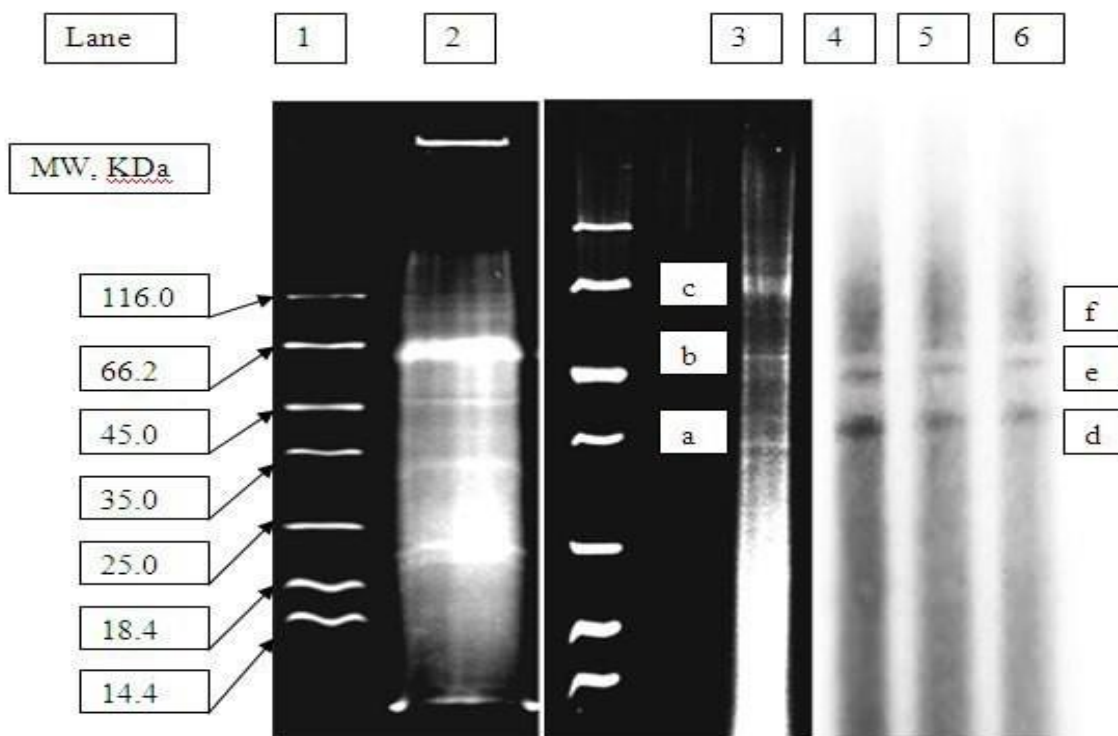


Figure 4.12: SDS-PAGE and NATIVE-PAGE analysis of the recovery of lignin peroxidase by 12 % gel. The molecular weight of the Bio Basic BSM043 used was ranging from 14.4 kDa to 116 kDa. SDS-PAGE-lane 1: protein molecular marker; lane 2: crude enzyme; lane 3: top phase. Native-PAGE-lane 4, 5, 6-top phase enzymes.

Note: The letters a, b, and c denoted lignin peroxidase (LiP) with different molecular weights. a-apparently 38 kDa; b-45-46 kDa; c-apparently 66 kDa.

Table 4.8: The lignin peroxidase (LiP) tests for three excised bands in NATIVE-PAGE.

Band's Name	Crude Enzyme	A	B	C	Enzyme Activity Retained after native-PAGE(%)
Lignin peroxidase activity (U/ml)	24.33 U/ml	4.67 U/ml	6.02 U/ml	6.02 U/ml	68.68%

CHAPTER 5 DISCUSSION

5.1 Preliminary plate screening

The preliminary plate screening was an important and economic way to assess for the reliability of the research being carried out. It is one of the important criteria to ensure the successful of an experiment.

From the result, the highest laccase and lignin peroxidase activities were on 11th to 12th day and 13th to 14th day of subculture respectively (Table 4.2). Thus, submerged fermentation for laccase and lignin peroxidase were carried out only at the selected times to ensure high enzymes productivities during the submerge fermentation.

Nilaveni and Prema (2005) reported that plate assay with substrate guaicol as the effective primary screening method to detect lignolytic enzymes such as laccase, lignin peroxidase and manganese peroxidase from 20 actinomycetes cultures. In this study, plate screening provides a simple and fast detection method for laccase using syringaldazine and sodium tartrate buffer initiated by hydrogen peroxide (H₂O₂) for lignin peroxidase. Cheng *et al.* (2009) reported the highest laccase production on PDA plate added with guaiacol at day 11 of *Pleurotus ostreatus*.

5.2 Submerge liquid fermentation

5.2.1 Optimization of culture medium for submerged fermentation.

In order to obtain high laccase and lignin peroxidase activities, different types of media are chosen with different level of carbon and nitrogen sources. Rogalski *et al.* (2001) stated that the lignolytic enzymes production by white rot fungi is highly dependent on the growth conditions. However, result showed that none of the media was able to produce laccase in this study (Table 4.2). This might due to lignin peroxidase (LiP) in *Amauroderma rugosum* involve in taking a major role in growth and development.

From all the media tested, it can be concluded that when both the carbon and nitrogen concentration increased, the enzyme productivities of LiP would be increased. Massadeh *et al.* (2010) reported that addition of carbon source (glucose) in the media increased the lignolytic enzyme production such as laccase, LiP and manganese peroxidase (MnP) in *Pleurotus sajor-caju*. Both LiP and MnP have different level of sensitivity toward carbon and nitrogen content, the high concentration of nitrogen suppressed the MnP production but increased the LiP production (Bonnarme *et al.*, 1990) in white rot fungi.

Hence, the defined medium reported by Ding *et al.* (2012) containing high carbon and nitrogen sources gave the highest lignin peroxidase (LiP) activity. However, this medium was unstable from batch to batch which may due to the lack of continuous supply of nitrogen sources. Thus the medium contains of potato dextrose broth (PDB), 1 % of saw dust and 0.5 % of yeast was chosen for LiP production and for further experiments.

The medium contains of PDB, 1 % of saw dust and 0.5 % of yeast was supplemented with a natural nitrogen source which is yeast. Chae *et al.* (2001), reported that the yeast extract is enriched with peptides, amino acids, nucleotides and the soluble components of yeast cells thus acts as better nitrogen sources .

Besides, the used of lignocellulosic compounds enhanced the LiP production. Kapich *et al.* (2004) stated that a lignocellulosic substrate such as wheat straw and hemp woody core promoted the production of LiP of *Phanerochaete chrysosporium* under the submerge culture with non-limiting nitrogen and carbon sources. This explained that the media added with rubber wood saw dust (C:N=95:1) or bamboo leaves produced higher lignin peroxidase (LiP).

Besides, the *Amauroderma rugosum* was found to grow on hardwood forest and often grow on old stump suggested it might be a secondary decomposer. According to Elevitch (2004), the secondary decomposer mushroom relies on partially break down substrate by primary fungi species for growth and development. Thus the rubber wood saw dust that contained a lower percentage of lignin content compared to the bamboo leaves enabled higher production of lignin peroxidase (LiP).

