PRODUCTION AND RECOVERY OF LIGNIN PEROXIDASE FROM *PLEUROTUS PULMONARIUS* (FR) QUEL BY AQUEOUS TWO PHASE SYSTEM

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2014

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

UNIVERSITI MALAYA

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Name of Degree: **MASTER OF BIOTECHNOLOGY**

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ABSTRACT

Pleurotus pulmonarius is an edible mushroom, which secretes lignocellulolytic enzymes, like laccase and lignin peroxidases. These enzymes enable the fungus to grow on a variety of different substrates such as lignocellulosic waste. In this study white rot fungi *Pleurotus pulmonarius* was tested for lignin peroxidase production in a submerged liquid fermentation. To enhance the enzyme production the influence of different parameter such as culture composition, inoculum size and agitation speed were investigated. The optimum cultivation condition for highest lignin peroxidase activity of 95.54± 2.26 U/ml was obtained in the presence of 1% (w/v) yeast, 1% (w/v) glucose and 1% (w/v) sawdust at agitation speed of 120 rpm and inoculum size of 2 discs.

An aqueous two-phase system composed of recyclable random copolymer of ethylene oxide (EO)-propylene oxide (PO) and potassium phosphate salt was employed for the recovery of *Pleurotus pulmonarius* lignin peroxidase from submerged liquid fermentation. Lignin peroxidase partitioned in ATPS system was examined under various parameters such as polymer molecular weight, phase composition, volume ratio (V_R), system pH and addition of sodium chloride. The result showed that the highest enzyme purification factor was achieved by ATPS composed of 18.80% (w/w) EOPO 3900, 7.11% (w/w) potassium phosphate with volume ratio of 0.82 at pH 7. Furthermore, the result showed that purification of lignin peroxidase is not influenced significantly by addition of sodium chloride. The purification factor of 9.22±1.07 and yield of 80.47% were achieved from the bottom phase of this optimized ATPS system with enzyme activity of 22.37±2.30 U/ ml.

The recycling of EOPO was conducted at the end of recovery process. A recovery of more than 80% of the EOPO 3900 polymer was obtained from the ATPS. The result indicated that there is no significant difference in the purification factor and partitioning efficiency of purified lignin peroxidase in the ATPS system using fresh or recycled polymer.

ABSTRAK

Pleurotus pulmonarius merupakan sejenis cendawan yang boleh dimakan dan merembeskan enzim-enzim lignocellulolytic terutamanya enzim laccase dan lignin peroxidase. Enzim-enzim ini membolehkan fungus untuk bertumbuh dalam pelbagai substrat seperti bahan-bahan buangan lignocellulosic. Dalam kajian ini, keupayaan fungus white rot *Pleurotus pulmonarius* untuk menghasilkan lignin peroxidase dalam keadaan penapain cecair secara bertenggelam telah dikaji. Untuk meningkatkan produksi enzim, parameter pengaruhan seperti komposisi kultur, saiz inokulum dan kelajuan pengocakan turut dikaji. Keadaan optimum pemupukan enzim lignin peroxidase yang mencatatkan hasil sebanyak 95.54± 2.26 U/ml telah diperoleh dengan penggunaan 1% yis, 1% glucose dan 1% habuk kayu dalam kelajuan pengocakan sebanyak 120 rpm dan saiz inokulum 2 x 10mm.

Satu kaedah aqueous two-phase yang terdiri daripada ko-polimer rawak yang boleh dikitar semula, ethylene oxide (EO)-propylene oxide (PO) dan garam potassium phosphate telah diaplikasikan bagi pemulihan enzim lignin peroxidase *Pleurotus pulmonarius* daripada penapain cecair secara bertenggelam. Lignin peroxidase yang berpetak dalam kaedah ATPS telah dikaji di bawah beberapa parameter seperti jisim molekul polimer, composisi fase, nisbah jumlah(V_R), sistem pH dan penambahan of garam semula jadi. Faktor purifikasi sebanyak 9.22±1.07 dan hasil sebanyak 80.47% telah dicapai pada fasa bawah yang telah dioptimasikan; fasa bawah juga mencatatkan aktiviti enzim sebanyak 22.37±2.30 U/ ml.

Kitaran semula EOPO telah dilakukan pada peringkat akhiran proses pemulihan enzim. Kadar pengitaran yang melebihi 80% daripada polimer EOPO 3900 telah

berjaya diperoleh daripada kaedah ATPS. Kajian juga menunjukkan bahawa tiada perbezaan signifikasi dalam faktor purifikasi dan kecekapan pemetakan dalam pemulihan enzim lignin peroxidase berasaskan kaedah ATPS yang mengaplikasikan polimer baru atau polimer kitaran semula.

DEDICATION

This dissertation is dedicated to my parents, Mr. Majid Mohebali, and Mrs. Zohreh Alibaba, to whom I owe everything

ACKNOWLEDGMENT

"In the name of God, most merciful, praise be to God, the cherisher and sustainer of the worlds, for without him nothing is possible.

I would like to sincerely acknowledge my supervisors Professor Dr. Ling Tau Chuan and Dr. Tan Yee Shin for providing me a chance to do this project and for their invaluable guidance and supports. I am also grateful to Professor Dr. Vikineswary Sabaratnam for her support and practical advice.

My most sincere thanks also go to Dr. Show Pau Loke, Dr. Jegadeesh Raman and Mr Lin Yu Kiat for their valuable guidance.

Many thanks to my laboratory mates and friends including Nor Hamizah Binti Md Non, Zeinab Masoudian, Linda Jong Wan Yng, and the entire members of Mycology and Plant Pathology Lab for the invaluable support and cooperation throughout the project. Besides, I would like to thank Madam Chang May Heng and Mr Karim, for their technical assistance towards this project.

I am deeply grateful to my family, my lovely father, mother and brothers for their endless support, patience, love and understanding they provided me throughout my life.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation and symbol	Full Title
ATPs	Aqueous two-phase system
ANOVA	Analysis of variance
EOPO	Ethylene oxide propylene oxide
EO	Ethylene oxide
PO	Propylene oxide
PEG	Polyethylene glycol
cm	Centimeter
℃	Degree Celsius
PEG	Polyethylene glycol
TLL	Tie lie line
NaCl	Sodium chloride
nm	nanometer
rpm	Rotation per minute
μmole	micromole
K_2HPO_4	Di-potassium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
Mw	Molecular weight
VAD	Veratraldehyde
VA	Veratryl alcohol
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
PDA	Potato dextrose agar

PDB	Potato dextrose broth
P_{FT}	Top phase purification factor
V_R	Volume ratio
K	Partition coefficient
C_T	Activities in top phase
C_B	Activities in bottom phase
SA	Specific activity
V_T	Volume of top phase
V_B	Volume of bottom phase
Y_T	Yield of top phase
w/v	Weight per volume
w/w	Weight per weight
U/ml	Unit/millimeter
U/mg	Unit/milligram
LiP	Lignin peroxidase
MnP	Manganese peroxidase
H_2O_2	Hydrogen peroxide
pH	Hydrogen ion concentration
OD	Optical density
kDa	Kilo dalton

CHAPTER 1: INTRODUCTION

1.1. Introduction

Mushrooms have been used throughout the world since ancient times (Wasser and Weis, 1999) due to their ability to produce a wide range of valuable products with different characteristics such as anticoagulant and antifungal protein, anti-tumour compounds and ligninolytic enzymes. The white-rot fungus *Pleurotus pulmonarius* belongs to the Pleurotaceace family and is well known as oyster mushroom (Toyama and Ogawa, 1974).

Pleurotus pulmonarius has been widely studied because of its ability to produce variety of ligninolytic and cellulolytic enzymes such as laccase, lignin peroxidase, manganese peroxidase, xylanase and cellulose (Massadeh and Modallal, 2007). Lignin peroxidase (LiP) is one of the enzymes that have been extensively studied. This enzyme was first discovered in 1983 from *Phanerochaete chrysosporium* during the studies on the mechanism of the lignin biodegradation (Glenn and Gold, 1985; Tien and Kirk, 1984).

Lignin peroxidase is gaining more attractive for biotechnological application because of the high potential to degrade varied range of compound such as lignocellulose; lignin related aromatic compounds and other non-lignin related compounds. Applications of LiP are biobleaching, bioremediation of variety of organic waste byproduct and biopulping (Glenn and Gold, 1985). In addition, lignin peroxidase has high potential application in cosmetic product as well as dermatological preparation for skin due to its ability to break the melanin structure in the skin (Roushdy *et al*., 2011). The wide application of lignin peroxidase however is being restricted; as there is

lack of the simple, cost effective and robust methods for lignin peroxidase production (Jing *et al*., 2007; Jing, 2010).

Submerged fermentation is the most economical methods to produce large amount of mycelial biomass and subsequently extracellular enzyme (Xu *et al*., 2011). Several agricultural substrate or by-product, which contains high concentration of soluble carbohydrates and enzyme inducer, has been used in submerged fermentation of different microorganism to accelerate the lignocellulolytic enzyme production (Elisashvili *et al*., 2001; Moldes *et al*., 2004; Reddy *et al*., 2003). Besides, it has been reported that the production of extracellular LiP can be influenced by culture condition such as growth media composition, heat shock, agitation speed and inoculum size (Darah and Ibrahim, 1990; Papagianni, 2004; Nigam *et al*., 2012; Conesa *et al*., 2002; Martinez, 2002; Valderrama *et al*., 2003).

The extraction and purification of desired product from fermentation broth is considered as the most expensive part of downstream process (Naganagouda and Mulimani. 2008). Ultrafiltration, chromatography and precipitation are some conventional methods, which have been widely used in bioproduct purification (Wood, 1980). However, these methods are not efficient for purification because of multi-step process, high operation cost, low yield, and long cycle time involved (Mayolo-deloisa *et al*., 2009). To overcome the disadvantages of conventional methods, aqueous two-phase system can be a suitable separation method with higher efficiency.

Aqueous two-phase system (ATPS) provide an ideal condition for recovery of several biomolecules such as protein, enzyme, amino acid, biopharmaceuticals product (Ratanapongleka , 2010; Sinha *et al.,* 1996; Chavez-Santoscoy *et al*., 2010) which have been broadly used in field of biotechnology. In addition, aqueous two-phase system can

be used for separation of cell organelles, viruses and biological membrane due to the high water concentration in both phases, which provide gentle environment for such biomolecules (Walter and Johansson, 1994; Azevedo *et al*., 2009). Aqueous two-phase method has some advantages over the conventional methods such as simplicity, biocompatibility, low operation cost and easy scale up process (Benavides and Ritopalomares, 2008).

Normally, for the large-scale isolation of macromolecules, polyethylene glycol (PEG)/salt or PEG/dextran systems are being used. However, an ineffectual recycling ability of phase-forming chemical in the mentioned systems is a main disadvantage of conventional ATPS. Moreover, it has been reported that additional operations such as filtration, diafiltration and crystallization, are needed to remove the phase forming chemical from the desired protein in conventional ATPS (Johansson *et al*., 1997). These drawbacks have limited the application of conventional systems in the large scale process.

Recently, thermoseparating ethylene oxide-propylene oxide copolymer and potassium phosphate has been successfully applied for primary purification of biomolecules The ATPS could be easily recycled at low cost when the EOPO is used as a copolymer. For this aim, the EOPO from primary system subjected to the temperature greater than the LCST (lower critical solution temperature) to induce the termoseparation (Johansson *et al*., 1997). Afterward the recovered EOPO can be reused in new primary ATPSs. Moreover, the use of recyclable polymer makes this system more economical and environmental friendly (Show *et al*., 2011).

1.2. Objective

The objectives of this research were:

1. to optimize the production of lignin peroxidase in submerged fermentation
2. to investigate the application of aqueous two-phase system (ATPSs) for lignin peroxidase purification and recovery.
3. to investigate the recycling of phase component in an aqueous two-phase system.

CHAPTER 2: LITERATURE REVIEW

2.1. *Pleurotus pulmonarius* (Fr.) Quel

Pleurotus pulmonarius (synonym *Pleurotus sajor-caju*), a white-rot fungus, are an edible mushroom (Wasser and Weis, 1999) that belongs to the order Agaricales and family Pleurotaceace. (Toyama and Ogawa, 1974). *Pleurotus pulmonarius* (Figure 2.1) is first being discussed by Lucien Quélet on 1872. It is known as "Houbitake" and "Feng Wei Gu" in Japanese and Chinese, respectively (Toyama and Ogawa, 1974). The Indian scholar, Yan Dai Ke, found this fungus for the first time at the foot of Himalayan Mountain. It was then distributed in China from Indian and Australia (Zhuang *et al*., 1993).

Naturally, this fungus grows on tissues of *Euphorbia royleans,* in the foothills around Himalayas (Jandaik and Kapoor, 1976). *Pleurotus pulmonarius* grows on stumps and trunks of a wide range of deciduous trees, usually in the form of overlapped leafs (Wasser and Weis, 1999). The optimum temperature for *Pleurotus pulmonarius* in order to fruit faster and produce larger mushrooms is 25℃, however it is able to survive in tropical temperature of 28-30℃ as well (Quimio, 2001). Southeast Asian countries (tropical areas) such as India and Malaysia are popular places for this species to grow.

Yeast extract, artificial logs, rubber wood sawdust (Gern *et al.,* 2008), using bark and trunks of banana trees, cereal straw (Bano *et al*, 1988; Mizuno and Zhuang, 1995) are among several substrates which can grow oyster mushroom.

Figure 2-1: The picture of *Pleurotus pulmonarius* (Y.S.Tan)

Table 2-1: Scientific classification of *Pleurotus pulmonarius (http://en.wikipedia.org)*

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Pleurotaceace
Genus	*Pleurotus*
Species	*Pleurotus pulmonarius*

Pleurotus pulmonarius contains vitamin C (33mg), vitamin B_1 (0.2 - 0.3 mg), vitamin B_2 (1.1 - 1.4 mg) and niacin (18.2 – 21.3 mg) per 100g, in the dry matter (Mizuno and Zhuang, 1995). It also contains protein (21%) and eight kinds of amino acids essential for humans, including high level of lysine and threonine. Fresh fruiting bodies of *Pleurotus pulmonarius* contain low level of glycogenic polysaccharide (lipid and starch) and 80-90% moisture. Rich nutrient content, flavor and taste of *Pleurotus pulmonarius* has made it one of the most treasured fungus (Mizuno and zhuang, 1995). Apart from its good taste, it might also be useful in antitumor drugs development and other pharmaceutical applications (Mizuno and zhuang, 1995).

Pleurotus pulmonarius produces different kind of ligninolytic and cellulolytic enzymes such as laccase, lignin peroxidase, manganese peroxidase, xylanase and cellulose. Ligninolytic enzymes have made *Pleurotus pulmonarius* one of the most studied fungus among its family (Massadeh and Modallal, 2007).

An important ability of white rot fungi is to produce effective enzymes in degrading lignin, such as extracellular polyphenol oxidases particularly lignin peroxidases, manganese peroxidases and laccases (Revankar and Lele, 2007). The isolated lignin peroxidases and manganese peroxidases from *Pleurotus pulmonarius* are widely used in bioremediation of different kind of organic waste byproducts such as textile dyes, polyethylene, pesticides and herbicides, dynamite, PAHs, dioxins and oil-contaminated soil (Glenn and Gold, 1983). *Pleurotus pulmonarius* is also capable of processing lignocellulose, which is highly present in agroindustrial wastes.

2.2. Lignin

Lignin is complex, heterogeneous, three- dimensional, natural polymer, which is the main component of wood and provides a structural support for woody plants (Higuchi, 1990; Whetten and Sederoff, 1995). After cellulose, lignin is the most abundant aromatic polymer in biosphere. Wood comprised primarily of 45% cellulose, 25-30% hemicellulos and 25% lignin (Perez *et al*., 2002). These three types of polymers are strongly linked together by non-covalent forces. Lignin acts as a cementing component to connect cells and harden the cell walls of xylem tissue and provides water transportation through vessels and tracheids from root to branches (Higuchi *et al*., 1994). The process of impregnated of the wood cellulose with lignin is called lignification, which greatly increased the hardness and strength of the cell wall and give the necessary rigidity to the tree.