5.2.2 Effects of shaking conditions for lignin peroxidase production

In static conditions, no enzyme production was being detected. The fungus mycelia were found to grow on the surface of the submerge culture in form of floating colonies with increasing surface area exposing to air (Thammajaruk *et al.*, 2011) suggested that sufficient amount of oxygen was needed for the species to produce lignin peroxidase .

Hence in case of static condition, a higher partial pressure of oxygen is needed to ensure sufficient oxygen supply to submerge hyphae in static culture (Leisola *et al.*, 1983, Michel *et al.*, 1992). The increased oxygen tensions of the culture would then increased the lignin peroxidase production (Dosoretz *et al.*, 1990; Faison and Kirk, 1985).

The shaking condition at 120 rpm was found to promote the enzyme activities where both media, the potato dextrose broth (PDB) with 1 % of saw dust and the potato dextrose broth (PDB) with 1 % of saw dust and 0.5 % of yeast produced high lignin peroxidase (LiP) activities. This might due to the constant shaking allowed even distribution of oxygen and nutrients. While at low rpm (50 rpm), the oxygen and nutrients were not distributed evenly and resulted in low production of lignin peroxidase. Moreover, Hadibarata *et al.* (2012) reported the anthracene degradation efficiency of *Polyporus sp.* was optimum at agitation speed of 120 rpm.

5.2.3 Effects of copper (Cu²⁺) for lignin peroxidase (LiP) production

The lignolytic enzymes production in most basidiomycetes is highly regulated by nitrogen, copper and manganese (Patrick *et al.*, 2011). Collins and Dobson (1997) reported that the induction of ligninolytic enzymes by adding copper induce the enzyme production.

Giardina *et al.* (1999), reported that addition of the copper (150 µM) into the media gave the maximum enzyme activity. However, the optimum amount of copper addition was species dependent. For example, *Trametes multicolor* required only addition of 100 µM of copper for maximum laccase production (Hess *et al.*, 2002).

However, any addition of the copper beyond the threshold value of the particular species type will lead to decrease in enzyme production. The threshold value for copper addition in our media was 150 µm, thus any copper addition after this will acts as potent inhibitor of fungal growth (Chen *et al.*, 2003).

5.3 Factor affecting the partition behaviours of ATPs

ATPs has been successfully applied in single step extraction and purification of extracellular enzymes of several mushroom species such as laccase from residual compost of *Agaricus bisporus* (Mayolo-Deloisa *et al.*, 2009), laccase from *Lentinus polychrous* (Ratanapongleka and Phetsom, 2011), α -galactosidase from *Aspergillus oryzae* (Naganagouda and Mulimani, 2008) and lipase from *Burkholderia pseudomallei* (Ooi *et al.*, 2009). To our knowledge, there has been no report on the extraction and purification of extracellular lignin peroxidase from *Amauroderma rugosum* using ATPs method.

The molecular weight and molecular length of the polymer; sizes of the enzyme; hydrophobicity; crude enzyme loading mass; pH and ionic composition of the top phase were factors that affecting the partitioning of the biomolecules (Banik *et al.*, 2003).

5.3.1 Influence of the different PEG molecular weight on purification factor and yield of lignin peroxidase (LiP).

The molecular weight of the PEG is one of the determining factors for purification factor and yield. According to Yang *et al.* (2008), the composition of the polymer and salt needs to be selected in order to obtain high yield and purification factor with minimum contaminants for the desired biomolecules.

For all the 25 systems tested with different range of PEG molecular weights, lignin peroxidase (LiP) showed top phase or PEG preference; with majority of the partition coefficient values above 1 (Table 4.4). Such partitioning behavior indicated that the lignin peroxidase was a hydrophobic enzyme and interacted well with the PEG-rich phase. The molecular weights of the PEG impact the protein partitioning by altering the hydrophobic interactions between the PEG and the hydrophobic areas of the targeted protein (Mohamadi *et al.*, 2007; Yücekan and Önal, 2011).

Albertsson (1986) stated that the biological molecules selectively partition on the PEG phase with reduced PEG molecular weight. Zaslavsky (1995) concluded that the most preferable PEG for most proteins were range from PEG 1000 to PEG 3000 with the upper limit usually at PEG 3000, however some protein may showed top phase preference mainly at PEG 600 or bottom phase preference mainly at PEG 8000. In this study, the top phase purification factor (P_{FT}) and yield from the low molecular weight PEG such as PEG 600, PEG 1000 and PEG 1500 gave better results (Table 4.4) as compared to the intermediate to high molecular weight PEG (PEG3350-PEG8000). Similar report was found for the recovery of laccase from the residual compost of *Agaricus bisporus* using the ATPs method where the best PEG molecular weight selected for enzyme recovery was PEG 1000.

The high molecular weights of PEG were not a suitable option in terms of protein hydrophobicity (Franco *et al.*, 1996) and phase excluded volume (Benavides and Rito-Palomares, 2004). Hachem *et al.* (1996) reported that the interaction between PEG and the hydrophobic areas of the enzyme became weaker in the presence of high molecular weights. Patil and Raghavarao (2007) reported when the polymer molecular weights increased indicated an increase in polymer length; and when the polymer became compact, it would reduce the free volume available on the top phase and hence lead to poor recovery of enzyme. However, Ratanapongleka and Phetsom (2011) reported in certain cases, higher molecular weights of PEG such as PEG 8000 were more preferable as there was higher interfacial tension between the phases and hence produced desired products with higher purity. Despite any of the reports regarding the best molecular weight on enzyme partitioning, different types of enzyme might have different patterns of enzyme partitioning.

5.3.2 Influence of the TLL on purification factor and yield of lignin peroxidase (LiP).

The TLL was increased when the PEG and salt concentration increased in the system 1-3 of PEG 1000 (Table 4.3). The increase in PEG concentration would increase the hydrophobic interaction between the PEG phase and the surface protein (Bassani *et al.*, 2007) which led to higher enzyme purity and yield.

Moreover, the increase in TLL indicated the increase of salt concentration in the bottom phase when there were more ions that act with the oppositely charged group of the protein and thus a double layer of ionic group would be formed (Ooi *et al.*, 2009). This double layer of ionic group caused protein dehydration and more protein hydrophobic surface was exposed (Bonomo *et al.*, 2006), hence improved the PEG and targeted protein interaction (Vojdani, 1996) and shifting protein partition to top phase.

For system 9 and 10 of PEG 1000 (Table 4.6), the top phase purification factor decreased when the TLL increased. As the TLL increased, the free volume in bottom phase would be decreased (Aguilar *et al.*, 2006) and all the contaminants from the bottom phase might shifted to the top phase and affected the targeted protein purity. For PEG 600 with TLL of 34.10 % (w/w), there was a fine balance between the PEG hydrophobicity and the salting-out capability of the salt.

5.3.3 Effect of V_R on P_{FT}

The top phase purification factor (P_{FT}) and $\log K$ for system 2 of PEG 600 was 1.33 ± 0.62 and 0.43 ± 0.19 respectively. The top phase purification factor (P_{FT}) increased only slightly as compared to the huge elevation in $\log K$ (Appendix B).

Theoretically, any changes in the volume ratio would not altered the partitioning behavior of the protein as the relative partitioning of the protein itself was constant (Walter and Johansson, 1994; Hustedt *et al.*, 1985). However, when the volume ratio decreased, the free volume on the top phase decreased and the protein partition tends to take place in the intermediate phase. For example, at lower volume ratio such as 0.52, protein precipitation can be easily observed in intermediate phase of ATPs and lead to reduced top phase and bottom phase enzyme purity. Thus, both the top phase purification factor and bottom phase purification factor were low. Ooi *et al.* (2009) reported that the low purification factor in low volume ratio was happened when there was a great reduction in free volume and hence causes the losses of the lipase from *Burkholderia pseudomallei* at the top phase.

At higher volume ratio of 0.79 to 1.77, the top phase free volume was too high and caused the enzyme diluted and participated in these phases, hence gave low enzyme purity

with small purification factor and log K readings. While at volume ratio of 0.62, there was fine balance between the top phase and bottom phase. In this volume ratio, the free volume needed for enzyme partitioning was suitable.

5.3.4 Effect of pH on P_{FT}

The system failed to be carried out at phosphate salt at pH 6.0 as the salt formed precipitations at room temperature and thus was not continued to other experiment. According to Benavides and Rito-Palomares (2008), PEG -phosphate systems showed greater stability when phosphate salt was at pH above pH 7.0 while PEG-sulphate system was most suitable when sulphate was at pH below 6.5.

The lignin peroxidase attained stability at pH 2.5 to pH 3.0 (Fakoussa and Hofrichter, 1999) and isoelectric point (pI) of 3.2-4.0 (Renganathan *et al.*, 1985; Leisola *et al.*, 1987). Lignin peroxidase has been reported to have isoelectric point at 3.2-4.0 (pI 3.2-4.0) indicated that the enzyme was an acidic enzyme. Abbott and Hatton (1988) described that the negatively charge protein prefers to partition at top phase while positively charge protein prefer to partition at bottom phase.

Thus, when the pH of the ATPs increased above the isoelectric (pI) value, the enzyme's surface charges become more negative. When the enzyme becomes more negatively charged, the positively charged top phase PEG would attracted the enzyme in top phase and lead to higher partition coefficient and better purity (Naganagouda and Mulimani, 2008). In our study, the purity of the enzyme was increased when pH were in the range of pH 6.5 to pH 8.0. Moreover, the top phase of LiP activity increased to 58.47 ± 17.32 U/ml while the crude enzyme of LiP activity was at 22.56 ± 5.87 U/ml at pH

8.0. The highest top phase purification factor of 6.25 ± 3.21 was recorded (Appendix B, Table B2).

However, the purification factors were dropped for both pH 8.5 and pH 9.0 indicated that there was a gradual loss of the enzyme activity in extreme pH environment perhaps due to lower solubility and changes on the surface properties.

5.3.5 Effect of crude enzyme addition on P_{FT}

In this study, the volume ratio of the system after adding the 10 % (w/w) crude enzyme and the 20 % (w/w) crude enzyme were 0.55 to 0.48 respectively indicated the decrease in the volume ratio. Such decrease caused the changes in the composition properties of ATPs and hence induced the precipitate accumulation at the interface causing the loss of both desired bio-molecules and contaminants (Minuth *et al.*, 1996).

The increment of the biomass loading has the impact on the phase volume ratio (Abbott and Hatton, 1988) and the partition behavior of the biomolecules (Brooks and Fisher, 1985). Show *et al.* (2012) reported that the increase of the biomass loading (>20% w/w) altered the composition/properties of the ATPs and led to a decrease in primary recovery of lipase from *Burkholderia cenocepacia*.

5.3.6 Effect of NaCl salt on Partition Coefficient and P_{FT} of ligninperoxidase (LiP).

Walter *et al.* (1985) believed that the salt addition into the ATPs system affected both enzyme partitioning and purification factor. However, in this study, it was believed that the addition of NaCl salt often drawn the desired protein and contaminants with similar hydrophobicity towards single direction and resulted in low purification factor (Chen *et al.*, 2008).

This was further supported by Ketnawa *et al.* (2010) that NaCl salt altered the enzyme structure and hence cause decreased the enzyme activity. Thus, the addition of NaCl salt to the system was not recommended for the recovery of LiP.

5.4 SDS-PAGE and native-PAGE analysis of purified lignin peroxidase from ATPS extraction.

An intensive brown coloured band was observed on both SDS-PAGE and native-PAGE. Vares *et al.* (1995) reported that the coloured compounds often appeared in lignin peroxidase enzyme and could only be partially removed by ultrafiltration method. The lanes with intensive brown staining affected the resolution of the enzyme bands.

Fakoussa and Hofrichter (1999) stated the range of subunit molecular mass of lignin peroxidase in white rot-fungi was at 38-47 kDa. Vares *et al.* (1995) also reported the lignin peroxidase produced by *Phlebia radiate* consisted of two distinct bands which were at 45 to 46 kDa and 44 kDa in SDS-PAGE. But in this study, three distinctive bands were observed at 38 kDa; 45-46 kDa; and 66 kDa.

The molecular weight of the 38 kDa and 45-46 kDa bands were between the ranges being reported for lignin peroxidase. However, the band with apparent band size of 66 kDa was never been reported so far in mushroom species. Parshetti *et al.* (2012) reported the purified lignin peroxidase from the bacterium *Kocuria rosea* MTCC 1532 with molecular weight of 66 kDa. This LiP was reported as a versatile peroxidase as it was able to decolorize a wide range of synthetic dyes.

The lignin peroxidase activity test of all the bands showed positive LiP activities. The band with molecular size at approximately 66 kDa presented as a clumps in native-page. According to Kurien and Scofield (2005), native-PAGE gave lower resolution as compared to SDS-PAGE but it retained the native structure of the enzyme.

This was yet to conclude that the band c with approximately band size of 66 kDa was the isomers of the LiP. Piontek *et al.* (1993) also reported an interesting feature of the lignin peroxidase crystal structure where there was an ordered carbohydrate residues that attached to the active site of the enzyme active site with unknown function. It was suspected that this carbohydrate residue might present as an artifact or may play some determining rules in the structure of lignin peroxidase (Schoemaker and Piontek, 1996). Kim and Shoda (1999) reported a peroxidase (DyP) produced by *Geotrichum candidum* Dec 1 gave a considerably larger molecular mass of 55-60 kDa compared to the reported molecular mass of 40-44 kDa reported; this enzyme was suspected to be spherical in structure or the sugar portion of the enzyme was interacting with the gel matrix of SDS-PAGE. However, further investigation is needed.