2.2.1. Structure of lignin

Lignin is a structurally complex aromatic biopolymer and non-water soluble. Aromatic rings of this biopolymer has made it resistant to microbial enzymes (Heim and Schmidt 2006). Lignin is made of many phenyl propanoid units which act as precursor in lignin polymerization. Propanoid units have three different types which are p-coumaryl alcohol, coniferyl alcohol (guaiacyl unit) and sinapyl alcohol (syringyl unit) (Ahammed, 2002). These three types of phenyl propanoid units are differing in the number of methoxyl group on the aromatic ring (Figure 2.2).In order to degrade lignin, oxidative attack on the carbon-carbon and either interunit bonds is required. Cellulose microfibrils are coated with lignin polymer especially within secondary walls. Depolymerization of lignin is necessary in order to enter the cellulose and hemicellulose. There are only few numbers of filamentous fungi with the ability of

using lignin as the only source of carbon and energy. The extracellular oxidase and peroxidase are the two enzymes believed to have an important role in the initial depolymerisation of lignin. Eventually, fragments with small molecular weight are then metabolized to water and carbon dioxide (Cullen and Kersten 2004).

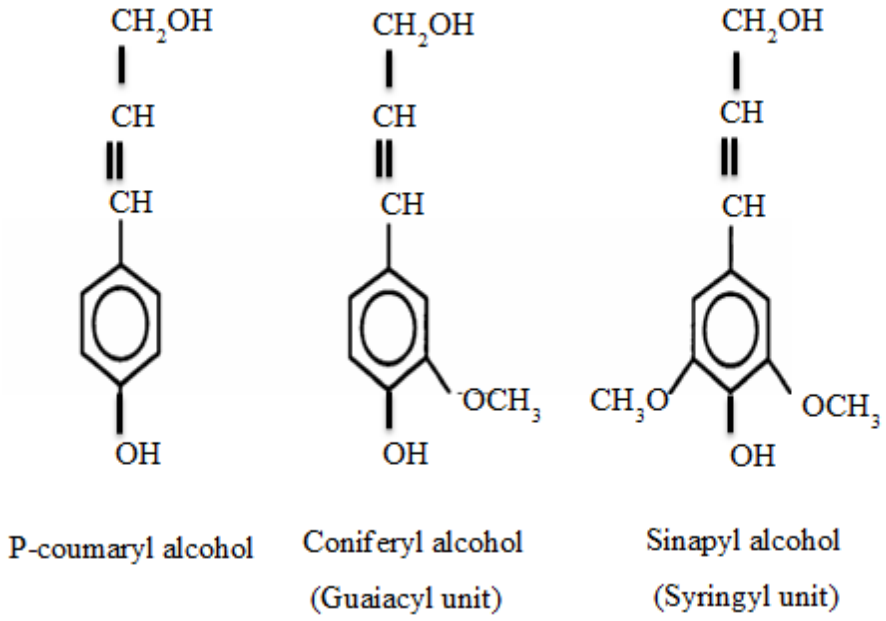

Figure 2-2: Structure of three phenyl propanoid precursors (Ahammed, 2002)

2.2.2. Lignin polymerization

In order to polymerize lignin, precursors of phenylpropanoid have to polymerize first in the cell wall (Higuchi 1985). These precursors (three different types) are different in the number of methoxyl group on the aromatic ring. After deposition of polysaccharides, the lignin will then polymerize. This process begins with conversion of phenylpropanoid precursor to phenoxy radicals by enzymatic oxidation of electrons. A complex cross linked-network of lignin polymer is then formed by coupling of radicals with each other and other radicals, resulting in the final structure of the lignin polymer. (Adler 1977).

2.2.3. Lignin degrading microorganisms

Lignin is an insoluble polymer; therefore the initial steps of its biodegradation must be occurred extracellular (Feijoo *et al.,* 1995). Due to its hydrophobicity, complex random structure and lack of regular hydrolysable bonds, lignin degradation process has a long time cycle and only few numbers of microorganisms such as white rot fungi and specific groups of bacteria are able to degrade it. (Buswell *et al*., 1987; Coll *et al*., 1993; Mester and Field 1998).

2.2.3.1. Lignin degrading fungi

The wood rotting fungi have capacity to degrade lignin efficiently (Eriksson *et al*., 1990). This group of fungi can be divided into three groups which are soft rot fungi, brown rot fungi and white rot fungi according to the morphology of wood decay.

a) Soft rot fungi

Soft rot fungi belong to the ascomycetes and euteromycete. Soft rot fungi are less important than white and brown rot fungi, due to their low ability to degrade the wood composition. Despite of high level of cellulose and hemicellulose degradation by these fungi, the amount of lignin degradation is little. The rate of decay on softwood may be low and the extent of the degradation is minimal compare to the decay by white or brown rot fungi in the same period of time (Eriksson *et al*., 1990).

b) Brown rot fungi

Brown rot fungi are members of the Basidiomycetes. They are the major components of forest soils and responsible for most of the destructive decay of wood (Worral *et al.*, 1997). They preferentially attack cellulose. The brown fungi rot is named

such because the decayed wood has brown color. The main features of brown rot fungi are rapid depolymerization of holocellulose. This ability seems to be normal for brown rot fungi (Eriksson *et al*., 1990).

c) White rot fungi

White rot fungi are the only specialized group of fungi, either pathogenic and/or saprophytic, are able to efficiently degrade lignin (Blanchette, 1984; Schwarze *et al*., 2007). White rot fungi include several member of species of basidiomycetes also some of the ascomycetes. These groups are the biggest agent of lignin decomposer in nature due to their capability to degrade all the main component of the wood. Lignin degradation by white rot fungi revealed that lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase are extracellular ligninolytic enzymes responsible for initiating the lignin depolymerization (Kondo *et al*., 1994; Ohkuma *et al*., 2001; Ikehata *et al*., 2004).

White rot fungi required a co- metabolisable carbon source in order to break down lignin. These sources of carbon are within wood as breakdown products of cellulose and hemicellulose (Blanchette 1984; Schwarze and Fink, 1997). Typically white rot appears as a spongy, stringy, or laminated structure in affected wood, where lignin and polysaccharides present in sound wood are removed in equal proportions. The white rot fungi are of considerable ecological interest because they play such an important role in decomposing woody material in forests.

2.2.3.2. Lignin degrading bacteria

Lignin-degrading enzymes may come from both bacteria and fungi. There are several other bacteria that produce enzyme to degrade lignin. For example,

Pseudomonas putida produce vanillate o-demethylase-oxido-reductase. While the more common lignin peroxidase and laccase are produced by *Streptomyces viridosporus* and *Bacillus subtilis,* respectively (Tuomela *et al*., 2000).

2.3. Lignin peroxidase (LiP)

Lignin peroxidase (LiP) was discovered in 1983 from *Phanerochaete chrysosporium*. This enzyme is extracellular metabolites that required hydrogen peroxide (H_2O_2) as an inducer to catalyze several reactions (Tien and Kirk 1984, Glenn and Gold 1985). Lignin peroxidase is a monomeric hemoglycoprotein (38-46 kDa)(Doyle and Smith 1996), which is used to degrade lignin. This extracellular enzyme is produced by many wood-degrading fungi (Kirk and Farrell, 1987).

According to Tien (1986), the size of lignin produced by *Streptomyces viridosporuscan* is approximately 37KDa which are similar to plant peroxidase in mechanism and structure. Lignin peroxidase produced by bacteria is able to utilize hydrogen peroxide and organic peroxide to oxidize a variety of substrates (Tien, 1986). Lignin peroxidase is a glycoprotein that contains about 15% carbohydrate and an iron protoporphyrin IX (heme). Typical lignin peroxidase is a protein with 38-46 kDa with an isoelectric point varies between pH 3 and 5 (Tuisel *et al*., 1990; Asther *et al*., 1992).

The lignin peroxidase contains multiple isoenzymes. The numbers of isoemzyme present are differing between species and also strain depending on culture condition and purification/fractionation techniques (Buswell *et al*., 1987; Kirk and Farrell, 1987). Lignin peroxidase has a very low pH optima and high redox potential in compare with other peroxidases. Lignin peroxidase has no substrate specificity, reacting with a wide range of phenolic and non-phenolic aromatic compounds. Analysis of decayed wood

showed that the oxidation of lignin resulting in cleavage of Cα-Cβ bond, the aryl Cα bond, hydroxylation of aromatic ring and side chain (Zavarzina *et al*., 2011). The enzyme is capable to oxidize lignin monomers, dimers and trimmers as well as polycyclic aromatic compound such as benzopyrene with reduction potential higher than 1.3 volts (Haemmerli *et al*., 1986).

2.3.1. Application of lignin peroxidase

Recently, using enzymes obtained from various plant and microbial sources for the treatment or removal of environmental and industrial pollution has attracted high attention. Enzymatic processes have various advantages over conventional biological, physical, and chemical treatment processes. The strong emphasis on the use of enzymes is because of their high efficiency, high selectively and environmentally benign reaction. Lignin peroxidase play important role in biotechnology due to its potential application in the biodegrading of lignin, phenolic compound, bioremediation of waste water and catalyzing difficult chemical (Doyle *et al*., 1998). In addition, lignin peroxidase has an application in food industry due to their ability to generate natural aromatic flavor (Lesage-Meessen *et al*., 1996; Lomascolo *et al*., 1999; Barbosa *et al*., 2008). The applications of lignin peroxidase in different sectors are shown in Table 2-2.

Table 2-2: Lignin peroxidase applications in different sectors

Sector	Lignin peroxidase (LiP) Application	References
Food industry	Source of natural aromatics Production of vanillin	Lesage-Meessen *et al*., 1996 ;Lomascolo *et al*., 1999; Barbosa *et al*., 2008
Bioremediation	Degradation of azo, heterocyclic, reactive and polymeric dyes. Mineralization of environmental contaminants Xenobiotic and pesticides degradation	Bumpus and Aust, 1987; Abraham *et al*., 2002; Ohtsubo *et al*., 2004; Robles-Hernández *et al*., 2008; Gomes et al. 2009; Wen et al. 2009
Organic synthesis, Medical, Pharmaceutical, Cosmetics and Nanotechnology	Functional compounds synthesis Cosmetics and dermatological for skin Bioelectro-catalytic activity at atomic resolution	Christenson *et al*., 2004; Higuchi, 2004; Belinky *et al*., 2005; Barbosa *et al*., 2008
Pulp and paper industry	Decolouriment of kraft pulp Mill effluents	Ferrer *et al*., 1991; Bajpai, 2004; Sigoillot *et al*., 2005

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

2.4. Aqueous two-phase systems (ATPS)

Aqueous two-phase systems (ATPS) are a purification system, which are formed by mutual incompatibility of two polymers or a polymer and a salt in aqueous solution (Albertsson, 1986). This system first reported by Dutch microbiologist M. Beijerinck who obtained a two-phase system after trying to mix certain properties of gelatin, agar

and water (Beijerinck, 1910). However, in 1956 Swedish biochemist, P. A. Albertsson achieved rediscovery of this system as an important separation technique by applying this technique to separate various biomolecules (Albertsson 1956). Later, ATPS has been successfully used for the separation of different biological products such as proteins, nucleic acids, microorganisms, plant and animal cells (Albertsson, 1986; Hatti-Kaul, 2000; Johansson, 1985).

Aqueous two phase systems involving two operation steps, which are equilibration and phase separation (Figure2-3). Rapid mixing of the phase components can create two equilibrium phases. This step is followed by separation of the liquid phases. The phase separation under gravity is not as rapid as in water-organic solvent system, and time required varies from a few minutes to a few hours (Hatti-kaul 2000). This is due to rather low differences in the densities of two liquid phases (about 0.05 to 0.15 g/cm^3) (Hustedt *et al.*, 1985), their viscosities and the time required by small droplet, formed during mixing, into larger droplets (Walter *et al*., 1994). In order to quicken the separation process, low speed centrifugation is commonly used (Hatti-kaul 2000).

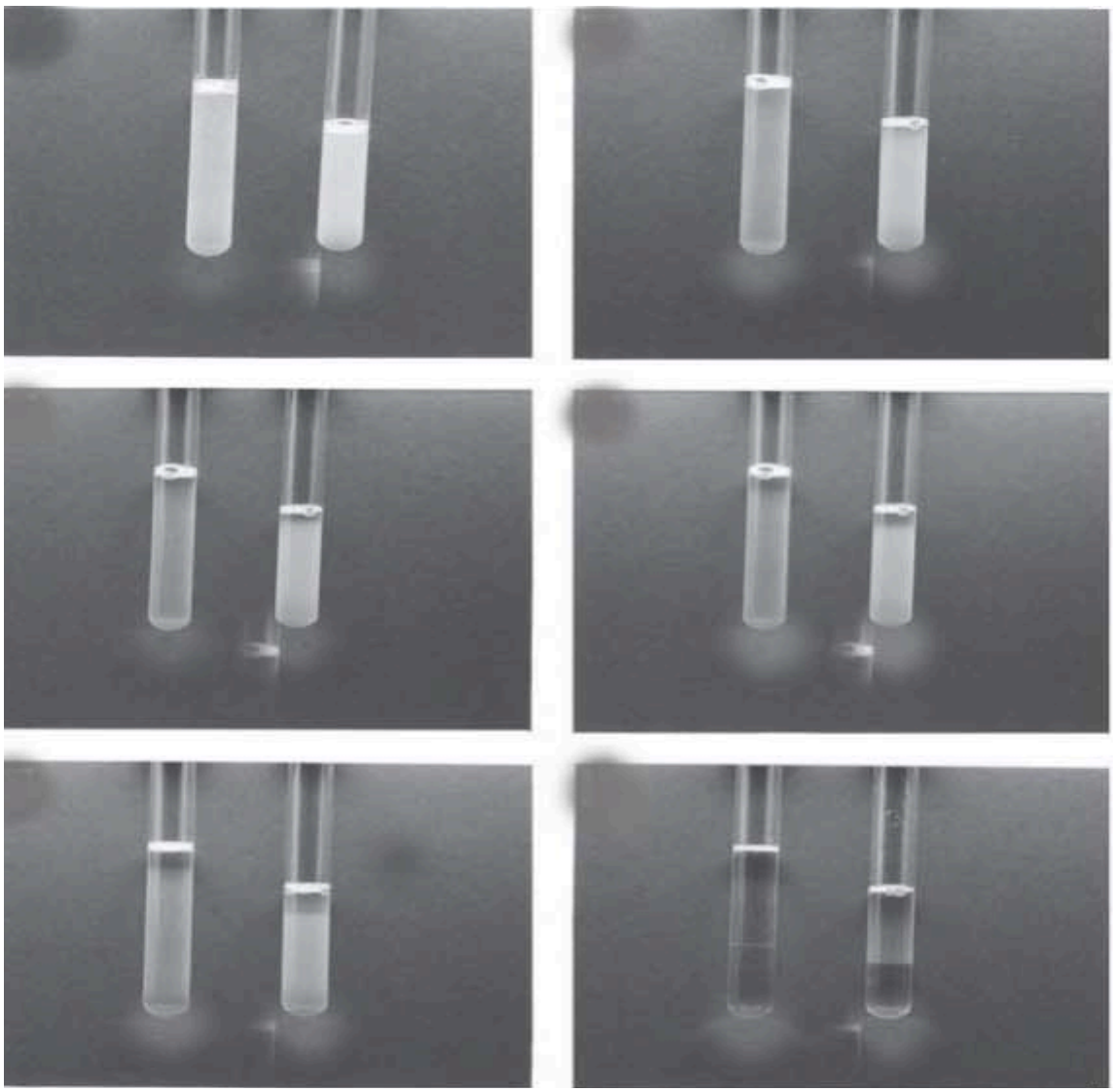

Figure 2-3 Photographic sequence of the phase separation phenomenon of PEG/Dextran system (Forciniti *et al*., 2000).

The distribution of biomolecules between the phases is variable and usually controlled by the properties of the partitioned biomolecule such as size, net charge and surface properties (Albertsson, 1986). In spite of small molecules, the macromolecules are not evenly distributed between two phases in the system and usually directed to one phase or to the interface. In addition to the physico-chemical properties of biomolecules

there are other factors that play a part in the partitioning of desiered biomolecules such as, electrostatic interaction, hydrophobicity, biospecific affinity interaction and conformational effects between the phase components and biomolecule (Albertsson *et al.*, 1990; Albertsson, 1986).

Generally, aqueous two-phases (ATPS) are formed when two incompatible water-soluble polymers differing in their chemical structure, or a polymer and a salt in water, are mixed above a certain critical concentration (Albertsson, 1986). Formation of two phases presented by binodial curve as shown in Figure 2-4. According to binodial curve, the mixture proportion above the curve gives two phases and below the binodial provides only one phase (Albertsson, 1986).