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusion

The aim of the study was to culture the *Amauroderma rugosum* lignin peroxidase in submerge fermentation and evaluate the application of the ATPs method in single step extraction and purification of the enzyme. The best medium for submerge fermentation was found to be by combination of potato dextrose broth (PDB), 0.5 % of yeast and 1 % of saw dust. This medium with the high carbon content, natural nitrogen source, lignocellulosic compounds and 120 rpm shaking speed became the best inducer for the production of lignin peroxidase from *Amauroderma rugosum* with the lignin peroxidase (LiP) of 26.70 ± 3.70 U/ml. The used of rubber wood saw dust utilized the agricultural by products and was more environmental friendly. Moreover, the addition of copper of 150 μ M increased the lignin peroxidase (LiP) to 105.68 ± 5.87 U/ml.

The cultivated enzyme was then subjected for ATPs purification. From the study, the enzyme lignin peroxidase (LiP) showed top phase or PEG preferences by partition mainly at top phase indicated the enzyme was an acidic and hydrophobic enzyme. After several optimization of the ATPs parameters in term of volume ratio, pH, crude enzyme adding and salt adding, it was found that TLL 34.10, volume ratio of 0.62, pH8.0, and 10 % crude enzyme gave the best result. The optimal top phase purification factor and yield gained from the optimization were 6.25 ± 3.21 with yield of 81.08 ± 4.92 % respectively.

The SDS-PAGE analysis of the crude enzyme of lignin peroxidase (LiP) gave multiple bands. The SDS-PAGE analysis after the aqueous two-phase system (ATPs) was able to give the purified lignin peroxidase (LiP) by eliminating non-desirable bands and

impurities. The SDS-PAGE analysis gave a total of 3 bands at molecular weights of 38 kDa; 45-46 kDa and 66 kDa. Native-PAGE analysis indicated all the bands contained lignin peroxidase (LiP) activities and the LiP activities retained after native-PAGE was 68.68 %. Hence, ATPs method is a promising method for enzyme extraction and purification.

6.2 Recommendation for future research

The lignin peroxidase (LiP) from various white rot fungi such as *Phanerochaete chrysosporium* and *Phlebia radiata* were so far reported for research purpose and yet to large scale industrial used. The difficulties of set up an appropriate large scale apparatus such as fermentor; the accurate temperature, pH and aeration control; the maintenance of stable and continuous production of enzymes and the needs of trained personnel restricted the large scale production of enzyme. However, these obstacles should be overcome as there are needs to optimize the industrial scale *Amauraderma rugosum*'s lignin peroxidase cultivation since there are increasing demanding in this enzyme. Thus, the parameters affecting ATPs extraction and purification are needed to be carried out in industrial-scale instead of lab-scale to enable commercial applications.

The used of biodegradable and non-toxic PEG and phosphate is one of the advantages of the ATPs system. However, PEG is non-recyclable and less economics, the salt disposal also creating the eutrophication phenomena in water (Ratanapongleka, 2010). Thus, it is recommended to use the recyclable polymer such as ionic liquid which is stable and non-flammable.

The purified lignin peroxidase can then be tested for biological activities such as fibrinolytic and anti-coagulant properties. Moreover, the enzyme extracted can be tested for melanin degradation since the lignin peroxidase from the *P. chrysosporium* reported to successfully decoloured the melanin.

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APPENDIX A

A.1: Determination of Lignin Peroxidase Activity

Reagent

Hundred mM of Sodium Tartrate buffer, pH 3.0; Thirty mM of veratryl alcohol; zero point five mM H₂O₂. (Have *et al.*, 1997)

Procedure for Preparation of Veratraldehyde (VAD) Standard Plot

Veratraldehyde (VAD) was used as the standard curve to determine the LiP activity. The veratraldehyde (VAD) solution containing 10- 200 µg VAD in a volume up to 1.5mL was pipette into 12 different test tubes. Each test tube was added with buffer until reached a final volume of 3.0mL and the test tubes were mixed with a vortex. Finally, 0.5mL of 0.5 mM hydrogen peroxide was added and the reaction was read at wavelength of 310nm.

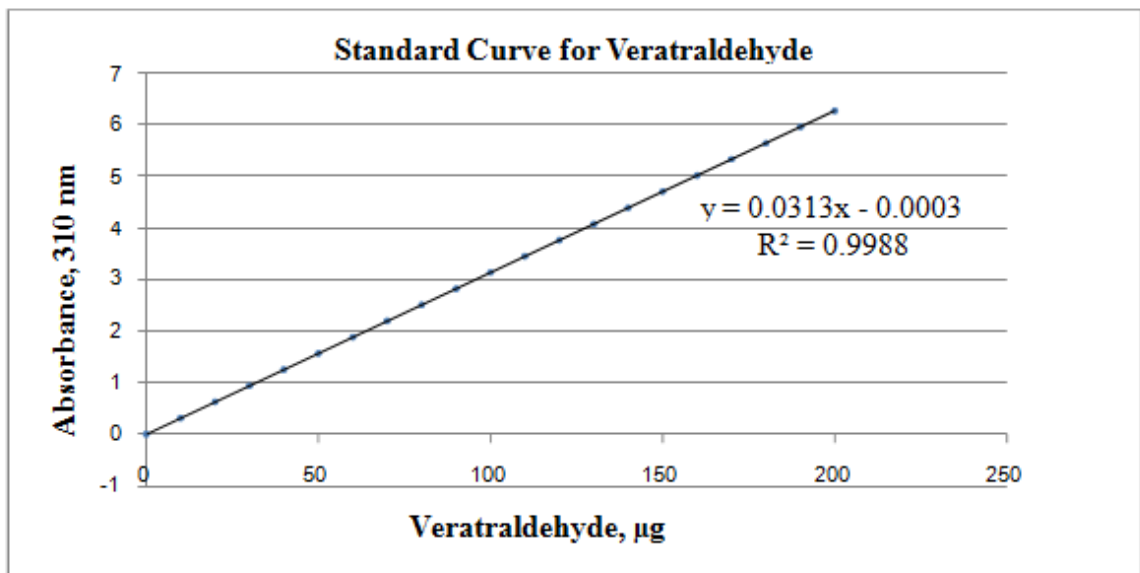


Figure A.1: Standard curve for veratraldehyde.

Note: The linear correlation and its R² are depicted in the figure.

From the standard curve, the lignin peroxidase activity was determined as the unit of activity in µmol of VAD released per minute, thus

Lignin peroxidase activity (U/mL) =

$$\left(\frac{\text{Final absorbance} + 0.003}{0.0313} \right) \left(\frac{\text{dilution factor}}{0.2} \right) \left(\frac{1}{5 \text{ min}} \right) \left(\frac{1000}{1 \text{ mg}} \right) \left(\frac{1 \text{ } \mu\text{mole}}{166.18 \text{ } \mu\text{g}} \right)$$

Procedure for Determination of Lignin Peroxidase Activity

The reaction mixture consisted of 2.4mL 100mM Sodium Tartrate buffer pH 3.0, 0.2mL of enzyme sample and 0.2mL of 30 mM veratryl alcohol pH3.0. LiP activity was measured by oxidation of veratryl alcohol to veratrylaldehyde in the presence of H₂O₂ at pH 3.0. The reaction was initiated using 0.2mL of 0.5mM H₂O₂. The enzyme activity was measured at the wavelength of 310nm after 5 minutes. The usual reagent blank and enzyme blank were included. Reagent blank consisted of 2.6mL buffer, 0.2mL substrate and 0.2mL H₂O₂, whereas enzyme blank consisted of 2.8 buffer, 0.2mL of enzyme sample.

Table A1: Assay mixtures for determination of lignin peroxidase activity

Column	Volume
Substrate blank	2.6mL sodium tartrate buffer+ 0.2mL of 30mM veratryl alcohol+ 0.2mL of 0.5mM H ₂ O ₂
Enzyme blank	2.8mL sodium tartrate buffer+ 0.2mL Of enzyme
Reaction mixture	2.4mL sodium tartrate buffer+ 0.2mL of enzyme+ 0.2mL of 30mM veratryl alcohol+ 0.2mL of 0.5mM H ₂ O ₂

A.2: Determination of Laccase Activity

Reagent

Fifty mM of sodium citrate buffer, pH4.8. Zero point one mM of syringaldazine in 50percent ethanol. The syringaldazine was dissolved in 50percent ethanol after one hour of stirring (Harkin and Obst, 1973; Leonowicz and Grzywnowicz, 1981).

Procedure for Determination of Laccase Activity

The reaction mixture consisted of 3.2mL of 50mM sodium citrate buffer, 0.2mL of enzyme and 0.2ml of syringaldazine substrate. The substrate blank consisted of 3.2mL of 50mM sodium citrate buffer and 0.2mL of syringaldazine substrate. While the enzyme blank consisted of only 3.2mL of 50mM sodium citrate buffer and 0.2mL of enzyme. The test was carried out in room temperature and all the test tubes were mixed with vortex before test. The initial rate of colour change was measured on a spectrophotometer at wavelength of 525nm. The changes of the reagent from colourless to pink colour 1 minute after the substrate adding indicated the presence of laccase.

Table A2: Assay mixtures for determination of laccase activity

Column	Volume
Substrate blank	3.2mL of 50mM sodium citrate buffer +0.2mL of syringaldazine substrate
Enzyme blank	3.2mL of 50mM sodium citrate buffer + 0.2mL of enzyme
Reaction mixture	3.2mL of 50mM sodium citrate buffer + 0.2mL of enzyme + 0.2mL of syringaldazine substrate

Calculation of Unit of Laccase Activity

One unit was defined as the enzyme producing one unit of absorbance change per minute. Laccase activity on the sample was calculated as followed:

$$\text{Laccase activity} = (\text{Final absorbance}) \left(\frac{\text{Dilution factor}}{0.2\text{ml}} \right) \left(\frac{1}{1 \text{ minute}} \right)$$

A.3: Determination of Soluble Protein Concentration

Preparation of Reagent

Hundred milligrams of Coomassie Brilliant Blue G-250 was dissolved in fifty millilitre of ninety five percent ethanol. Then, hundred millilitre of eighty five percent (w/v) phosphoric acid was added and the solution was made to a final volume of one litre using distilled water (Bradford, 1976).

Procedure for Preparation of Protein Calibration Plot

The Bovine Serum Albumin (BSA) solution with the protein concentration ranging from 10 to 100 µg in a volume ranging from 0.1mL to 1.0mL was pipetted into ten different test tubes. Each test tube was added with distilled water until reached the final volume of 1.0mL. The blank with only 1.0ml of distilled water was prepared separately. Then, 5mL of the Coomassie Brilliant Blue reagent was added in each tubes and the reagent was mixed thoroughly using the vortex prior to read at wavelength of 595nm using spectrophotometer. The readings were read only 2 minutes after the mixing and the whole process shall not exceed 1 hour. The weight of protein was measured against corresponding absorbance resulting in a standard curve.

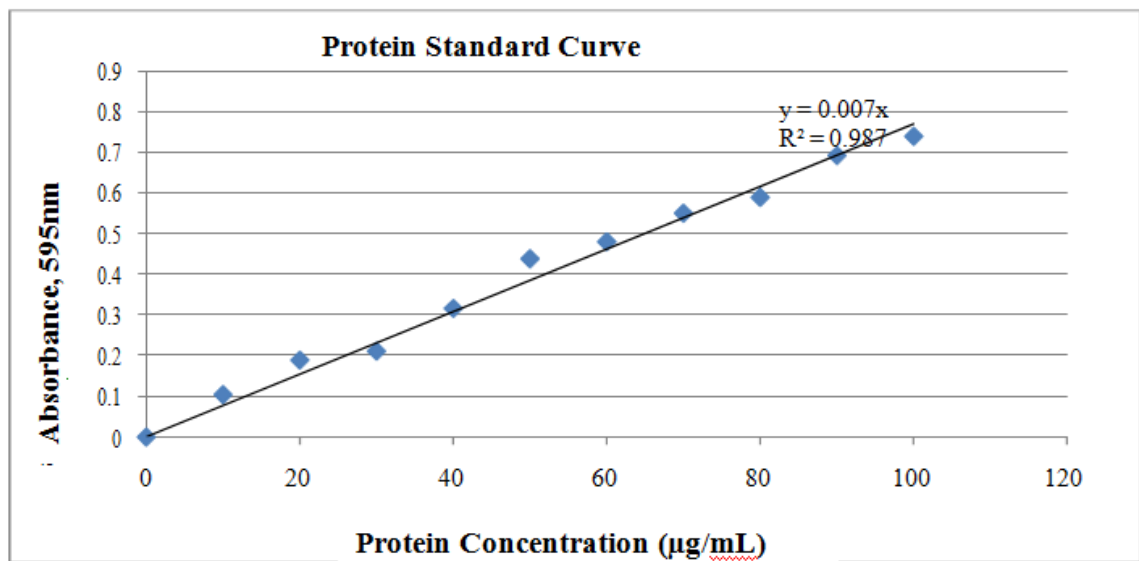


Figure A.2: Protein Standard curve.

Note: The linear correlation and its R^2 are depicted in the figure.

$$\text{Soluble Protein (mg/mL)} = \left(\frac{\text{Final absorbance}}{0.007} \right) \left(\frac{1}{0.2} \right) \left(\frac{1\text{mg}}{1000\mu\text{g}} \right)$$

Note: BSA stock solution = 100 µg/mL

Procedure for Determination of Soluble Protein Concentration

The enzyme sample in a volume of 0.2mL was pipette into a test tube and about 5mL of Coomassive Brilliant Blue reagent was added later. The solution in the test tube was mixed using the vortex mixer and the absorbance was read at wavelength of 595nm. The total soluble protein was then calculated using the formulated formula from the graph.

A4: Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The casting and running protein gels according to Laemmli and Favrel, (1973).

12% resolving gel (~12 mL) for 2 gels and 5% stacking gel (~6mL) for 2 gels

	Resolving gel	Stacking gel
Distilled water (ddH ₂ O)	5.22mL	3.59 mL
Resolving gel (1.5M Tris-HCL, pH8.8)	3.00 mL	-
Stacking gel (0.5M Tris HCL, pH6.8)	-	1.50 mL
10% Sodium Dodecyl Sulphate (SDS)	0.12 mL	0.06 mL
40% Bis-acrylamide	3.60 mL	0.75mL
10% Ammonium Persulphate	50 µL	20 µL
TEMED	10 µL	10 µL

Preparation of Resolving gel Buffer (1.5 M of Tris-HCL; pH 8.8)

The Trizma base (27.23 g) was dissolved in 100mL of distilled water. The pH was adjusted to pH 8.8 by using 1M of HCl. Then, the solution was brought to a final volume of 150 mL by using distilled water and the buffer was kept in 4±2 °C.