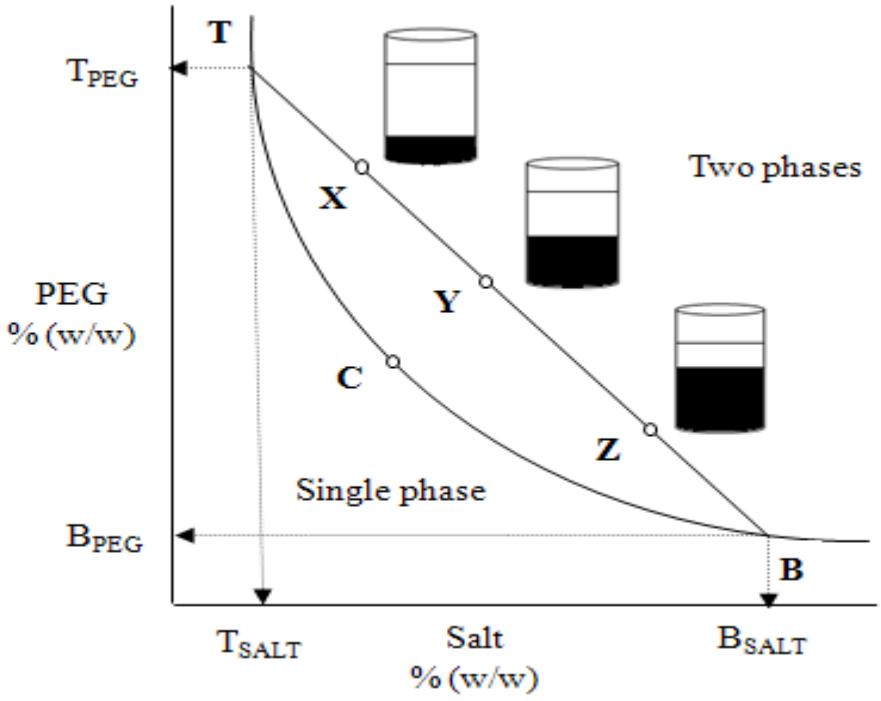

Figure 2-4 Schematic phase diagram of ATPS (Raja *et al*., 2011)

Successful microbial fermentation process depends upon proper recovery of desired biomolecule from the mixture of a large number of interfering substances. Some

byproduct like protein, enzyme, nucleic acid, cell organelles, antibiotics, antigens are sensitive to pH, temperature, surface charge and osmotic pressure, therefore, to enhance the recovery and functionality of target biomolecule, the extraction methods should be compatible to the product (Banik *et al*., 2003). The conventional purification techniques such as chromatography, ultrafiltration and precipitation are time-consuming, expensive and have a multi-steps protocol. According to Scope (1993), these separation techniques such as precipitation and ultrafiltration may result in undesired purity (Scop, 1993).

Aqueous two-phase system (ATPS) is an alternative method, which has been broadly used in field of biotechnology for purification and recovery of sensitive biomolecules (Ratanapongleka, 2010). In addition, aqueous two-phase system can use to separate of cell organelles, viruses and biological membrane due to the high water concentration in both phases (Walter and Johansson, 1994; Azevedo *et al*., 2009).

2.4.1. Advantages of aqueous two-phase (ATPS)

ATPS has been reported as an interesting method for isolation of desired biomolecule compared to conventional methods for several reasons. ATPS provides the mild environment condition for separation of desired product since it contains high water content in each of the two-phases (70 to 90%) (Ratanapongleka and Phetsom, 2011) which allows the biomolecule retain its biological conformation (Walter and Johansson, 1994). Moreover, ATPS is known as a convenient method to substitute the time-consuming purification methods since it has the ability to combine the early downstream steps into the single steps process (Madhusudhan *et al*., 2008; Mazzola *et al*., 2008). Aguilar et al., (2006), compared ion exchange chromatography with ATPS composed of PEG/phosphate for purification of penicillin acylase, the result indicated the significant reduction of operation steps by using ATPS.

Other advantages of applying ATPS include lower investment cost, high yield and ease of scaling up (Albertsson, 1986). The study conducted by Nit-sawang *et al.,* (2006) showed the high papain recovery yield of 88% from wet *Carica papaya* by using PEG/ammonium sulphate. However, the reduction of papain recovery yield to 49% was observed using multiple step precipitation method.The whole process of recovery can be considered as an economical and environmental friendly method due to the simple and low cost phase's component. Furthermore, using recyclable phase component can make the whole system more economical and may also minimize the problem of downstream pollution (Hustedt 1985; Veide *et al.,* 1989; Louwrier, 1999).

2.4.2. Factors effecting partition behavior

The biomolecule characteristic such as size, electrochemical properties, surface hydrophobicity and hydrophilicity, and conformational characteristics can alter partition behavior of target compound as intrinsic properties. Furthermore extrinsic properties such as type, molecular weight and concentration of phase forming components, ionic strength, pH and temperature, have an effect on the partition behavior of target compound (Albertsson, 1987). Therefore, the optimum ATPs purification system can be achieved by manipulating the extrinsic and intrinsic properties (Rito-Palomares, 2004)

2.4.3. Polymer/salt ATPS

Aqueous two-phase system has several types such as polymer-polymer, polymer-salt, surfactant based and alcohol-salt. Most of the ATPS systems are based on polymer/salt. Polymer/salt composition are the most cost effective in comparison to polymer/polymer system, as a result, this form of ATPS has a wide application in biotechnology. The most common form of polymer/salt system is polyethylene glycol

(PEG)/potassium phosphate. The PEG/Phosphate is more preferred for industrial application due to their advantage such as lower viscosity, needing a short time of phase separation and also the low cost (Hatti-Kaul, 2000). Several types of other polymers have been studied such as dextran, starch derivatives and hydroxypropyl starch. Another alternative is using recyclable polymers such as thermo sensitive, pressure sensitive, pH sensitive and light sensitive polymers, which are shown in Table 2.3.

Table 2-3: List of different recyclable polymers using in ATPS

Polymer	Type of Polymer	Extractive	Polymer Recycle Rate (%)	Reference
EOPO[a]	Thermo sensitive	L-asparaginase	81.3-84.7	Zhu, 2007.
HM-EOPO[b]		Apolipoprotein A1	--------	Johansson, 1999.
NIPAMVI/ HM-EOPO[c]		BSA	53/92	Persson, 2000.
PEG/NH_4NH_2CO	Pressure-sensitive	Amino acide	--------	Van Berlo *et al*., 2000.
PNBC/P ADB[d]	pH-sensitive	Lysozyme, BSA	98/97	Biao *et al*., 2009.
PNNC/PADB[e]	Light sensitive	BSA, L-Tyr	98/97	Wang *et al*., 2008

(Liu *et al.,* 2011)

a. EOPO: ethylene oxide–propylene oxide polymers. b HM-EOPO: a hydrophobically modified random polymer of EO and PO with aliphatic C14H29- groups coupled to the end of the polymer.c NIP AM-VI: copolymers of 1-vinylimidazole (VI) with N- isopropylacrylamide (NIPAM).d PNBC: copolymer synthesized by using n-isopropylacrylamide, n-Butyl acrylate, chlorophyllin sodium copper salt as monomers; PADB: copolymer synthesized by using acrylic acid, 2-(dimethylamino) ethyl methacrylate, and n-butyl methacrylate as monomers, and ammonium persulfate and sodium hydrogen sulfite as initiators.)e PNNC: copolymer synthesized by using N-isopropylacrylamide, N-vinyl- 2-pyrrolidone, chlorophyllin sodium copper salt as monomers, and 2,2'-azo- bisisobutyronitrile as initiator.

2.4.4. Application of aqueous two-phase system (ATPS)

It has been reported that ATPS has been widely applied for purification of extracellular macromolecule such as pectinases, β -galactosidase, α-amylase and cellulose from various fermentation process (Antov and Pericin, 2000; Johansson and Reczey, 1998; Anderson *et al*., 1985; Alam *et al*., 1989; Stredansky *et al*., 1993; Persson *et al*., 1984). Apart from using ATPS techniques to purify different protein from bacterial and fungal culture, this method has been successfully applied for recovery of viral or plasmid gene therapy vector (Garca-Perez et al., 1998), inclusion bodies and viral coat protein for the protein vaccines (Rito-Palomares, 2004) (Table 2.4).

Table 2-4: Application of ATPSs

ATPS	Application	Reference
PEG/dextran	Separation of polymerase chain reaction (PCR) inhibitory	Lantz *et al*., 1996
	Substances from bacterial cells	Cole, 1991
	Recovery of viral coat proteins from recombinant *E. coli*	Rito-Palomares and Middelberg, 2002
PEG/phosphate	Isolation of membrane proteins	
	Preparation of highly purified fractions of small inclusion bodies	Walker and Lyddiatt, 1998
	Recovery of aroma compounds under product inhibition conditions	Rito-Palomares *et al*., 2000
PEG/sulphate	Drowning-out crystallisation of sodium sulphate	Taboada *et al*., 2000
	Recovery of metal ions from aqueous solutions	Rogers *et al*., 1996
	Recovery of food coloring dyes from textile plant wastes	Huddleston *et al*., 1998
	Partition of small organic molecules	Rogers *et al*., 1998
EOPO/Phosphate	Recovery of lipase derived from *Burkholderia Cenocepacia*	Show *et al*., 2012
	Extraction of Lysozyme from hen egg white	Dembczynski *et al*., 2010

Modified from (Dreyer, 2008)

2.5. Properties of ethylene oxide -propylene oxide (EOPO)

Random copolymers of ethylene oxide (EO) and propylene oxide (PO) are water soluble and thermo stable that can form two phases with other polymers and different salts which allowed the replacement of PEG in ATPS system. (Show *et al.,* 2012). EOPO copolymer has a lower critical solution temperature (LCST) of 60℃ and can

used for recovery of protein. When EOPO is used in ATPS, the process divided in two recovery stages, which are called primary recovery and secondary recovery. In primary EOPO/salt ATPS the target protein is partition in the top phase (EOPO rich phase) .In the secondary recovery stage the EOPO rich phase removed from the system and heated up to the lower critical solution temperature(LCST). The new two phases is formed where the bottom phase contains of the concentrated EOPO and top phase consist of target protein and water. The thermoseprating copolymer from the secondary ATPS can be reused in further ATPS system (Show *et al*., 2012).

2.6. Concluding remarks

Different purification methods are available for biomolecules purification. However, ATPS is a better choice for the separation and purification of protein since the extraction of ATPS is relatively rapid and the processing capacity of ATPS is relatively high as compared to other existing purification methods.

Although ATPS have been successfully applied for the industrial recovery of wide variety of enzyme from different natural sources such as mushroom and bacteria (Ratanapongleka, 2010) to our knowledge, there is no report on extraction and purification of lignin peroxidase from *Pleurotus pulmonarius* using polymer/salt systems.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

Ethylene oxide-propylene oxide (EOPO) with different molecular weights (2500, 3900 and 12000 g/ml), veratryl alcohol (3, 4-dimethoxy benzyl alcohol) and bovine serum albumin were purchased from Sigma-Aldrich Company (USA). Di-potassium hydrogen phosphate (K_2HPO_4) and potassium di-hydrogen phosphate (KH_2PO_4) were obtained from Merck. All chemicals used were analytic grade.

3.2. Fungi strain

The mycelium of *Pleurotus pulmonarius* was obtained from Mycology laboratory, Institute of Biological Science, University of Malaya. The mycelium plate were incubated at the ranged of 27± 2℃ for 14 days. The mycelia stock culture were maintained in to potato dextrose agar (PDA) plate and subcultured routinely. The pure culture was kept on potato dextrose agar slant and liquid paraffin oil for long-term storage.

3.3. Preliminary plate assay

Three wells were made in the fungi plate and labeled as A, B and C. Each wells were filled with different solutions. Well A was filled with 95% (v/v) ethanol which act as a control, well B was filled with 0.1% (v/v) syringaldazine to determine the presence of laccase activity and a mixture of 1% (v/v) pyrogalic acid and 0.4% (v/v) hydrogen peroxide with 1:1 ratio was added to the well C to test on the presence of lignin peroxidase (Figure 3.1). The experiment was done in three replicates. The changes of the colors after 30 minutes were observed and recorded.

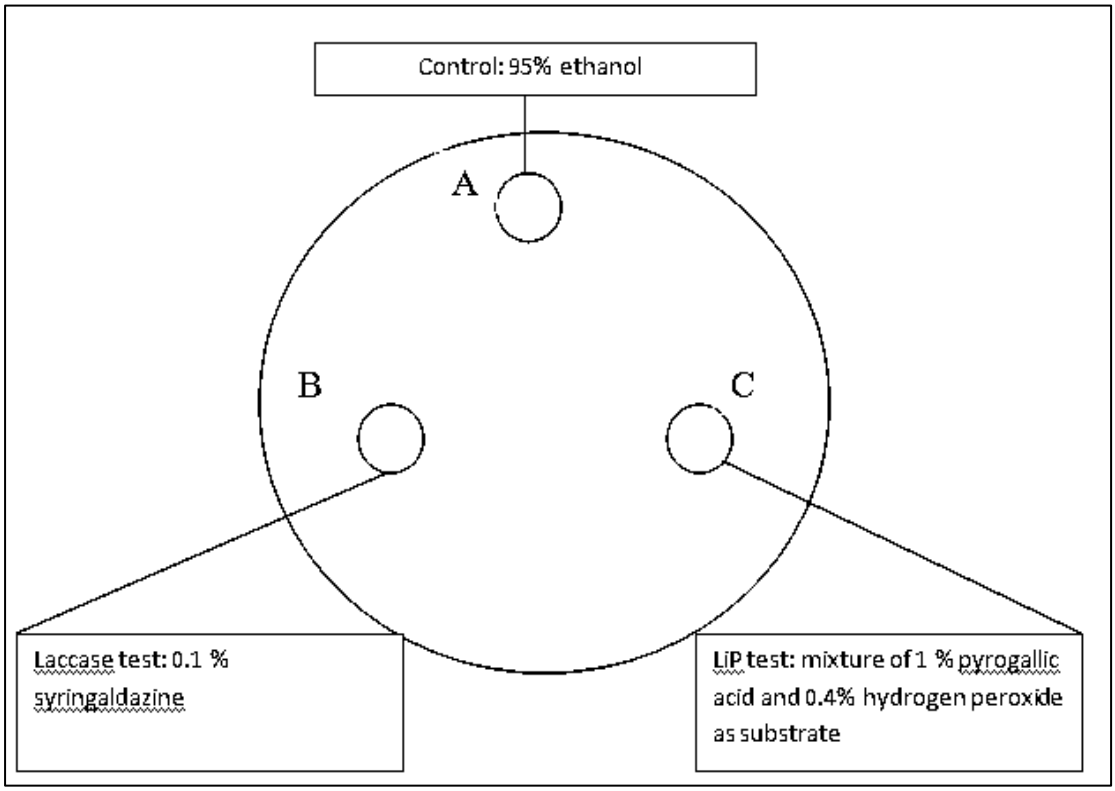

Figure 3-1: Preliminary plate screening for lignin peroxidase and laccase activity

3.4. Submerge liquid fermentation

3.4.1. Optimization of different media for submerged fermentation

Submerge fermentation is the best technique to obtain mushroom extracellular product (Xu *et al.,* 2011). The submerge culture of *Pleurotus pulmonarius* was performed by inoculating mycelia agar blocks (1×1 cm) taken from 14 days old culture of fungus in 250ml Erlenmeyer flasks filled with 100 ml different media composition (Figure 3.2). Different type of culture media contains different type of substrates have been developed in order to obtain the optimum lignin peroxidase production (Table 3.1).

Table 3-1: Different media composition for lignin peroxidase production

PDB + 1% Glucose
PDB + 1% Sawdust
PDB + 1% Yeast + 1% Sawdust
PDB + 1% Glucose + 1% Sawdust
PDB + 2% Glucose1+ 1% Sawdust
1% Yeast + 1% Glucose +1% Sawdust
1% Yeast + 0.5% Glucose 0.5% Sawdust

All the flasks were incubated in a shaking incubator at 120 rpm and 27±2 ºC for 9 days. All the experiments were done in triplicate. After 9 days of submerged fermentation, the media were filtered through Whatman filter paper (No.1) to obtain the filtrated liquid. The filtrate was kept in sterilized polypropylene tubes and stored at 4℃ for further use.

3.4.2. Effect of agitation speed on lignin peroxidase production

The effects of different shaking speed at 50rpm, 80 rpm and 120 rpm on the lignin peroxidase (LiP) production were tested for the culture media.

3.4.3. Effect of inoculum size on lignin peroxidase production

The liquid culture media contained three different inoculum sizes of 2%, 5% and 10 % (v/v) mycelia agar blocks were evaluated at incubation length of 9 days, 120 rpm and 27±2ºC.