Preparation of Stacking gel Buffer (0.5M of Tris-HCL; pH 6.8)

The Trizma base (6.10 g) was dissolved in 80 mL of distilled water. The pH was adjusted to pH 6.8 using 1M of HCl. Then, the solution was brought to a final volume of 100 mL by using distilled water and the buffer was kept in 4±2 °C.

Preparation of 10% of Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS powder (10g) was dissolved in 100 mL of distilled water.

10% (w/v) Ammonium persulphate (APS)

The Ammonium Persulphate (0.01g) was dissolved in 100 µL distilled water.

SDS-PAGE Electrophoresis Running Buffer (Tank buffer, 10 x conc.)

The trizma base (3.03 g), 14.40 g of glycine and about 10 g of 10% SDS powder was dissolved in 1000 mL of distilled water.

SDS-PAGE Coomassie Staining solution

The Coomassie Brilliant Blue R-250 (1 g) was dissolved in 450 mL of methanol. Then 100 mL of glacial acetic acid was added to this solution and the whole solution was brought to a final volume of 1000 mL using distilled water.

SDS-PAGE Coomassie Destaining solution

The methanol (100 mL), acetic acid (100 mL) and 800 mL of distilled water was mixed in the beaker and stored in Schott bottle.

APPENDIX B

Table B.1: Results for top phase purification factor, partition coefficient, selectivity and log k for different volume ratio of PEG 600. Formulas for calculation were showed in section 3.8.

Different Volume Ratio (V_R)	Top Phase Purification factor	Selectivity	K(Enzyme)	Log k
(1) 0.52	1.89±0.33	14.11±2.67	8.03±1.68	0.90±0.09
(2) 0.62	2.66±0.14	23.69±6.49	12.07±1.86	1.08±0.07
(3) 0.79	1.18±0.22	4.79±1.31	3.24±0.62	0.51±0.08
(4) 1.00	1.33±0.62	8.40±6.64	2.85±1.28	0.43±0.19
(5) 1.30	1.35±0.06	3.19±2.06	2.09±0.48	0.31±0.11
(6) 1.77	1.67±0.53	3.78±1.08	2.92±0.76	0.46±0.11

Table B2: ATPs results foreffect of different pH. Formulas for calculation were showed in section 3.8.

pH	LiP Crude Activity (U/m)	LiP Top Activity (U/ml)	LiP Bottom Activity (U/ml)	Top Purification Factor	Bottom Purification Factor	Partition coefficient (Enzyme)	Selectivity	Top Recovery Yield	Bottom Recovery Yield
6.5	41.41±7.56	63.38±11.27	25.00±2.93	2.41 ±0.11	0.50±0.04	2.53±0.24	4.79±0.17	60.97±2.29	39.03±2.29
7.0	87.93±9.67	106.28±2.06	8.92±1.11	2.66±0.14	0.12±0.04	12.07±1.86	23.69±6.49	88.07±1.50	11.93±1.50
7.5	22.56±5.87	59.75±4.81	15.77±1.11	5.71±1.78	0.90±0.33	3.80±0.42	6.52±0.75	70.10±2.21	29.90±2.21
8.0	22.56±5.87	58.47±17.32	8.08±1.11	6.25±3.21	0.34±0.09	7.38±2.72	17.72±4.46	81.08±4.92	18.92±4.92
8.5	66.46±1.92	46.44±7.83	0.83±1.44	1.05±0.15	0.01±0.01	6.49±11.23	18.12±31.39	30.78±53.32	2.55±4.42
9.0	66.46±1.92	46.29±2.06	0.00±0.00	0.84±0.12	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table B3: ATPs results for effects of sodium chloride (NaCl) salt adding. Formulas for calculation were showed in section 3.8.

NaCl Adding (%)	LiP Crude Activity (U/m)	LiP Top Activity (U/ml)	LiP Bottom Activity (U/ml)	Top Purification Factor	Bottom Purification Factor	Partition coefficient (Enzyme)	Selectivity
0	22.56±5.87	58.47±17.32	8.08±1.11	6.25±3.21	0.34±0.09	7.38±2.72	17.72±4.46
1	66.46±1.92	34.54±4.00	0.00±0.00	0.59±0.14	0.00±0.00	0.00±0.00	0.00±0.00
2	66.46±1.92	54.41±3.33	0.00±0.00	1.86±0.68	0.00±0.00	0.00±0.00	0.00±0.00
3	66.46±1.92	72.99±6.75	0.00±0.00	1.59±0.25	0.00±0.00	0.00±0.00	0.00±0.00
4	66.46±1.92	55.84±2.69	0.00±0.00	1.05±0.10	0.00±0.00	0.00±0.00	0.00±0.00

APPENDIX C

SPSS Statistical Analysis

C1:SPSS Data for Media Optimization

Descriptives

Lip

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	3.1333	.83966	.48478	1.0475	5.2192	2.17	3.71
2.00	3	13.5167	.19502	.11260	13.0322	14.0011	13.32	13.71
3.00	3	4.8000	.29462	.17010	4.0681	5.5319	4.48	5.06
4.00	3	26.7033	3.70082	2.13667	17.5100	35.8967	22.43	28.84
5.00	3	24.6133	1.50533	.86911	20.8739	28.3528	23.22	26.21
6.00	3	3.2633	.22898	.13220	2.6945	3.8322	3.08	3.52
7.00	3	7.1933	.18771	.10837	6.7270	7.6596	6.99	7.36
8.00	3	7.2267	.03512	.02028	7.1394	7.3139	7.19	7.26
9.00	3	4.5433	.29023	.16756	3.8224	5.2643	4.29	4.86
10.00	3	5.4400	.19000	.10970	4.9680	5.9120	5.25	5.63
11.00	3	29.4600	.69735	.40262	27.7277	31.1923	28.67	29.99
Total	33	11.8085	9.89793	1.72301	8.2988	15.3181	2.17	29.99

ANOVA

Lip

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3100.032	10	310.003	194.999	.000
Within Groups	34.975	22	1.590		
Total	3135.007	32			

For One Way Anova, the p-value was less than 0.05, which showed that there were significant differences between the groups.

Homogeneous Subsets

Lip

Media	N	Subset for alpha = 0.05				
		1	2	3	4	5
Tukey HSD ^a						
1.00	3	3.1333				
6.00	3	3.2633				
9.00	3	4.5433	4.5433			
3.00	3	4.8000	4.8000			
10.00	3	5.4400	5.4400			
7.00	3		7.1933			
8.00	3		7.2267			
2.00	3			13.5167		
5.00	3				24.6133	
4.00	3				26.7033	26.7033
11.00	3					29.4600
Sig.		.502	.303	1.000	.632	.271

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Note: Media 1-9 are as stated in table in page 28.