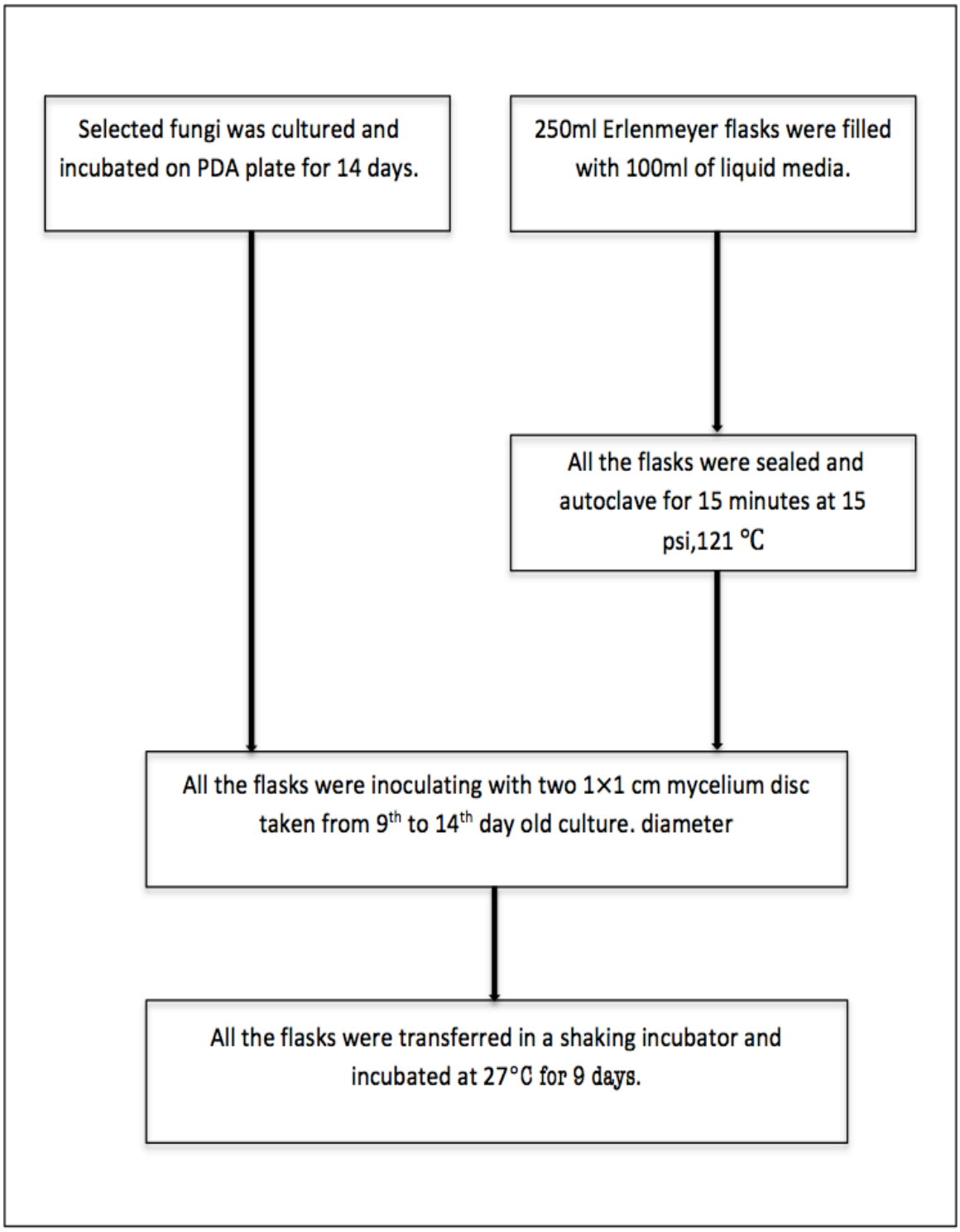

Figure 3-2: Flow chart of steps involved in submerged fermentation of *Pleurotus pulmonarius*

3.5. Determination of lignin peroxidase activity

The activity of lignin peroxidase was determined followed the method by Have *et al*., (1998) veratraldehyde (VAD) in the presence of hydrogen peroxide (H_2O_2) measured at 310 nm wavelength (Have *et al*., 1998).

The enzyme reaction mixture contained 0.2ml of crude enzyme in 2.4ml of 100mM sodium tartrate (pH 3.0) and 0.2ml veratryl alcohol (30mM). The reaction was initiated by adding 0.2ml of freshly prepared 0.5mM H_2O_2. The formation of veratraldehyde was measured after 5 minutes of incubation at room temperature and the absorbance were measured at wavelength 310 nm by using spectrophotometer. The reagent blank contained 2.6 ml 100 mM sodium tartrate buffer, 0.2 ml of 30 mM veratryl alcohol and 0.2ml of 0.5 mM hydrogen peroxide, H_2O_2. The enzyme blank contained 2.4ml of 100 mM sodium tartrate buffer and 0.2ml of tested enzyme. The lignin peroxidase activity was calculated using the following formula (Equation 1). Thus, one unit of LiP was defined as a μmol of VAD released per minute.

$$LiP\ activity\left(U/mL\right) = \left(\frac{final\ absorbance + 0.0003}{0.0313}\right)\left(\frac{dilution\ factor}{0.2}\right)\left(\frac{1}{5\,\text{min}}\right)\left(\frac{1000}{1mg}\right)\left(\frac{1\mu mole}{166.18\mu g}\right) \quad (1)$$

Veratraldehyde stock solution = 100μg/ml Veratraldehyde MW = 166.18 μg

3.6. Measurement of soluble protein

The protein concentration of crude enzyme was measured using method described by Bradford (1976). This method is based on the reaction between Coomassie Brilliant Blue G-250 reagent and proteins in the solution and evaluated by measuring the absorbance of the solution at 595 nm wavelengths with spectrophotometer.

In this method, 0.2 ml of crude enzyme was pipetted into 5 ml Coomassie Briliant Blue reagent. The blank contained 0.2 ml of distilled water and Coomassie Brilliant Blue reagent .The mixture then mixed by vortex and the absorbance was read within 1 hour at wavelength of 595nm. The bovine serum albumin (BSA) was used as standard to determine the protein concentration. The final soluble protein concentration was calculated as followed (Equation 2)

$$\text{Soluble protein}\left(mg/mL\right)=\left(\frac{final\ absorbance}{0.007}\right)\left(\frac{1}{0.2ml}\right)\left(\frac{1mg}{1000\mu g}\right) \qquad (2)$$

BSA stock solution = 1000 μg/10 ml

3.7. Aqueous two-phase system

3.7.1. Ethylene oxide propylene oxide phase diagram

The binodial phase diagram for the ethylene oxide propylene oxide **(**EOPO) 12000, 3900, 2500 and phosphate was determined by cloud point method (Albertsson, 1986). Predetermine amount of EOPO (50% (w/w)) stock solution was weighted into the centrifuge tube and 40% potassium phosphate (KH_2PO_4=18.3 g/100 ml; K_2HPO_4=21.70 g/100 ml) at pH 7 was added to the tube and mixed. The turbid mixture become clear by adding the distilled water and the mass of mixture was measured. The binodial phase diagram divided a region of component concentrations that formed two immiscible aqueous phases, which were above the curve and those that formed one phase which were located below the curve (Selvaraj, 2011).

3.7.2. Tie lie length (TLL)

The TLL was deliberated by analyzing the top and bottom phase composition. The salt concentration and polymer concentration were determined by conductivity and refractive index measurement, respectively.

The TLL that was depicted the two phase composition and calculated as below (Equation 3):

$$TLL = \sqrt{\Delta p^2 + \Delta c^2} \quad (3)$$

In this equation Δp and Δc showed the difference between polymer and salt concentration, respectively, at the top and bottom phase.

3.8. Preparation of aqueous two-phase system (ATPS)

In this study, EOPO/phosphate was used to investigate the partitioning behavior of lignin peroxidase. Two phase system were prepared in a 15 ml centrifuge tube and 50% (w/w) EOPO stock solution and 40 % (w/w) potassium phosphates was added followed by 1 g crude enzyme extract. The final weight of system was adjusted to 10 g by addition of distilled water. The systems were thoroughly mixed and then centrifuged at 4000 rpm for 10 minutes to accelerate the formation of two phases. After formation of two phases with volume ratio 1:1, the top phase and bottom phase were pipetted out and measured the concentration of lignin peroxidase. The protein concentration in each phase was determined by Bradford assay. The system parameters of the ATPS such as TLL, V_R and pH can be then manipulated to get an optimum purification performance (Rito-Palomares, 2004; Rosa *et al.*, 2010). All experiment was carried out in triplicate.

3.9. Optimization of different parameters in aqueous two phase system

3.9.1. Optimization of volume ratio (V_R)

The effect of V_R on P_{FT} , five different systems lying on the same TLL with different V_R (0.29, 0.82, 1.0, 2.1 and 3.75) were tested. The final concentration of the phase component in all these V_R were the same.

3.9.2. Optimization of pH

The effects of different pH values (pH 6-9) on the purity of lignin peroxidase were tested. The pH in ATPS was measured according to Lin *et al*., (2012). This was controlled by mixing different compositions of two potassium phosphates (KH_2PO_4 and K_2HPO_4) with an accuracy of pH $\pm$0.5.

3.9.3. Optimization of NaCl concentration

The effect of the addition of natural salt (sodium chloride NaCl) from 1% to 4% (w/w) on P_{FT} were evaluated.

3.10. Determination of partition coefficient (k), specific activity (SA), volume ratio (V_R), purification factor (p_{FT}) and yield

The partitioning activity of lignin peroxidase was assessed by parameter including partition coefficient, specific activity, purification factor and yield. The partition coefficient (K) was calculated as activity of Lignin peroxidase in two phases (Equation 4):

$$K = \frac{C_T}{C_B} \tag{4}$$

Where C_T and C_B shows the LiP activities (U/ml) in top and bottom phase, respectively.

In order to estimate the purification process, specific activity calculated as below (Equation 5):

$$\mathrm{SA}\left(\mathrm{U/mg}\right) = \frac{\text{Total enzyme activity}\left(\mathrm{U/ml}\right)}{\text{Total protein concentration}\left(\mathrm{mg/ml}\right)} \tag{5}$$

The purification factor defined as an enzyme specific activity at the top phase divided by specific activity of crude enzyme before ATPS. The P_{FT} was calculated according to (Equation 6):

$$\mathrm{P_{FT}} = \frac{\text{Specific activity of collected phase}}{\text{Specific activity of crude enzyme}} \tag{6}$$

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 7):

$$V_R = \frac{V_T}{V_B} \tag{7}$$

The recovery yield in enzyme rich phase calculated as (Equation 8):

$$Y_T\left(\%\right) = \left(\frac{C_T V_T}{C_B V_B + C_T V_T}\right) \times 100\% \tag{8}$$

Where C_T and C_B shows the LiP activities (U/mL) in top and bottom phase respectively, V_T and V_B are the volume ratio in top and bottom phase

3.11. Recycling of the phase component

For recycling of EOPO, the polymer rich phase was taken out from the centrifuged tube and transferred into the new tube. The top phase was diluted with distilled water (ratio1:1) and incubated in water bath at 65 ℃ for 15 minutes to induce the thermosepration. At the next step the diluted top phase sample was centrifuged for 10 min at 4000rpm. Once the phase separation of the phase sample was attained the secondary ATPS contained water at the top phase and concentrated EOPO at the bottom phase formed. The water phase was withdrawn from the copolymer phase and then the new fresh polymer was added, plus salt and protein to provide the optimum condition for first recovery in further studies (Show *et al*., 2012). The schematic diagram for this ATPS is exhibited in Figure 3.3.

The mass of EOPO bottom phase was weighted and recorded to determined polymer recovery. The concentration of EOPO polymer recovered was measured in refractive index by using refrectometer. The polymer recovery (R_{pol}) is calculated as (Equation 9)

$$\mathrm{R}_{pol} = \frac{M_{thermosep}}{M_{initial}} \times 100\% \qquad (9)$$

Where, $M_{thermosep}$ represent the mass of EOPO recovered in lower phase after thermoseparation process and $M_{initial}$ is the total EOPO mass in the top phase of the primary system .

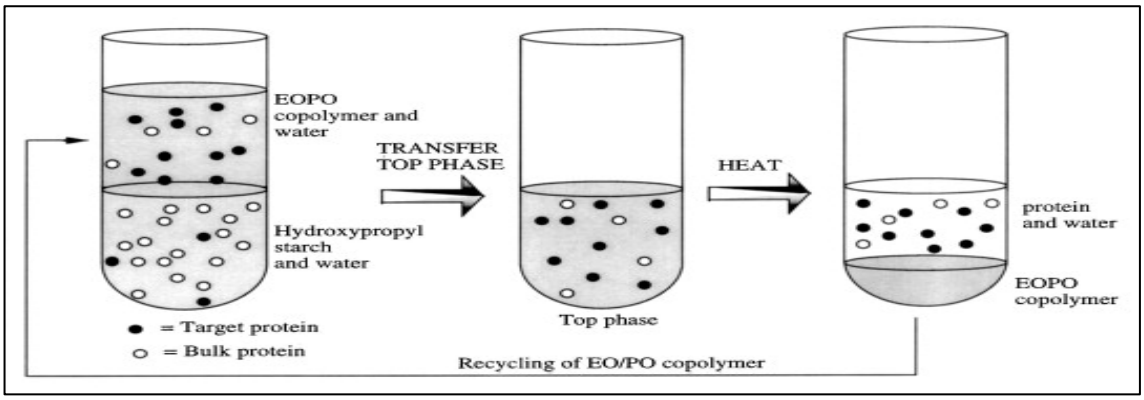

Figure 3-3: Schematic diagram of the recycling EOPO in an aqueous two-phase (Persson *et al*., 2000)

3.12. Characterization of protein

The molecular weight of protein that obtained from ATPS was characterized by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli and Favrel (1973) .The experiment was carried out by acetone precipitation of protein samples in order to remove the salt which affects the electrophoresis process. One milliliter of partially purified protein sample and crude enzyme were mixed with 4 ml of cold acetone solution. The mixture was vortexed and allowed to precipitate at -20℃ for one hour. This was followed by centrifugation at 4000 rpm for 10 minutes. The supernatant was decanted and the pellet was resuspended in 100 μl of distilled water. The concentrated sample was mixed with 10X sample buffer in 17:3 ratio followed by heating the sample in boiling water bath for 2 minutes. A heated sample solution (20μl) was loaded per well on the gels. The samples were analyzed in an acrylamide gel consisting of 12% (w/v) resolving gel and 4.5% (w/v) stacking gel (Appendix A). Electrophoresis was run at 110V and 36mA for approximately 85 minutes.

Unstained protein molecular weight markers ranged from 14.4 to 116.0 KDa were loaded into the gel as a standard. After electrophoresis, the gel was stained with solution consisted of 0.05 % (v/v) Coomassie Brilliant Blue R-250, 10 % (v/v) methanol and 10 % (v/v) acetic acid. It was then destained using the same buffer solution without Coomassie Brilliant Blue. The native-PAGE was then carried out to determine the lignin peroxidase (LiP) activity. The method for the native-PAGE was to leave out the SDS and β-mercaptoethanol from the SDS-PAGE protocol. The bands from the native-PAGE were excised and subjected to lignin peroxidase (LiP) activity test (Have *et al*., 1998).

3.13. Statistical analysis

Mean values of triplicate data for all the parameters tested were obtained and objected to one-way analysis of variance (ANOVA). The statistical significance was accepted at $p<0.05$ using Duncan's multiple range test (Appendix B).

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Preliminary plate screening

The preliminary plate-screening assay is an important method to assess whether the selected fungi are able to produce desired enzyme. This procedure conducted for the mycelium from 10^{th} till 14^{th} day to determine the best day for lignin peroxidase production for submerged fermentation. The colour change of the substrate from colourless to dark pink and dark brown after 30 minutes illustrated the high productivity of laccase and lignin peroxidase, respectively in selected fungi (Figure 4.1).

According to the result, laccase activity was strongest at day 10^{th} and 11^{th} but after that the enzyme lost their activity (Table 4.1). For lignin peroxidase activity, the result showed the obvious increase in color intensity from the 10^{th} to 14^{th} day of mycelium; however the enzyme lost their activity after day 14^{th}. Therefore, the 14^{th} days old mycelium with high potential of enzyme production was chosen for submerge fermentation.

Table 4-1: The preliminary screening of the plate to determine laccase and lignin peroxidase activity

Day	10^{th}	11^{th}	12^{th}	13^{th}	14^{th}
Control	___	___	___	___	___
Laccase	++	++	___	+	___
LiP	+++	+++	+++	+++	++++

(colour intensity determined by + (low), ++ (intermediate), +++ (high), ++++ (very high), - indicates no colour changes).

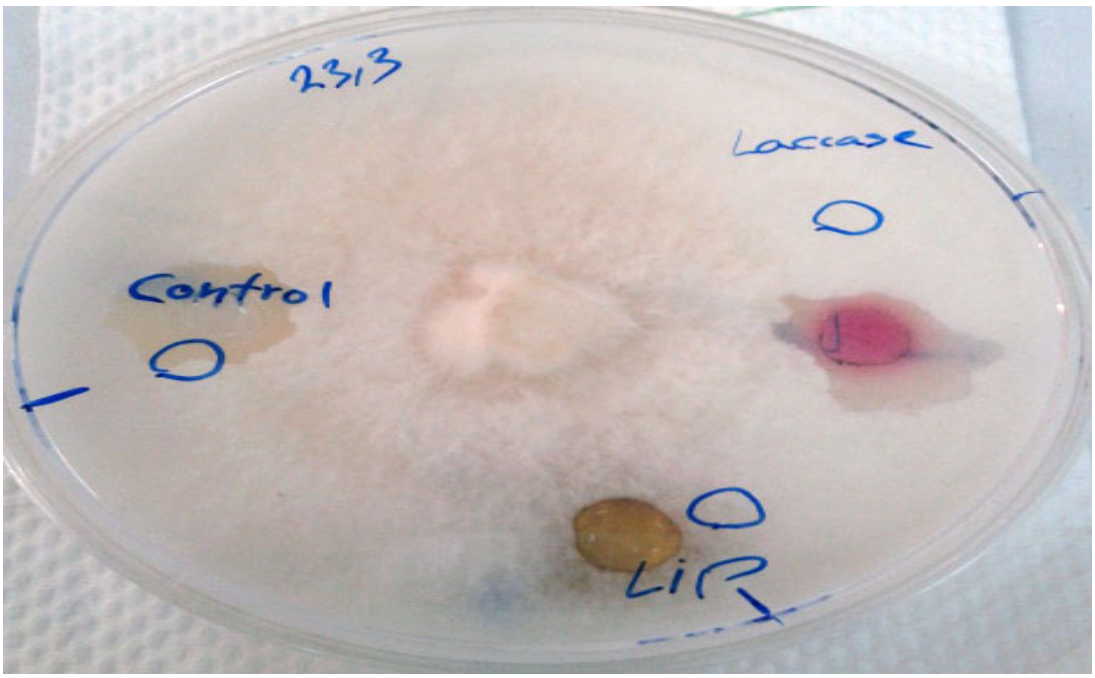

Figure 4-1: Preliminary plate screening of *Pleurotus pulmonarius*. The changes of well colour to yellow indicate the presence of lignin peroxidase and the changes to pink-purple colour indicates the presence of laccase

4.2. Optimization of lignin peroxidase (LiP) production in submerge fermentation

The effects of different media, agitation speed and inoculum size were investigated to optimize the LiP production. These factors were selected based on previous studies which were shown to have an important influence on lignin peroxidase production in submerge fermentation (Sing and Chen, 2008)

4.2.1. Effect of media composition on LiP production

The effect of different media composition was studied .The experiment was conducted for a total of 14 days in the 120 rpm at 27℃ and the crude enzyme was harvested on 3, 7, 10 and 14 th days after incubation.

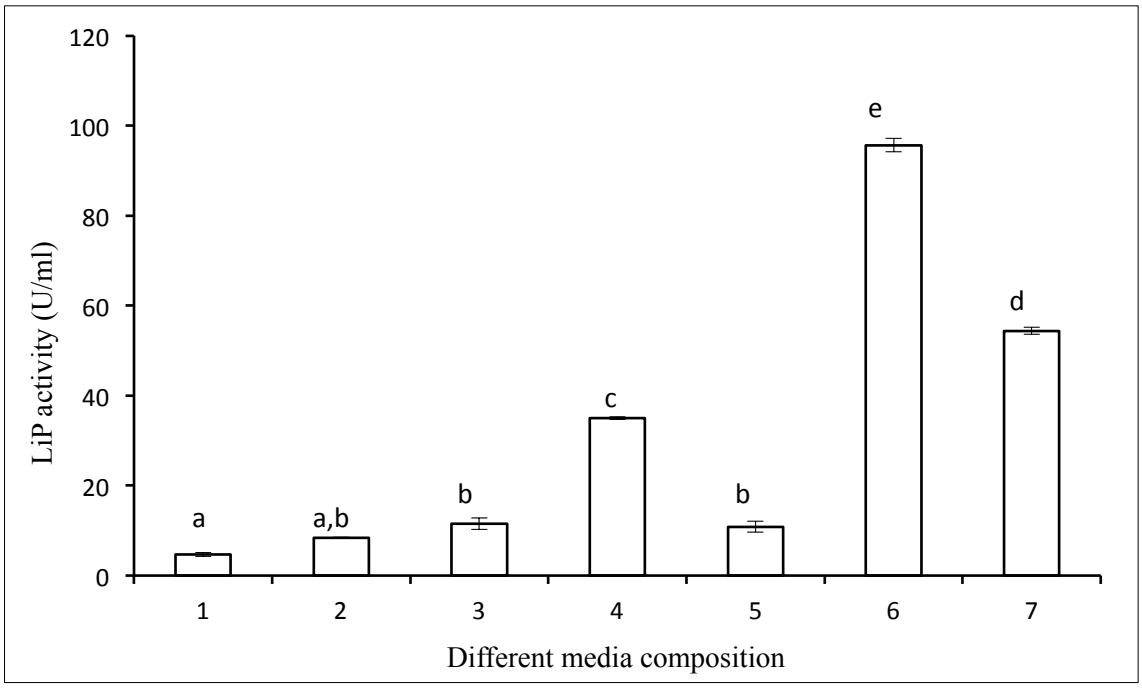

Figure 4-2:Lignin peroxidase activities of *Pleurotus pulmonarius* cultivated in different media.

(*1=PDB + 1% Glucose, *2=PDB + 1% Sawdust, *3=PDB + 1% Yeast + 1% Sawdust, *4=PDB + 1% Glucose +1% Sawdust, *5 =PDB + 2% Glucose + 1% Sawdust, *6=1% Yeast + 1% Glucose +1% Sawdust, *7=0.5Yeast + 0.5% Glucose +1% Sawdust).

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Uses Harmonic Mean Sample Size=3.00.

From Figure 4.2, the media contained 1% yeast + 1% glucose +1% sawdust showed the highest enzyme activity of 95.54 ± 2.26 U/ml in submerge fermentation. This medium provided the optimal condition for lignin peroxidase production as having appropriate amount of glucose as a carbon source and yeast as a nitrogen source and

sawdust. Massadeh *et al*., (2010) showed that addition of glucose as a carbon source induced the lignolytic enzyme production such as laccase, LiP and manganese peroxidase (MnP) in *Pleurotus pulmonarius.*

Kapich *et al.*, (2004) stated that lignocellulosic substrate such as wheat straw and hemp woody core induced the production of *P. chrysosporium* lignin peroxidase under submerge culture with no limitation in carbon and nitrogen source. Moreover, lignocellulosic waste such as sawdust might contain significant amount of soluble carbohydrates (C:N = 95:1), which enhances the enzyme production (Elisashvili *et al*. 2002). Besides, *Pleurotus* species generally live in nature on dead wood as saprophytes and served as a primary degrader and wood decomposer (Nieto and Chegwin, 2008). Hence, it is believed that the sawdust, which added to the liquid media, may provide a similar environment as their natural growing habitat of *Pleurotus pulmonarius,* to induce the production of lignin peroxidase.

4.2.2. Effect of agitation speed on LiP activity

The productions of lignin peroxidase by *Pleurotus pulmonarius* were carried out at different agitation speeds of 50 rpm, 80 rpm and 120 rpm. The results revealed that the highest lignin peroxidase activity of 71.50 ± 2.14 U/ml was observed at 120 rpm agitation speed (Figure 4.3). At lower speed of 50 rpm and 80 rpm, enzyme activity was found to be 57.61±1.65 U/ml and 33.52±1.90 U/ml, respectively. According to Purwanto *et al.,* (2009), low agitation speed reduced fungi enzyme production due to insufficient amount of oxygen supply and uneven distribution of nutrient (Akhavan sepahy and Jabalameli, 2011).

Agitation speed plays an important role in productivity of system since it facilitates the mixing of fermentation broth as well as increases the oxygen transfer. Agitation not only has the beneficial effect but also some negative influences on production broth such as cell destruction, changing the cell morphology and foam production at high agitation speed (Nigam *et al*., 2012).

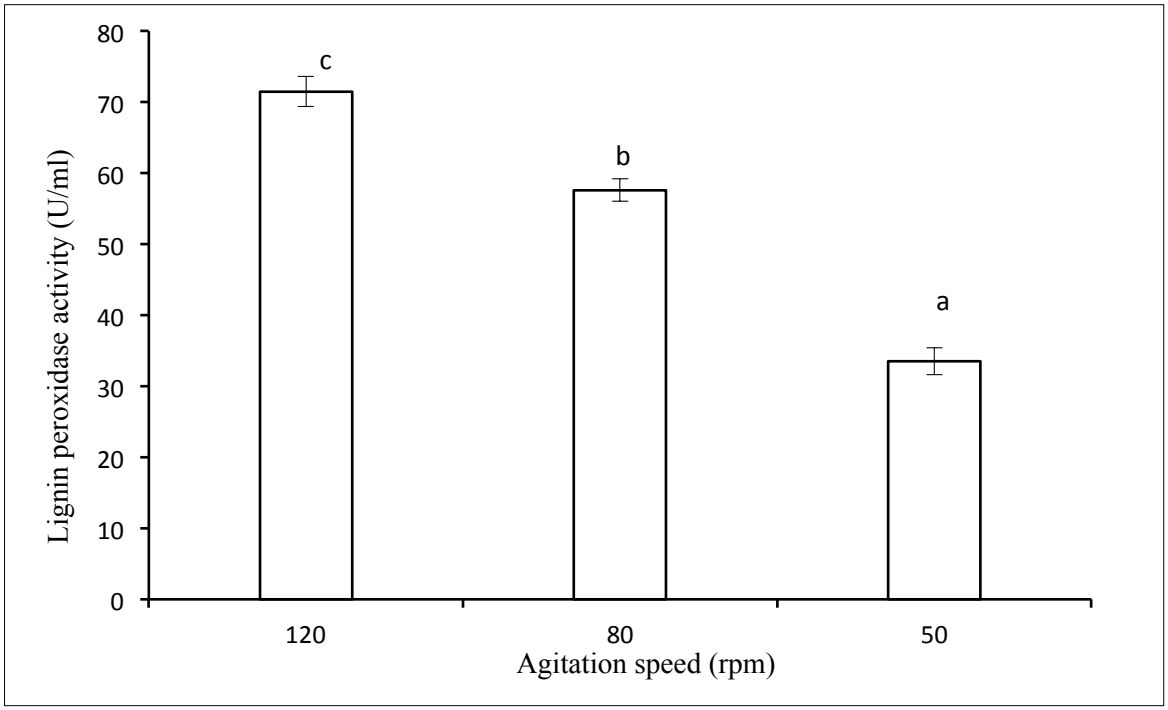

Figure 4-3: Effect of agitation speed on lignin peroxidase production

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Sample Size=3.00

4.2.3. Effect of inoculum size on LiP activity

Inoculum size is one of the factors affected lignin peroxidase production. The fungal culture morphology, fungal growth and enzyme activity, are influenced by inoculum concentration (Darah and Ibrahim, 1996; Papagianni, 2004).

In this study, different inoculum sizes (2%, 5% and 10% (v/v)) were evaluated to obtain optimum level of enzyme activity. The highest lignin peroxidase activity of 91.44 ± 4.65 U/ml (Figure 4.4) was observed with inoculum size of 2 % (v/v). The result showed that lignin peroxidase production was increased with decrease at inoculume size. Shafique *et al*., (2009) reported that inoculum size have an undesired effect on the enzyme activity. The reason might be due to the mycelia overgrowth, anaerobic condition forms that suppress the enzyme production. In addition, high amount of inoculum size resulted in competitive consumption of substrate for growth and metabolic processes.

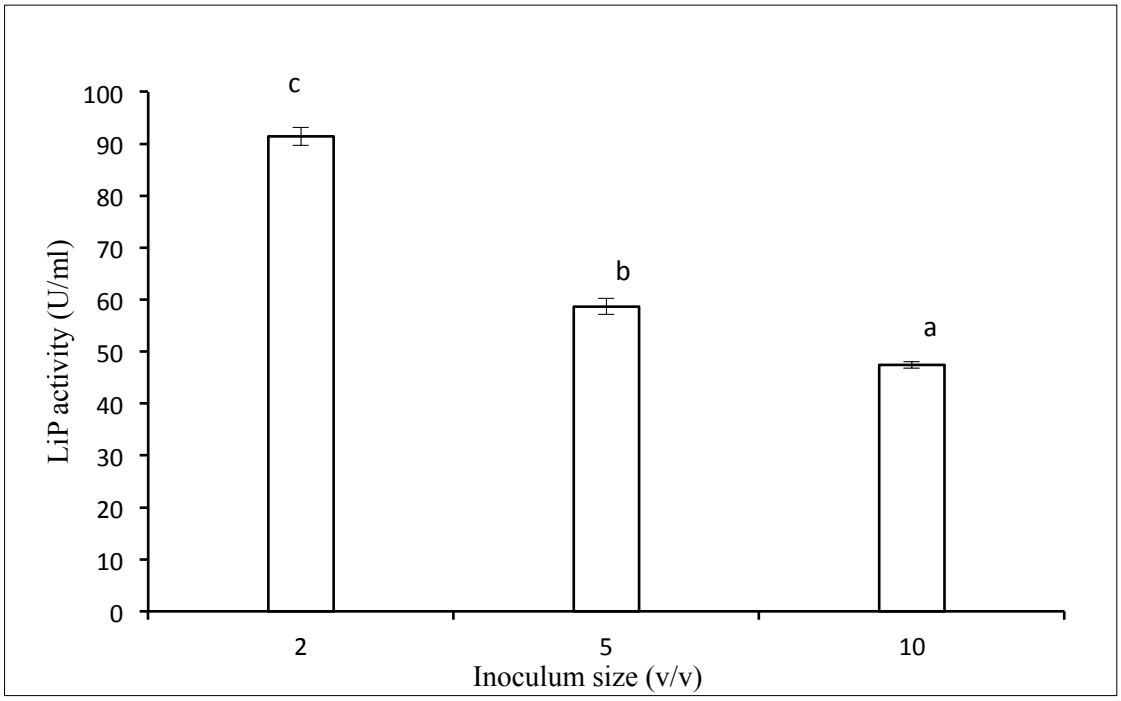

Figure 4-4: Effect of inoculums size on lignin peroxidase production

Note: Lignin peroxidase activity (U/ml) with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Sample Size=3.00.

4.3. The phase diagrams of ethylene oxide-propylene oxide (EOPO)-phosphate system

In this study, ethylene oxide-propylene oxide with three different molecular weights (EOPO 2500, 3900 and 12000) were tested. The phase diagrams of EOPO-phosphate with comparable tie-line lenght (TLL) are shown in Figures 4.5, 4.6 and 4.7. These systems were constructed using different molecular weights of EOPO 2500, 3900 and 12000 g/mol with an increasing trend of TLL at constant volume ratio (V_R=1) at pH 7. The curved line separates the working area into two-phase areas called binodial curve. All the points above this line construct two-phase mixture and the points below the curve do not give two phase. As the phase diagrams showed the binodials of all three copolymer are positioned toward lower polymer concentration and closed to the salt axis, indicated that the EOPO copolymer is not included in the salt-rich phase (Show *et al*., 2012).