C2: SPSS Data for Shaking Speed

Oneway

[DataSet0]

Descriptives									
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
PDB+S.D	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	1.6020	.77695	.44857	-.3280	3.5320	.77	2.31
	3.00	3	13.5167	.19502	.11260	13.0322	14.0011	13.32	13.71
	Total	9	5.0396	6.40809	2.13603	.1139	9.9653	.00	13.71
PDB+S.D+Y	1.00	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	2.00	3	4.7633	1.50041	.86626	1.0361	8.4906	3.08	5.96
	3.00	3	26.7033	3.70082	2.13667	17.5100	35.8967	22.43	28.84
	Total	9	10.4889	12.49508	4.16503	.8843	20.0935	.00	28.84

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
PDB+S.D	Between Groups	327.226	2	163.613	764.925	.000
	Within Groups	1.283	6	.214		
	Total	328.509	8			
PDB+S.D+Y	Between Groups	1217.121	2	608.560	114.482	.000
	Within Groups	31.895	6	5.316		
	Total	1249.015	8			

For One Way Anova, the p-values were less than 0.05, which showed that there were significant differences between the groups.

Homogeneous Subsets

PDB+S.D

Shaking speed	N	Subset for alpha = 0.05		
		1	2	3
Tukey HSD ^a				
1.00	3	.0000		
2.00	3		1.6020	
3.00	3			13.5167
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

PDB+S.D+Y

Shaking speed	N	Subset for alpha = 0.05	
		1	2
Tukey HSD ^a			
1.00	3	.0000	
2.00	3	4.7633	
3.00	3		26.7033
Sig.		.098	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

C3: SPSS Data for Copper Adding

➔ Oneway

[DataSet0]

Descriptives

LiP activity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
.00	3	3.1338	.83802	.48383	1.0520	5.2155	2.17	3.71
50.00	3	58.8940	2.93674	1.69553	51.5988	66.1893	56.33	62.10
100.00	3	95.4224	4.43993	2.56340	84.3930	106.4518	92.86	100.55
150.00	3	105.6760	5.87348	3.39105	91.0855	120.2665	100.55	112.08
200.00	3	39.0277	1.92255	1.10998	34.2518	43.8036	37.11	40.95
300.00	3	56.9715	4.00210	2.31061	47.0297	66.9132	52.49	60.18
Total	18	59.8542	35.34806	8.33162	42.2760	77.4324	2.17	112.08

ANOVA

LiP activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21074.753	5	4214.951	303.779	.000
Within Groups	166.501	12	13.875		
Total	21241.254	17			

*0.00 to 300.00 denoted different amounts of copper adding, i.e: 0 µg, 50 µg, 100 µg, 150 µg, 200 µg, 300 µg.

For One Way Anova, the p-values was less than 0.05, which showed that there was significant differences between the groups.

Homogeneous Subsets

LiP activity

Copper adding (µM)	N	Subset for alpha = 0.05				
		1	2	3	4	5
Tukey HSD ^a						
.00	3	3.1338				
200.00	3		39.0277			
300.00	3			56.9715		
50.00	3			58.8940		
100.00	3				95.4224	
150.00	3					105.6760
Sig.		1.000	1.000	.986	1.000	1.000
Duncan ^a						
.00	3	3.1338				
200.00	3		39.0277			
300.00	3			56.9715		
50.00	3			58.8940		
100.00	3				95.4224	
150.00	3					105.6760
Sig.		1.000	1.000	.539	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

C4: SPSS data for PEG 600-PEG 8000 Top Purification factor (PFt)

```
ONEWAY Pft BY PEG
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=TUKEY DUNCAN LSD ALPHA(0.05).
```

Oneway

[DataSet0]

Descriptives

Top Phase Purification factor

	N	Mean	Std. Deviation	Std. Error	95 % Confidence Interval for Mean	
					Lower Bound	Upper Bound
601.00	3	.6300	.12767	.07371	.3128	.9472
602.00	3	1.3333	.62003	.35797	-.2069	2.8736
603.00	3	1.2033	.19604	.11319	.7163	1.6903
604.00	3	1.0667	.24583	.14193	.4660	1.6773
605.00	3	.8967	.18009	.10398	.4493	1.3440
1001.00	3	.3667	.11930	.06888	.0703	.6630
1002.00	3	.8667	.16166	.09333	.4651	1.2682
1003.00	3	.9067	.49863	.28789	-.3320	2.1453
1004.00	3	.4600	.13115	.07572	.1342	.7858
1005.00	3	.2600	.12288	.07095	-.0453	.5653
1501.00	3	.9633	.15695	.09062	.5734	1.3532
1502.00	3	.8133	.23352	.13482	.2332	1.3934
1503.00	3	.8133	.12858	.07424	.4939	1.1327
1504.00	3	.8333	.24132	.13932	.2339	1.4328
1505.00	3	.7367	.09713	.05608	.4954	.9779
3351.00	3	.2600	.04359	.02517	.1517	.3683
3352.00	3	.2367	.01155	.00667	.2080	.2654
3353.00	3	.1533	.04933	.02848	.0308	.2759
3354.00	3	.1833	.04509	.02603	.0713	.2953
3355.00	3	.3733	.04933	.02848	.2508	.4959
8001.00	3	.7933	.34443	.19886	-.0623	1.6489
8002.00	3	.4633	.11015	.06360	.1897	.7370
8003.00	3	.4800	.13077	.07550	.1552	.8048
8004.00	3	.6100	.27000	.15588	-.0607	1.2807
8005.00	3	.0500	.02646	.01528	-.0157	.1157
Total	75	.6301	.38799	.04480	.5409	.7194

ANOVA

Top Phase Purification factor

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.646	24	.360	7.222	.000
Within Groups	2.494	50	.050		
Total	11.140	74			

*601.00 to 605.00 denoted system 1 to 5 of PEG 600, 1001-1005 denoted system 1 to 5 of PEG 1000, 1501-1505 denoted system 1 to 5 of PEG 1500, 3351-3355 denoted system 1 to 5 of PEG 3350 and 8001-8005 denoted system 1-5 of PEG 8000.

For One Way Anova, the p-value was less than 0.05, which showed that there was significant differences between the groups.

Top Phase Purification factor

PEG	N	Subset for alpha = 0.05							
		1	2	3	4	5	6		
Tukey HSD ^a	8005.00	3	.0500						
	3353.00	3	.1533	.1533					
	3354.00	3	.1833	.1833	.1833				
	3352.00	3	.2367	.2367	.2367	.2367			
	1005.00	3	.2600	.2600	.2600	.2600	.2600		
	3351.00	3	.2600	.2600	.2600	.2600	.2600		
	1001.00	3	.3667	.3667	.3667	.3667	.3667	.3667	
	3355.00	3	.3733	.3733	.3733	.3733	.3733	.3733	
	1004.00	3	.4600	.4600	.4600	.4600	.4600	.4600	
	8002.00	3	.4633	.4633	.4633	.4633	.4633	.4633	
	8003.00	3	.4800	.4800	.4800	.4800	.4800	.4800	
	8004.00	3	.6100	.6100	.6100	.6100	.6100	.6100	
	601.00	3	.6300	.6300	.6300	.6300	.6300	.6300	
	1505.00	3	.7367	.7367	.7367	.7367	.7367	.7367	
	8001.00	3		.7933	.7933	.7933	.7933	.7933	
	1502.00	3		.8133	.8133	.8133	.8133	.8133	
	1503.00	3		.8133	.8133	.8133	.8133	.8133	
	1504.00	3		.8333	.8333	.8333	.8333	.8333	
	1002.00	3			.8667	.8667	.8667	.8667	
	605.00	3				.8967	.8967	.8967	
	1003.00	3				.9067	.9067	.9067	
	1501.00	3					.9633	.9633	
	604.00	3							1.0667
	603.00	3							
	602.00	3							
	Sig.		.066	.072	.069	.083	.052	.054	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Top Phase Purification factor

PEG		Subset for alpha = 0.05				
		7	8	9	10	11
Tukey HSD ^a	8005.00					
	3353.00					
	3354.00					
	3352.00					
	1005.00					
	3351.00					
	1001.00					
	3355.00					
	1004.00					
	8002.00					
	8003.00					
	8004.00	.6100				
	601.00	.6300	.6300			
	1505.00	.7367	.7367			
	8001.00	.7933	.7933			
	1502.00	.8133	.8133			
	1503.00	.8133	.8133			
	1504.00	.8333	.8333			
	1002.00	.8667	.8667			
	605.00	.8967	.8967			
	1003.00	.9067	.9067			
	1501.00	.9633	.9633			
	604.00	1.0667	1.0667			
	603.00	1.2033	1.2033			
	602.00		1.3333			
	Sig.	.215	.052			

Means for groups in homogeneous subsets are displayed.
 a. Uses Harmonic Mean Sample Size = 3.000.

Top Phase Purification factor

PEG		N	Subset for alpha = 0.05					
			1	2	3	4	5	6
Duncan ^a	8005.00	3	.0500					
	3353.00	3	.1533					
	3354.00	3	.1833	.1833				
	3352.00	3	.2367	.2367	.2367			
	1005.00	3	.2600	.2600	.2600			
	3351.00	3	.2600	.2600	.2600			
	1001.00	3	.3667	.3667	.3667	.3667		
	3355.00	3	.3733	.3733	.3733	.3733		
	1004.00	3	.4600	.4600	.4600	.4600	.4600	
	8002.00	3	.4633	.4633	.4633	.4633	.4633	.4633
	8003.00	3	.4800	.4800	.4800	.4800	.4800	.4800
	8004.00	3		.6100	.6100	.6100	.6100	.6100
	601.00	3			.6300	.6300	.6300	.6300
	1505.00	3				.7367	.7367	.7367
	8001.00	3				.7933	.7933	.7933
	1502.00	3					.8133	.8133
	1503.00	3					.8133	.8133
	1504.00	3					.8333	.8333
	1002.00	3					.8667	.8667
	605.00	3						.8967
	1003.00	3						
	1501.00	3						
	604.00	3						
	603.00	3						
	602.00	3						
	Sig.		.052	.052	.074	.050	.066	.050

Means for groups in homogeneous subsets are displayed.
 a. Uses Harmonic Mean Sample Size = 3.000.

Top Phase Purification factor

PEG		Subset for alpha = 0.05				
		7	8	9	10	11
Duncan ^a	8005.00					
	3353.00					
	3354.00					
	3352.00					
	1005.00					
	3351.00					
	1001.00					
	3355.00					
	1004.00					
	8002.00					
	8003.00	.4800				
	8004.00	.6100	.6100			
	601.00	.6300	.6300			
	1505.00	.7367	.7367	.7367		
	8001.00	.7933	.7933	.7933	.7933	
	1502.00	.8133	.8133	.8133	.8133	
	1503.00	.8133	.8133	.8133	.8133	
	1504.00	.8333	.8333	.8333	.8333	
	1002.00	.8667	.8667	.8667	.8667	
	605.00	.8967	.8967	.8967	.8967	
	1003.00	.9067	.9067	.9067	.9067	
	1501.00		.9633	.9633	.9633	.9633
	604.00			1.0667	1.0667	1.0667
	603.00				1.2033	1.2033
	602.00					1.3333
	Sig.	.054	.111	.134	.062	.068

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

C5: SPSS data for PEG 1000- TLL

Descriptives

PEG 1000 PFT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	.3667	.11930	.06888	.0703	.6630	.27	.50
2.00	3	.8667	.16166	.09333	.4651	1.2682	.72	1.04
3.00	3	.9067	.49863	.28789	-.3320	2.1453	.47	1.45
4.00	3	.4600	.13115	.07572	.1342	.7858	.34	.60
5.00	3	.2600	.12288	.07095	-.0453	.5653	.17	.40
Total	15	.5720	.34798	.08985	.3793	.7647	.17	1.45

ANOVA

PEG 1000 PFT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.053	4	.263	4.095	.032
Within Groups	.643	10	.064		
Total	1.695	14			

For One Way Anova, the p-value was less than 0.05, which showed that there was significant differences between the groups.

Homogeneous Subsets

PEG 1000 PFT

	TLL	N	Subset for alpha = 0.05	
			1	2
Tukey HSD ^a	5.00	3	.2600	
	1.00	3	.3667	
	4.00	3	.4600	
	2.00	3	.8667	
	3.00	3	.9067	
	Sig.			.065
Duncan ^a	5.00	3	.2600	
	1.00	3	.3667	
	4.00	3	.4600	.4600
	2.00	3		.8667
	3.00	3		.9067
	Sig.			.378

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

C6: SPSS data for different pH of PEG 600

Oneway

[DataSet0]

Descriptives

Top Purification Factor

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
6.50	3	2.4100	.11269	.06506	2.1301	2.6899	2.34	2.54
7.00	3	2.6633	.14154	.08172	2.3117	3.0149	2.50	2.75
7.50	3	5.7100	1.77542	1.02504	1.2996	10.1204	4.32	7.71
8.00	3	6.2533	3.21083	1.85377	-1.7228	14.2295	4.33	9.96
8.50	3	1.0467	.15011	.08667	.6738	1.4196	.90	1.20
9.00	3	.8400	.12124	.07000	.5388	1.1412	.73	.97
Total	18	3.1539	2.51173	.59202	1.9048	4.4029	.73	9.96

ANOVA

Top Purification Factor

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80.186	5	16.037	7.111	.003
Within Groups	27.063	12	2.255		
Total	107.249	17			

For One Way Anova, the p-value was less than 0.05, which showed that there was significant differences between the groups.

Homogeneous Subsets

Top Purification Factor

	pH	N	Subset for alpha = 0.05	
			1	2
Tukey HSD ^a	9.00	3	.8400	
	8.50	3	1.0467	
	6.50	3	2.4100	2.4100
	7.00	3	2.6633	2.6633
	7.50	3		5.7100
	8.00	3		6.2533
	Sig.			.678
Duncan ^a	9.00	3	.8400	
	8.50	3	1.0467	
	6.50	3	2.4100	
	7.00	3	2.6633	
	7.50	3		5.7100
	8.00	3		6.2533
	Sig.			.193

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.