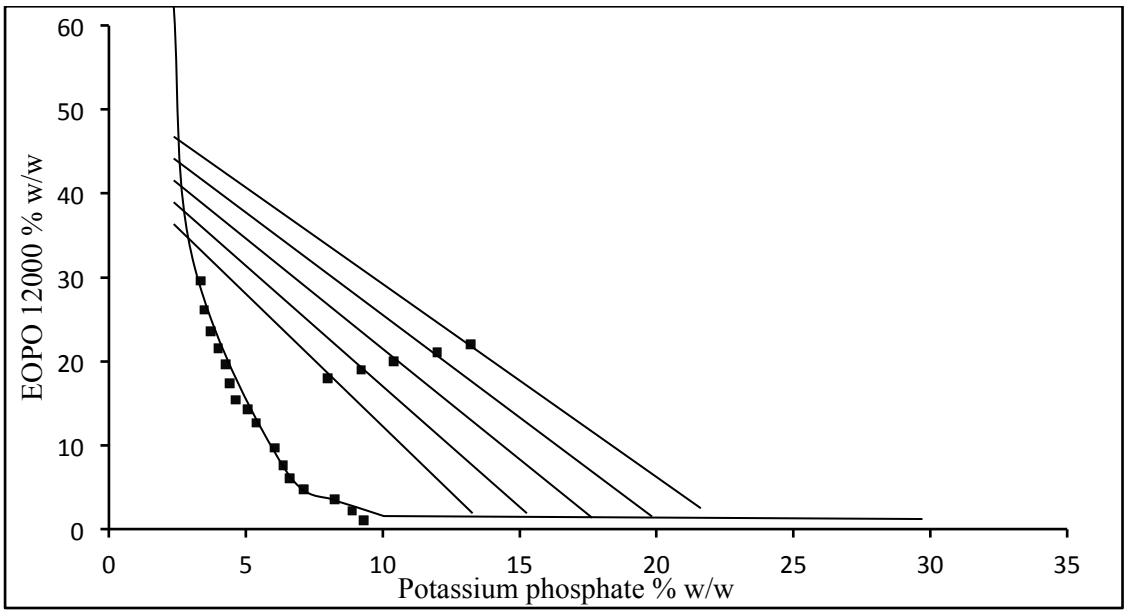

Figure 4-5: Phase diagram for EOPO12000/potassium phosphate system at pH 7 and room temperature

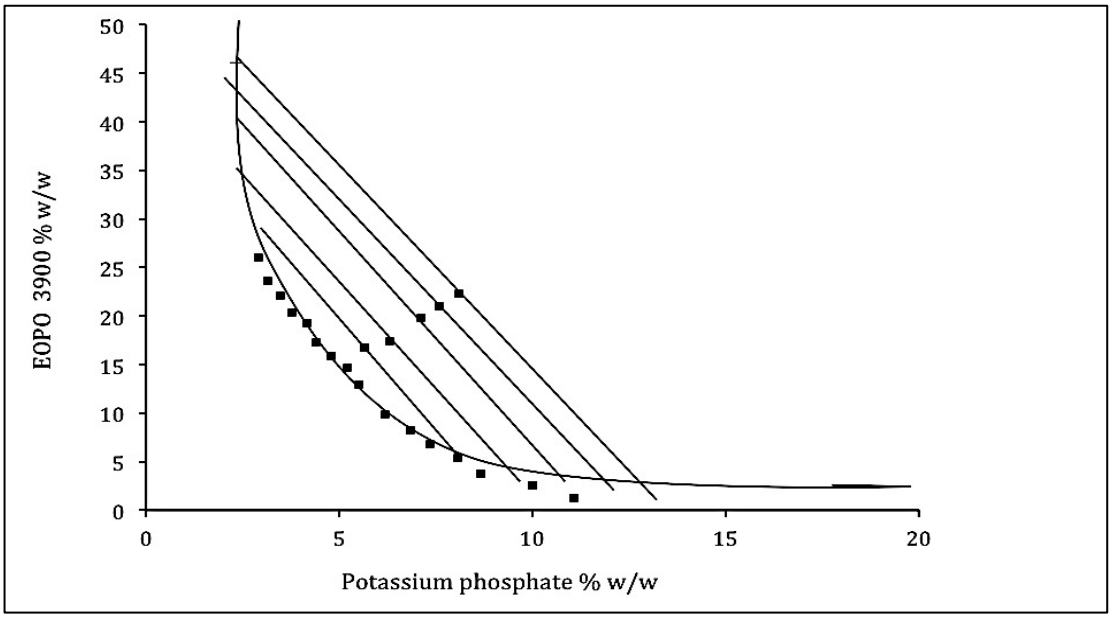

Figure 4-6: Phase diagram for EOPO 3900/potassium phosphate system at pH 7 and room temperature

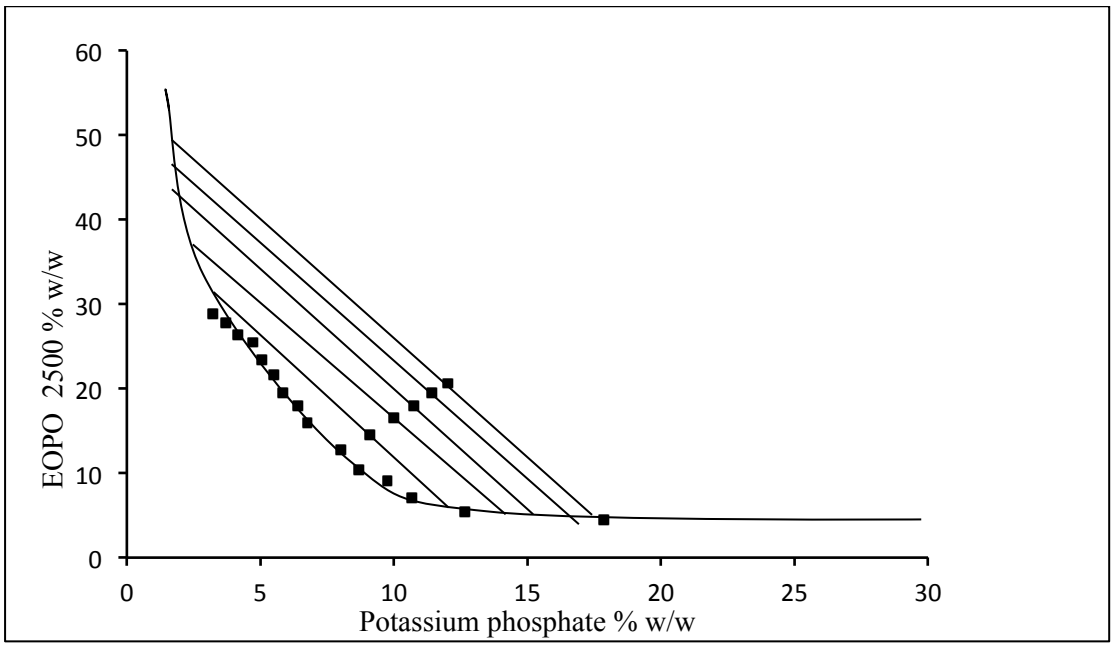

Figure 4-7: Phase diagram for EOPO 2500/potassium phosphate system at pH 7 and room temperature**.**

For the recovery of lignin peroxidase, a total of 15 systems composed by EOPO of molar mass 2500, 3900, 12000 and potassium phosphate were selected from the phase diagram. For each molecular weight, 5 systems with different TLL were selected to determine the lignin peroxidase recovery by ATPS. The pH of potassium phosphate and the volume ratio were kept constant during the experiment at pH7.0 and volume ratio at 1.0. The system was prepared in final weight of 10 g. The system composition and TLL of all 15 tested systems are shown in Table 4-2.

Table 4-2: System selected for the evaluation of the lignin peroxidase recovery

System	Molecular Weight of EOPO g/mol	EOPO % w/w	PO_4 % w/w	Tie Lie Length (TLL)
1	2500	14.50	8.90	24.41
2		16.50	10.00	34.00
3		18.00	10.75	39.05
4		19.50	11.40	41.78
5		20.60	12.00	45.02
6	3900	16.90	5.64	28.98
7		17.45	6.15	34.71
8		18.80	7.11	42.08
9		21.00	7.60	45.23
10		22.32	8.11	48.31
11	12000	18.00	8.00	32.66
12		18.20	9.20	37.76
13		20.00	10.40	42.53
14		21.00	12.00	43.46
15		22.00	13.20	46.21

The enzyme partitioning in the ATPS depended upon the size of the biomolecule, hydrophobicity, and ionic composition of the phases, molecular length and molecular mass of polymer (Banik *et al*., 2003). Thus, altering the conditions such as pH, ionic strength and concentration of phase component, can modify the partition coefficient of protein, and then obtain highest lignin peroxidase purification factor and yield.

4.4. The effect of EOPO molecular weight on purification factor

Tanaka *et al*., (1991); Kavakçıoğlu and Tarhan (2012), reported that the partitions of the biomolecules in the ATPS extremely depends on the polymer molecular mass and the system composition. These parameters changing the number of hydrophobic interactions between the polymer and the hydrophobic area of target protein as well as hydrophobic interactions between the polymer and other biomolecules which are being partition in the system.

The changes in concentration and EOPO molecular masses would affect the purification of a lignin peroxidase in ATPS system. In order to study the influence of both concentration and EOPO molecular mass on LiP purification, the other parameters such as pH and volume ratio of all systems were kept constant at 7.0 and 1.0, respectively. As shown in Table 4.3, for all 15 phase composition (ATPSs) with different molecular mass of EOPO the desired enzyme has shown more tendency to partition in salt-rich phase (partition coefficient is lower than 1.0). The highest purification factor was observed in EOPO 3900 with 3.69±0.22 in bottom phase and recovery yield of 90.40%.

From the studies by Ng *et al*., (2012) and Show *et al.,* (2012), EOPO (3900 MW) with 50% of propylene oxide (PO) content were more suitable for protein partitioning as compared to EOPO with 80% PO content (12000, 2500 MW). Ng *et al.,* (2012), has reported that EOPO polymers exhibit different degree of hydrophobicity by varying PO content. As the PO content increases, the hydrophobicity of EOPO increases as a result of the longer hydrocarbon chain of PO monomers. Moreover, they reported that the EOPO with the 50% PO gave the highest cyclodextrin glycosyl transferase purification factor in ATPS.

However, in this study when the PO composition increased, there was no improvement in purification factors and the enzyme was participated at the bottom phase. This indicated that the lignin peroxidase was the hydrophobic enzyme but it contains only a few areas of hydrophobicity in surface. This can be evaluated by different methods such as reversed-phase chromatography (RPC), hydrophobic interaction chromatography (HIC), and ammonium sulfate precipitation, which was described by Hachem *et al.,* (1996).

In order to evaluate the hydrophobicity of LiP, ammonium sulfate precipitation (Appendix A) has been conducted. From the Figure 4.8, LiP is not soluble in ammonium sulfate salt which well indicating that LiP is not a strong hydrophobic enzyme and the hydrophobic bonding's between the EOPO polymer was less stronger compared to others interaction. Thus, the enzyme showed a totally different trend by partitioning in the bottom salt phase as compared to other strong hydrophobic enzyme, which will participate mainly in the top phase.

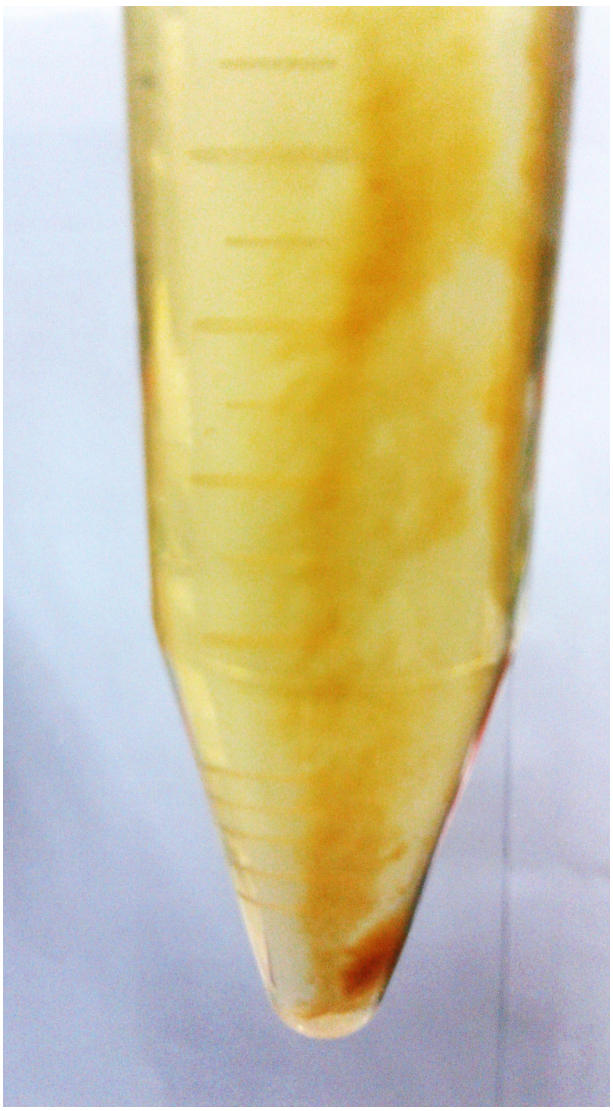

Figure 4-8: Lignin peroxidase precipitated in ammonium sulfate salt

Table 4-3: Partitioning of LiP in different concentrations of EOPO/phosphate system

EOPO Molecular Weights	System	Tie Lie Lenght	Purification Factor		Yield
			Top Phase Purification Factor	Bottom Phase Purification Factor	Bottom Phase Yield (%)
2500	1	24.41	0.00±0.00	0.00±0.00	78.5%
	2	34.00	0.60±0.12	1.52±0.34	75.3%
	3	39.05	0.00±0.00	0.00±0.00	0.00%
	4	41.78	0.00±0.00	0.00±0.00	0.00%
	5	45.02	0.00±0.00	1.11±0.60	71.0%
3900	6	28.98	**1.61±0.22**	1.47±0.02	89.5%
	7	34.71	0.24±0.04	2.79±0.06	85.0%
	8	42.08	0.78±0.30	**3.69±0.22**	90.4%
	9	45.23	0.00±0.00	2.06±0.10	90.5%
	10	48.31	0.00±0.00	1.58±0.30	90.9%
12000	11	32.66	0.05±0.00	0.58±0.0	88.0%
	12	37.76	0.03±0.00	0.50±0.04	87.9%
	13	42.53	0.12±0.02	0.33±0.03	64.5%
	14	43.46	0.12±0.02	0.79±0.06	26.8%
	15	46.21	0.05±0.00	0.50±0.02	30.3%

From our data, both top and bottom phase showed low purification factor suggested that the enzyme migrated towards the interface. Ibarra-Herrera *et al*., (2011) also reported that with the increase in polymer molecular weight, the highest recovery of alfalfa proteins shifted from top phase to interface and bottom phase and such a behavior can attribute to decrease in free volume of top phase. In this study, increase of EOPO molecular mass to 12000 and reduction in free volume at both top and bottom phase leading the enzyme partition mainly to interface

4.5. Effect of volume ratio on purification factor

To examine the effects of volume ratio value on partitioning of lignin peroxidase the best TLL of 28.98 % (w/w) at different V_R values were tested and shown in Figure 4.9.

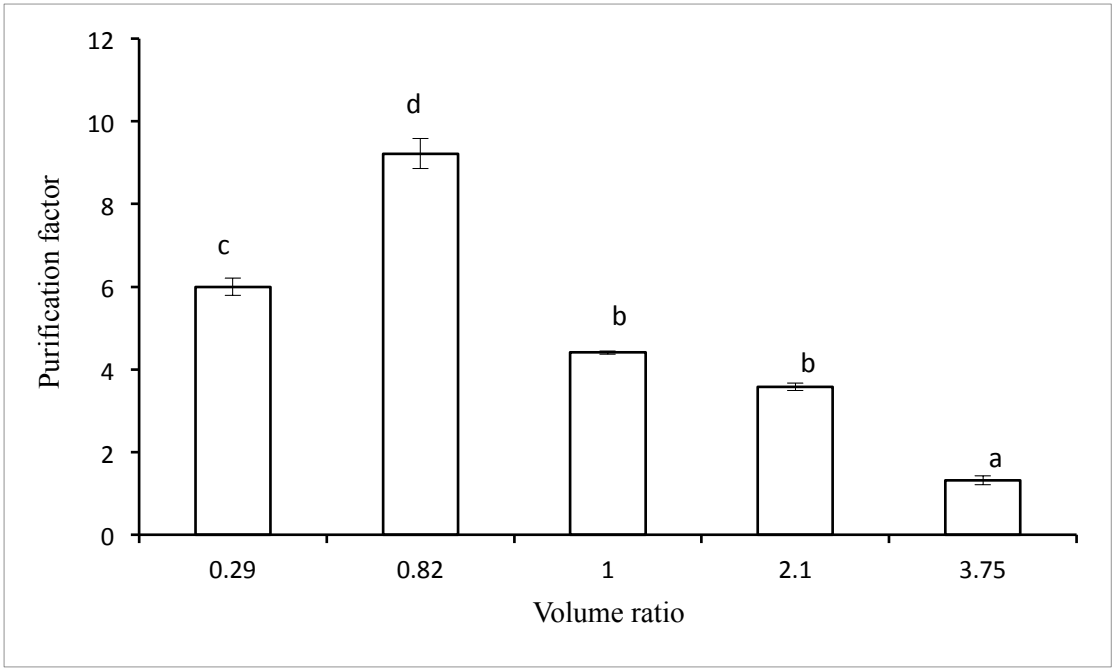

Figure 4-9: Influence of V_R on bottom phase P_{FT} of LiP. The V_R from 0.29 to 3.75 are shown

Note: purification factors with different letter(s) were significantly different by Turkey's HSD ($p<0.05$).

Basically, the partitioning behavior of protein will not change by altering the volume ratio as, the relative partitioning of each protein is constant (Ashipala and He, 2008). However, the result indicated that among the 5 different volume ratios tested (0.29, 0.82, 1, 2.1 and 3.75), the highest purity of LiP was observed at V_R of 0.82, with P_{FT} of 9.22± 1.07, and 22.37±2.6 U/ml LiP activity. This result well indicated that volume ratio 0.82 provided enough space for enzyme partitioning due to the fine balance between top and bottom phase volume ratio (Show *et al*., 2012).

Moreover, this might be due to the reduction of free volume in top phase which resulted in more protein partitioned toward salt-rich bottom phase. Besides, the contamination will concentrate more at top phase and decreased the protein purity (Benavides and Rito-Palomares, 2004). Therefore, as the concentration of contamination in top phase increased, the lignin peroxidase enzyme tends to partition into the salt-rich bottom phase. However, low P_{FT} was observed at V_R 0.29. In low volume ratio, the bottom phase density is higher and this is not a favorable condition for enzymes and hence protein will shift to intermediate phase. In high volume ratio of 3.75, the lowest purification factor of 1.32±0.33 was obtained due to low free volume in bottom phase. Therefore, the ATPS with volume ratio of 0.82 with notably high purification factor in bottom phase was selected for future study.

Furthermore, according to Ooi *et al*., (2009) the low purification factor would happened in low volume ratio, as there was great reduction in free volume which causes the loss of enzyme from the top phase. This also suggested that the protein had migrated to the intermediate phase or bottom phase and hence the purification factors of bottom phase in volume ratio of 0.82 are high.

4.6. Effect of pH on purification factor

The ATPS were tested at different pH ranges from pH 6 to pH 9 in order to obtain the best pH for lignin peroxidase purification. The pH in ATPS was measured according to Lin *et al*., (2012). This was achieved by mixing a different composition of potassium di-phosphate salt (KH_2PO_4) and di-potassium phosphate salt (K_2HPO_4) with an accuracy of $\pm$ 0.5 of pH. Figure 4.10 indicated that an increase in the system pH generally increased the purification fold of lignin peroxidase. However, this increase followed by a gradual drop at pH 8.0 and pH 9. 0.

Banik *et al.,* (2003) described that the changes in pH influences the ionizable group of molecules, which in return alter the surface charge of the molecule and hence its partition coefficient. Moreover, pH can be used to adjust the partition coefficient values, since the net charge of protein changes with acidity or basicity of the solution. Lignin peroxidase is an acidic enzyme with isoelectric points of 3.2-4.0 (Renganathan *et al*., 1985; Leisola *et al*., 1987). According to Abbot and Hatton, (1988), the protein with negative surface charge has more tendency to partition to the top phase while positively charge protein prefer to partition to the bottom. Therefore, when the pH of the system increased above the pI, the enzyme surface charge becomes negative and preferably migrated into polymer rich top phase whereas, positively charged protein would partition into salt-rich bottom phase (Forciniti *et al.,* 1992). Moreover, it has been reported that EOPO tends to have positive charge at pH above 7, which allows it to interact with the enzyme (Li *et al*., 2002). However, it was observed that if the pH higher than isoelectric point, the enzyme should be partition at top phase. Interestingly, at present experiment the enzyme participated at the bottom level revealing a very

important fact that ionic interactions are not responsible for partitioning of LiP toward the bottom salt-rich phase.

In order to explain this result, Flory-Huggins theory is used as a simple analytic expression for the partition coefficient. This theory demonstrated that any difference between top phase and bottom phase density would lead to uneven distribution of solute in the system. Under such a condition, solute shows more preference to the phase with high number of molecules per unit volume (density). The entropic effects on solute partitioning are larger for EOPO-salt system, when the polymer is effectively localized in the top phase, causing the density of the top phase to be lower than the salt-rich phase. Thus, a strong hydrophobic force driving the solute toward bottom polymer free phases (Johansson *et al*., 1998; Mageste *et al*., 2009). Rodriguez-Duran *et al*., (2012) reported tannase from *Aspergillus niger* also partitioned in bottom salt rich phase despite their negative surface charge (pI: 3.8) in system at pH 7. Therefore, the partitioning of tannase in ATPS is driven by entropic contribution rather than electrostatic interaction (Rodriguez-Duran *et al*., 2012).

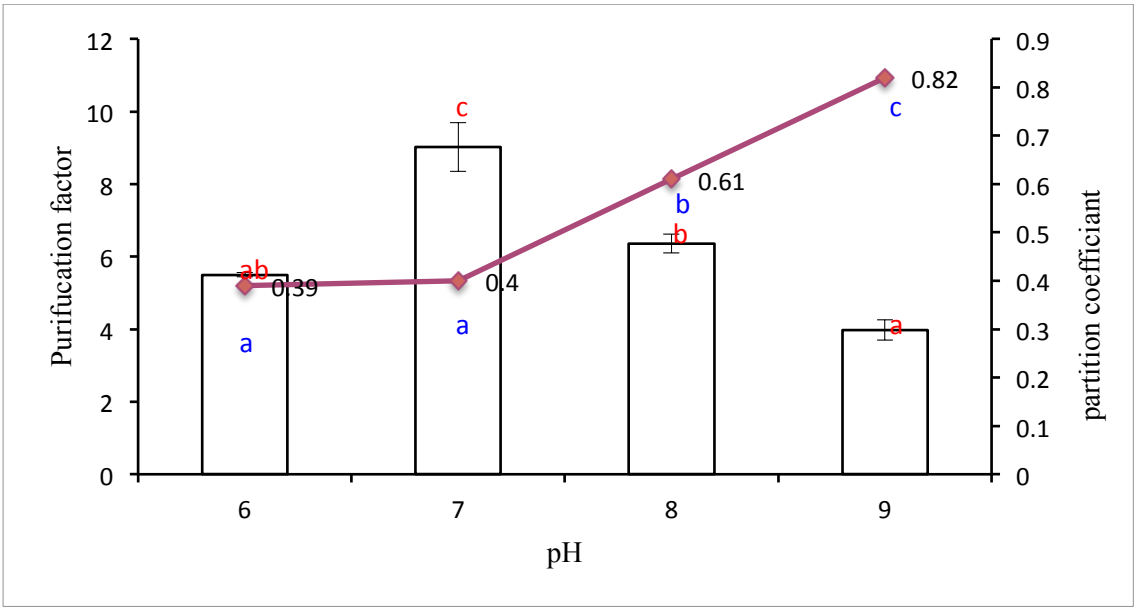

Figure 4-10: Influence of pH on bottom phase P_{FT} (■) and K_a (▲) of LiP. The pH from 6 to 9 is shown.

Note: Purification factors and partition coefficiant with different letter(s) were significantly different by Turkey's HSD ($p<0.05$).

4.7. Effect of NaCl on purification factor

The best ATPS system was used to study the effect of adding NaCl, ranging from 0% (w/w) to 4% (w/w), on purification factor of LiP. Table 4.4 showed the effect of NaCl concentration on the purification fold of LiP. It is observed that the purification fold of enzyme is almost remained constant in the range of 9.20 indicated that different concentrations of NaCl in the system does not changed the partitioning of LiP.

Table 4-4: Influence of NaCl concentration on P_{FT} on bottom phase.

NaCl concentration %(w/w)	P_{FT}
0	9.22 [(a)]
1	9.20 [(a)]
2	9.29 [(a)]
3	9.25 [(a)]
4	9.22 [(a)]

Note: Means with the same letter are not significantly different.

According to Walter *et al*., (1985), partitioning of biomolecule in two phases and subsequently the P_{FT} can be changed by addition of salt into ATPS. This was due to the unequal distribution of ion between phases which leading to an electrostatic potential difference between the phases. This phenomenon may increase the probability of changing the partition coefficient of specific protein according to their surface charge (Albertsson, 1977; Johansson *et al*., 1999). However, in this case, the results of purification factor suggested that NaCl could not promote the partitioning of LiP to the top phase of 18.80% EOPO / 7.11% PO_4 system.

Moreover, the ATPS phase diagram and phase component properties will be affected by addition of natural salt to the system, which lead to change in partitioning of target protein (Guo-qing *et al*., 2005; Abbott and Hatton.,1988). Though, the volume ratio of the system in this study did not change with variation in NaCl concentration, which indicated that the addition of NaCl have no significant influence on phase diagram. Naganagouda and Mulimani (2008) reported similar results that addition of NaCl in the PEG/phosphate system had no significant effect on α-galactosidase enzyme partitioning and purification factor.

Table 4.5: Summary of optimized parameters for ATPs

System parameters	P_{FT} (Optimum)	Yield	LiP activity U/ml
Crude	_____	_____	34.95±0.84
Molecular weight (EOPO 3900)	3.69±0.22	90.40%	27.37±0.04
Volume ratio (0.82)	9.22±1.07	80.47%	22.37±2.60
System pH (pH7.0)	9.22±2.04	80.47%	27.11±1.57

Note: The influences of ATPS parameters on partitioning of LiP were investigated. The table summarizes the optimum bottom phase LiP extraction results achieved in each parameter.

Results from this study have demonstrated that lignin peroxidase from *Pleurotus pulmonarius* can be purified using ATPS. Table 4.5 shows that the optimal parameters for LiP purification were EOPO-3900 MW, V_R of 0.82, and pH of 7.0. Thus, final bottom phase purification factor (P_{FT}) of 9.22±2.04 with recovery yield of 80.47% was obtained under such condition. The previous studies showed that another large-scale method for LiP purification involving DEAE column resulted in a purification fold of 5.63 purification fold and recovery yield of 31.86% (Yadav *et al*., 2009). Hence, ATPs method is an alternative to current conventional methods of LiP extraction.

4.8. Recycling of copolymer

The main objective of using thermoseparating copolymer is to recover the target protein in the solution free from copolymer and recycle the copolymer (Persson *et al* ., 2000). The recycling of EOPO from primary phase system was performed successfully.

Table 4.6 shows the LiP purification factor, volume ratio and total recovery of copolymer at the first extraction and primary system prepared from the recovered EOPO.

From the result, it could be seen that there is no difference between the V_R in the system prepared from fresh EOPO and the primary system prepared from recycled EOPO. This might be due to the fact that the EOPO and potassium phosphate concentration in the first extraction system were approximately the same as those in ATPS prepared from recycled EOPO (Persson *et al*., 2000). Moreover, the purification factor from the first extraction and system using the recycled EOPO.are in the range of 9.18 to 9.22.

Persson *et al*., (2010) reported recovery of more than 75% EOPO at first recycling process from the ATPS system composed of EOPO and sodium perchlorate. However, in this study the target enzyme partitioned in the bottom phase, the recycling of the copolymer has been successfully accomplished. The result showed the recovery of more than 80% of the EOPO from the ATPS system.

Table 4-5: The recovery percentage of EOPO and the P_{FT} and V_R of the LiP for the first extraction and the subsequent recycling step

	P_{FT}	V_R	EOPO Recovery (%)
First recovery	9.22 ± 1. 07	0.82	--------
First recycling	9.18 ± 0.62	0.80	81.25%

4.9. Characterization of lignin peroxidase by SDS-PAGE and native-PAGE analysis

The purity of lignin peroxidase recovered from bottom phase in ATPS was assessed by 12% SDS and native polyacrylamide gel electrophoresis analysis. As shown in Figure 4.11, the crude enzyme contained multiple bands, showing undesired proteins present in the original crude enzyme (Lane 1, Figure 4-11). The sample obtained from the bottom phase showed a band (Lane 2, Figure 4-11) both the gels carried out with SDS-PAGE and native-PAGE analysis with molecular mass of approximately 46-47 kDa. The band (lane 3, Figure 4-11) obtained from native-PAGE, was then assessed for lignin peroxidase (LiP) activity test as described by Have *et al*. (1998). The lignin peroxidase activity test of the band showed positive LiP activity of 5.35$\pm$ 1.19 U/ml. Lane 4 showed the purified LiP from the system conducted by recycled EOPO. The SDS-PAGE result showed that there was no variation in the molecular weight of LiP recovered from ATPS composed of fresh and recycled polymer.

Lignin peroxidase is a protein reported to have molecular weight in the range of 38-47 kDa (Tuisel *et al*., 1990; Asther *et al*., 1992). Vares *et al*. (1995) also reported the lignin peroxidase produced by *Phlebia radiate* consisted of two distinct bands at 45 to 46 kDa and 44kDa in SDS-PAGE. Yang *et al*., (2005) reported the purified lignin peroxidase from *Penicilluim decumbens* P6 possessed a subunit molecular weight of 46.3 kDa.

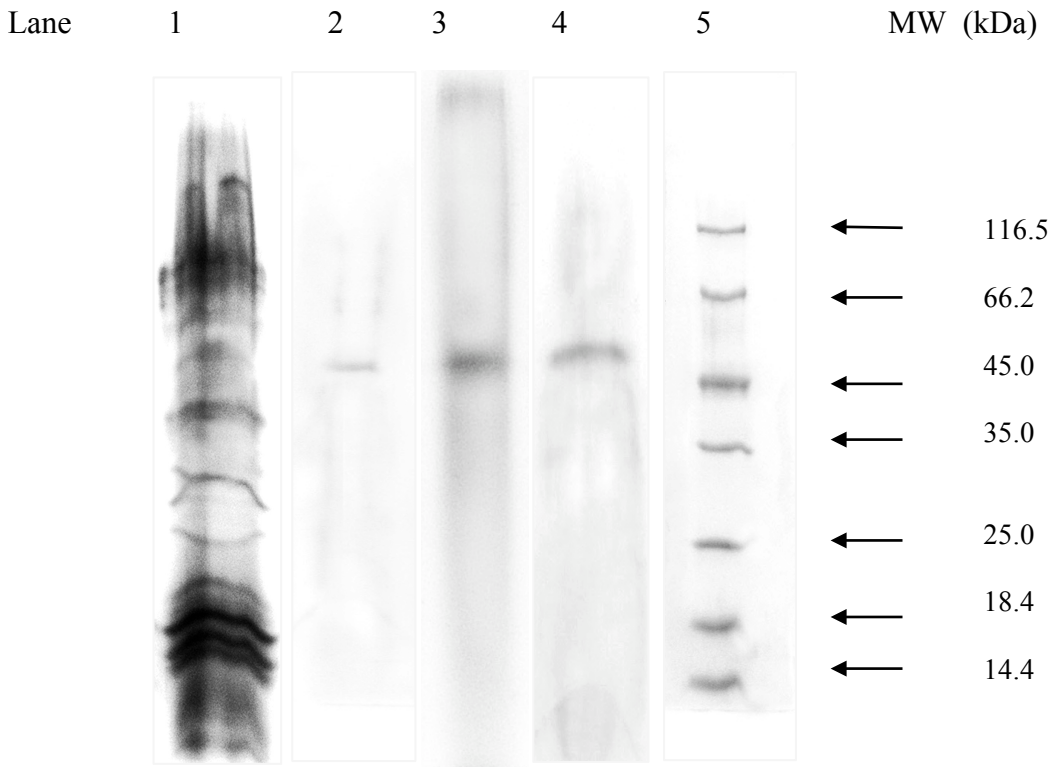

Figure 4-11: SDS-PAGE analysis on the recovery of lignin peroxidase by 12% gel. The molecular weight of standard protein marker ranged 14.4 to 116 kDa. Lane 1: crude enzyme; Lane2: Sample of first ATPS conducted by using fresh EOPO; Lane 3: native-PAGE of bottom phase sample; Lane 4: sample of recycling ATPS conducted by using recovered EOPO from first ATPS; Lane 5 standard protein marker

CHAPTER 5: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS FOR FUTURE STUDY

5.1 Conclusion

In conclusion, aqueous two-phase system was demonstrated to be a convenient purification method for lignin peroxidase from *Pleurotus pulmonarius*. After several optimizations the highest LiP activity of 95.54 ± 2.26 U/ml was observed in the liquid medium with 1% yeast, 1% glucose and 1% sawdust, 2 plugs (1x1 cm) of mycelium and with120 rpm agitation speed.

In order to purify the LiP from fermentation process, the partitioning of enzyme was investigating in the EOPO thermoseparating polymer/potassium phosphate two-phase mixture. The primary recovery of LiP was accomplished. Under optimized condition EOPO 3900/potassium phosphate system contained of 42.8 %(w/w) TLL, V_R of 0.82 at pH7.0 the desired enzyme with purification factor of 9.22 ±2.32 and yield of 80.47% was achieved in the salt-rich bottom phase. However, in the first extraction step the enzyme had partitioned in bottom phase of system. The result demonstrated that, the addition of natural salt to the system had no significant effect on partitioning of LiP.

5.2 Recommendations for future research

Lignin peroxidase has been successfully purified by using ATPSs in this study, further research works are still required to carry out in order to improve performance and its commercial applications.

1. Back-extraction step can be added to EOPO/phosphate ATPS for the recovery of lignin peroxidase from salt rich bottom phase. The high purity product able to be purified since further purification step has been proceeds.
2. As aqueous two phase system is the primary purification methods the ion exchange chromatoghrapy and fast protein liquid chromatography (FPLC) can use simultaneously for better extraction.
3. The larger volume of ATPS would allow higher amount of crude to be purified and thereby increases the efficiency. Hence, the ATPS could be further scaled-up with an aim to achieve a superior purification process of LiP.
4. The purified lignin peroxidase can then be tested for melanin decolorization since the lignin peroxidase from the *P. chrysosporium* was reported to successfully decoloured the melanin. Furthermore, the purified lignin peroxidase can be tested for biological activities such as fibrinolytic and anti-coagulant properties.

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APPENDIX A

A-1: Determination of Lignin Peroxidase Activity (Have *et al.*, 1997)

Reagent

- 100 mM sodium tartarate buffer pH 3.0
- 30mM of veratryl alcohol;
- 0.5 mM H_2O_2

Procedure for Preparation of Veratraldehyde (3, 4-Dimethoxybenzaldehyde) Standard Plot

Veratraldehyde (VAD) was used as the standard curve to determine the LiP activity. The veratraldehyde (VAD) solution containing 10- 200µg 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.

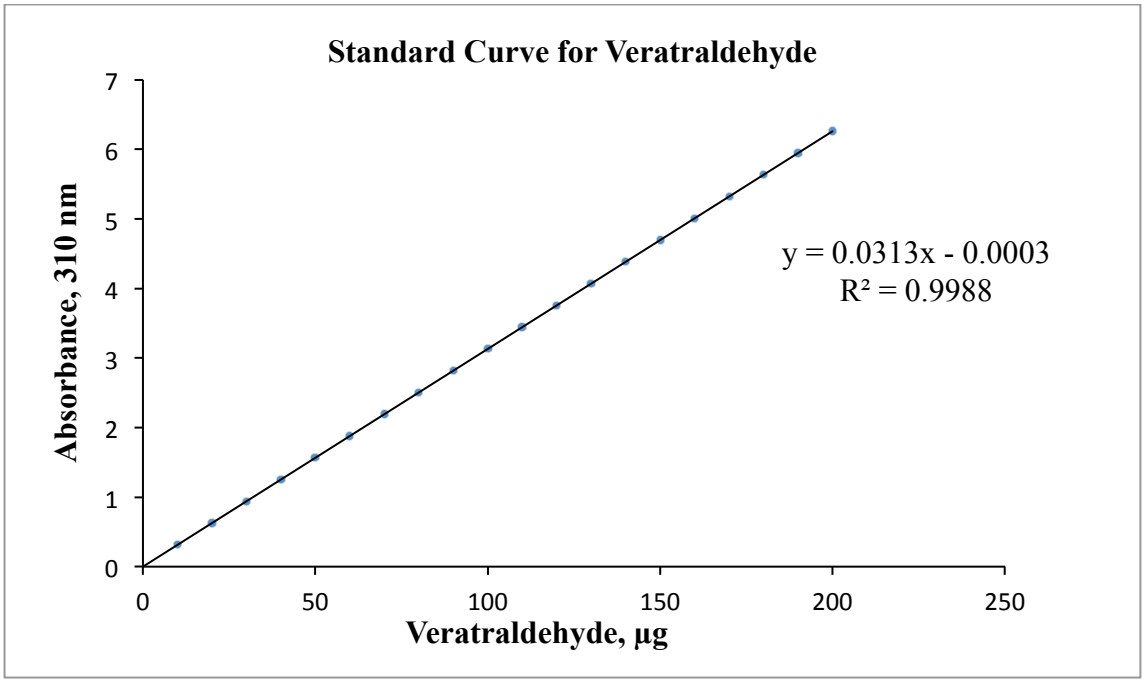

Figure A-1: Standard curve for veratraldehyde.

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

$$LiP\ activity\left(U/mL\right)=\left(\frac{final\ absorbance+0.0003}{0.0313}\right)\left(\frac{dilution\ factor}{0.2}\right)\left(\frac{1}{5\min}\right)\left(\frac{1000}{1mg}\right)\left(\frac{1\mu mole}{166.18\mu g}\right)$$

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 H_2O_2 at pH 3.0. The reaction was initiated using 0.2mL of 0.5mM H_2O_2. 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 H_2O_2, whereas enzyme blank consisted of 2.8 buffer, 0.2mL of enzyme sample.

Table A-1: 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_2O_2
Enzyme blank	2.8mL sodium tartrate buffer+ 0.2mL 0f enzyme
Reaction mixture	2.4mL sodium tartrate buffer+ 0.2mL of enzyme+ 0.2mL of 30mM veratryl alcohol+ 0.2mL of 0.5mM H_2O_2

A-2: Determination of Soluble Protein Concentration

Bradford Reagent

1. Coomassie Brilliant Blue G-250 (100mg) was dissolved in 50 mL of 95% ethanol.
2. 100 mL 85% (w/v) phosphoric acid was added to the above solution.
3. Resulting solution was made up to the final volume of 1 L.
4. The solution must be stored in a dark bottole and in a refrigerator.

Procedure for preparation of protein calibration plot (Bradford, 1976)

Bovine serum albumin solution containing 10 to 100μg/mL protein was prepared and the final volume was made up to 1 mL with distilled water in each test tube. Blank contained only 1 mL distilled water without bovine serum albumin. Coomassie briliant Blue reagent (5mL) was added to each test tube and mixed thoroughly. Absorbance was taken at 595 nm after 30 minutes and the weight of protein was plotted against the change of absorbance to generate a standard curve.

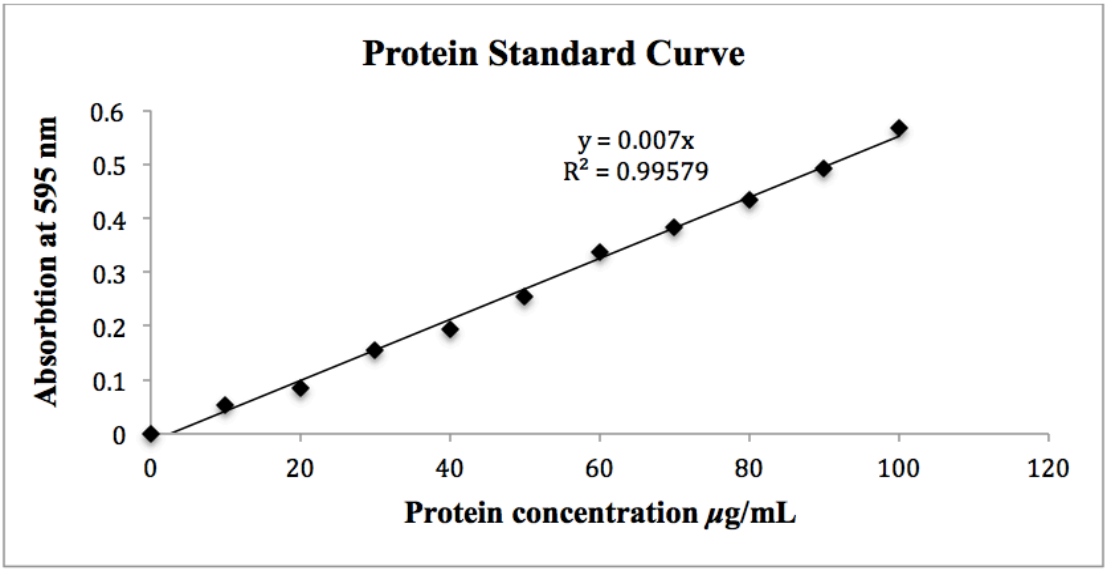

Figure A-2 Protein Standard curve

Determination of soluble protein (Bradford, 1976)

Sample (0.2mL) was mixed with 5 mL of Coomassie Briliant Blue reagent. Blank was only 0.2 mL of distilled water with 5 mL of coomassie Briliant Blue reagent. Absorbance was taken at 595 nm and amount of protein in the sample was calculated by using the protein calibration plot with the following formula:

$$\text{Soluble protein}\left(mg/mL\right) = \left(\frac{final\ absorbance}{0.007}\right)\left(\frac{1}{0.2ml}\right)\left(\frac{1mg}{1000\mu g}\right) \qquad (2)$$

BSA stock solution = 1000 μg/10 ml

A-3: Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

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

Resolving gel (12mL) for 2 gel

1. 3.60 ml 40% bis acrylamide
2. 3.00 ml resolving gel buffer
3. 0.12 ml of 10%SDS
4. 5.22 mL of dH2O
5. 50μl of APS
6. 10 μl of TEMED

Stacking gel (6 mL) for 2 gel

1. 0.75 mL 40% bis acrylamide
2. 1.50 mL Stacking gel buffer
3. 0.06 mL of 10%SDS
4. 3.59 mL of dH_2O
5. 20 μL of APS
6. 10 μL of TEMED

1.5 M Tris-HCL pH 8.8 (Resolving Gel Buffer)

1. 27.23g Tris-HCL in 80 mL of dH_2O
2. Adjust the pH to pH 8.8 by using HCL
3. Top up to 150 mL using dH_2O

0.5 M Tris-HCL pH 6.8 (Stacking Gel Buffer)

1. 6.1 Tris-HCL in 80 mL of dH_2O
2. Adjust the pH to pH 6.8 by using HCL
3. Top up to 150 mL using dH_2O

10% Ammonium Persulphate (SDS)

1. 1g of SDS in 10 mL of dH_2O

SDS-PAGE Running Buffer (Tank Buffer)

1. 3.03 g Trisbase
2. 14.4 g Glycine
3. 1 g SDS
4. Top up to 1 L using dH_2O

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

1. 0.011 g Ammonium persulfatte in 100 μL dH_2O

SDS-PAGE Coomassie Briliant Blue R-250

1. 0.1 g Coomassie Brilant Blue R-250
2. 255 mL of dH_2O
3. 255 mL of methanol
4. 50 mL glacial acetic acid

SDS-PAGE Coomassie Destaining Solution

1. 600 mL of dH_2O
2. 300 mL of methanol (30%)
3. 100 mL glacial acetic acid (10%)

APPENDIX B

SPSS Statistical Analysis

- SPSS data for media optimization

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

Descriptives

Lignin peroxidase activity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	4.6581	1.16602	.67320	1.7616	7.5547	3.32	5.42
2	3	8.3633	.39552	.22835	7.3808	9.3459	7.92	8.68
3	3	11.5567	3.87465	2.23703	1.9315	21.1818	7.15	14.43
4	3	34.9433	.83393	.48147	32.8717	37.0149	34.37	35.90
5	3	10.8567	3.57101	2.06173	1.9858	19.7276	7.53	14.63
6	3	95.6500	4.45815	2.57391	84.5753	106.7247	92.26	100.70
7	3	54.3733	2.30261	1.32942	48.6533	60.0933	52.01	56.61
Total	21	31.4859	31.85917	6.95224	16.9838	45.9880	3.32	100.70

ANOVA

LiP activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20189.824	6	3364.971	427.076	.000
Within Groups	110.307	14	7.879		
Total	20300.131	20			

Homogeneous Subsets

	Media	N	Subset for alpha = 0.05				
			1	2	3	4	5
Tukey B[a]	1	3	4.6581				
	2	3	8.3633				
	5	3	10.8567				
	3	3	11.5567				
	4	3		34.9433			
	7	3			54.3733		
	6	3				95.6500	
Duncan[a]	1	3	4.6581				
	2	3	8.3633	8.3633			
	5	3		10.8567			
	3	3		11.5567			
	4	3			34.9433		
	7	3				54.3733	
	6	3					95.6500
	Sig.		.128	.207	1.000	1.000	1.000

- SPSS data for agitation speed

For One Way Anova, the p-value <0.05, which showed that there were significant differences between the groups

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	73.3500	2.13937	1.23517	68.0355	78.6645	71.75	75.78
2	3	57.6133	2.01575	1.16379	52.6059	62.6207	55.63	59.66
3	3	33.5167	2.33415	1.34762	27.7183	39.3150	31.40	36.02
Total	9	54.8267	17.47557	5.82519	41.3938	68.2596	31.40	75.78

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2414.986	2	1207.493	257.126	.000
Within Groups	28.177	6	4.696		
Total	2443.163	8			

Homogeneous Subsets

	Agitation	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	3	3	33.5167		
	2	3		57.6133	
	1	3			73.3500
Duncan[a]	3	3	33.5167		
	2	3		57.6133	
	1	3			73.3500
	Sig.		1.000	1.000	1.000

- SPSS data for Inoculum size

For One Way Anova, the p-value <0.05, which showed that there were significant differences between the groups

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	91.4491	1.88697	1.08944	86.7616	96.1366	89.27	92.54
2	3	58.7001	4.65566	2.68795	47.1348	70.2654	54.75	63.83
3	3	47.3800	5.24002	3.02533	34.3631	60.3969	42.31	52.78
Total	9	65.8431	20.14981	6.71660	50.3546	81.3316	42.31	92.54

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3142.732	2	1571.366	89.462	.000
Within Groups	105.387	6	17.565		
Total	3248.120	8			

Homogeneous Subsets

	Inoculum	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	3	3	47.3800		
	2	3		58.7001	
	1	3			91.4491
Duncan[a]	3	3	47.3800		
	2	3		58.7001	
	1	3			91.4491
	Sig.		1.000	1.000	1.000

- SPSS data for volume ratio

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

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	6.0033	.61501	.35507	4.4756	7.5311	5.39	6.62
2	3	9.2233	1.06613	.61553	6.5749	11.8717	8.28	10.38
3	3	4.4133	.25502	.14723	3.7798	5.0468	4.16	4.67
4	3	3.5733	.13051	.07535	3.2491	3.8975	3.45	3.71
5	3	1.3200	.33045	.19079	.4991	2.1409	1.00	1.66
Total	15	4.9067	2.77202	.71573	3.3716	6.4418	1.00	10.38

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	104.165	4	26.041	76.316	.000
Within Groups	3.412	10	.341		
Total	107.577	14			

Homogeneous Subsets

	Volumeratio	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey B[a]	5	3	1.3200			
	4	3		3.5733		
	3	3		4.4133		
	1	3			6.0033	
	2	3				9.2233
Duncan[a]	5	3	1.3200			
	4	3		3.5733		
	3	3		4.4133		
	1	3			6.0033	
	2	3				9.2233
	Sig.		1.000	.109	1.000	1.000

- SPSS data for natural salt (NaCl)

For One Way Anova, the p-values was more than 0.05, which showed that there was no significant differences between the groups.

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	9.2233	1.06613	.61553	6.5749	11.8717	8.28	10.38
2	3	9.2033	.91183	.52645	6.9382	11.4684	8.26	10.08
3	3	9.2933	.62268	.35951	7.7465	10.8402	8.64	9.88
4	3	9.2467	.67892	.39198	7.5601	10.9332	8.64	9.98
5	3	9.2233	.30827	.17798	8.4575	9.9891	8.98	9.57
Total	15	9.2380	.64575	.16673	8.8804	9.5956	8.26	10.38

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.014	4	.004	.006	1.000
Within Groups	5.824	10	.582		
Total	5.838	14			

Homogeneous Subset

	Naturalsalt	N	Subset for alpha = 0.05
			1
Tukey B[a]	2	3	9.2033
	5	3	9.2233
	1	3	9.2233
	4	3	9.2467
	3	3	9.2933
Duncan[a]	2	3	9.2033
	5	3	9.2233
	1	3	9.2233
	4	3	9.2467
	3	3	9.2933
	Sig.		.896

-

- SPSS data for different pH

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

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	5.7000	.17349	.10017	5.2690	6.1310	5.51	5.85
2	3	9.0267	2.04216	1.17904	3.9537	14.0997	6.82	10.85
3	3	6.3600	.78886	.45545	4.4004	8.3196	5.67	7.22
4	3	3.9867	.85582	.49411	1.8607	6.1126	3.22	4.91
Total	12	6.2683	2.14366	.61882	4.9063	7.6303	3.22	10.85

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.437	3	13.146	9.465	.005
Within Groups	11.111	8	1.389		
Total	50.548	11			

Homogeneous Subset

	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	4	3	3.9867		
	1	3	5.7000		
	3	3	6.3600		
	2	3		9.0267	
Duncan[a]	4	3	3.9867		
	1	3	5.7000	5.7000	
	3	3		6.3600	
	2	3			9.0267
	Sig.		.113	.512	1.000