RECOVERY OF LIGNIN PEROXIDASE FROM *AMAURODERMA RUGOSUM* USING AQUEOUS TWO-PHASE SYSTEM

ZEINABSADAT MASOUDIAN

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

RECOVERY OF LIGNIN PEROXIDASE FROM *AMAURODERMA RUGOSUM* USING AQUEOUS TWO-PHASE SYSTEM

ZEINABSADAT MASOUDIAN

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

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

2014

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **ZEINABSADAT MASOUDIAN**

I/C/Passport No: **H95658571**
Registration/Matric No: **SGF110013**
Name of Degree: **Master of Biotechnology**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

"RECOVERY OF LIGNIN PEROXIDASE FROM *AMAURODERMA RUGOSUM* USING AQUEOUS TWO-PHASE SYSTEM"

Field of Study: **INDUSTRIAL AND ENVIRONMENTAL**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date

Subscribed and solemnly declared before,

Witness's Signature Date

Name: **PROF. DR. LING TAU CHUAN**
Designation:

Witness's Signature Date

Name: **DR. TAN YEE SHIN**
Designation:

DISCLAIMER

This Dissertation is prepared here in fulfilment of the requirement for the Master of Biotechnology by the University of Malaya. The Department of Biology, Faculty of Science and University of Malaya makes no responsible for any claim from the third party with regards to the production of this Dissertation.

2014

MOTHER, FATHER, & FAMILY

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude and appreciation to my project supervisor, Professor Dr. Ling Tau Chuan for giving me a chance to do this project and for providing his kind guidance to accomplish this project. I gratefully acknowledge the advice and guidance of my co-supervisor Dr. Tan Yee Shin who has thought me both consciously and unconsciously, how good experiment can be done. I appreciate all her supports, helps, ideas and providing motivation to me to fulfil this project.

I gratefully thank Professor Dr. Vikineswary Sabaratnam for providing the lab facilities. Many thanks go to Madam Chang May Heng and Mr Karim for their technical assistance towards the project. Special thanks also go to my laboratory mates and friends; Linda Jong, Nooshin Moheb Ali and all members of Mycology and Plant Pathology Lab for their kind contribution of supports, ideas and cooperation. My thanks also go to the Dr. Show Pau Loke for his advices and guidance in the ATPS field from the very early stage of this project.

Last but not the least I owe more than thanks to my family members, my parents and my brothers who have enlightened my way through their encouragement, support and blessing to surpass this degree.

ABSTRACT

A very important challenge in industrial biotechnology is development of an efficient downstream method for enzyme purification and separation due to the growing market of enzyme in biotechnology. Within the scope of this study, aqueous two-phase system based on imidazolium ionic liquid as an efficient downstream processing method was evaluated for primary purification of lignin peroxidase. The white-rot fungi *Amauroderma rugosum* is capable to produce lignin peroxidase as one of the ligninolytic enzymes. Lignin peroxidase (LiP) is an extracellular hem protein enzyme which is able to degrade lignin or lignin related aromatic compounds. The fungus cultivation in liquid media comprised of potato dextrose broth, 0.5% yeast and 1% sawdust gave the highest lignin peroxidase activity of 39.03±3.33 U/ml. The lignin peroxidase was then extracted by partitioning in aqueous two-phase system based on ionic liquid (IL-based ATPS) composed of 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$) and di-potassium phosphate salt (K_2HPO_4). The effect of tie-line length (TLL), volume ratio (V_R), pH and addition of more K_2HPO_4 on partitioning of LiP were studied. Experimental results showed that LiP was best partitioned in the ionic liquid rich top-phase with activity of 25.58±0.63U/ml, purification factor of 10.76±1.61 and 91.80%±0.30 of yield in the system with tie-line length (TLL) of 87.57 % (w/w), volume ratio (V_R) at 1.17, pH 8 and with addition of more K_2HPO_4 up to 5.85g. It was indicated that hydrophobic interaction, electrostatic interaction and salting out effect were the driving force for partitioning of LiP into top phase. Importantly the enzyme could retain its activity in presence of ionic liquid. Thus the IL-based ATPS is expected to render a suitable method for bio-product separation.

ABSTRAK

Pembentukan kaedah pemprosesan hiliran bagi purifikasi dan pemisahan enzim merupakan cabaran yang penting dalam industri bioteknologi selaras dengan pembangunan dalam bidang enzim. Selaras dengam skop kajian ini, *'aqueous two-phase*' sistem yang berasaskan '*imidazolium ionic liquid'* yang juga merupakan kaedah pemprosesan hiliran yang berkesan telah dinilai bagi mengkaji keberkesanannya dalam penulenan utama enzim *'lignin peroxidase'*. *Amauroderma rugosum* merupakan kulat pereput putih mempunyai keupayaan untuk menghasilkan *'lignin peroxidase*' sebagai salah satu enzim lignolitic. *'Lignin peroxidase*' (LiP) juga merupakan hem protein enzim luar sel dan berkemampuan untuk mendegradasi lignin atau bahan-bahan aromatic yang berkaitan dengan lignin. Pemupukan fungus dalam media cecair yang terdiri daripada '*potato dextrose broth'*, 0.5% yis dan 1% habuk kayu memberi aktiviti '*lignin peroxidase'* yang tertinggi sebanyak 39.03 ±3.33 U/ml. *'lignin peroxidase*' ini kemudiannya diekstrak melalui sistem '*aqueous two-phase'* yang berdasarkan cecair ionic (IL-based ATPS) yang terdiri daripada '*1-butyl-3-methylimidzolium chloride'* ([C_4mim]Cl) dan garam '*di-potassium phosphate'* (K_2HPO_4). Kesan '*tie-line length'* (TLL), nisbah jumlah (V_R), pH, dan penambahan K_2HPO_4 dalam proses purifikasi LiP telah dikaji. Keputusan eksperimen menunjukkan pemetakan terbaik LiP berlaku dalam fasa teratas yang kaya dengan cecair ionic dengan enzim aktiviti 25.58±0.63U/ml, factor purifikasi 10.76±1.61, hasil 91.80%±0.30 dalam sistem dengan '*tie-line length'* (TLL) sebanyak 87.57 % (w/w), nisbah jumlah(V_R) sebanyak 1.17, pH 8 dengan penambahan lebih K_2HPO_4 sebanyak 5.85g. Ini menunjukkan interaksi hydrophobic, interaksi elektrostatik, kesan '*salting out'* merupakan antara faktor yang mendorong pemetakan LiP ke fasa teratas. Secara keutamaan, enzim dapat mengekalkan aktivitinya dalam kehadiran cecair ionic. Secara keseluruhan, ATPs yang berasaskan IL telah dijangkakan menjadi kaedah yang sesuai bagi pemisahan produk.

TABLE OF CONTENTS

List of Figures

List of Tables

List of Appendix

Abbreviations

WRF	White-rot fungi
LiP	Lignin peroxidase
LMEs	Lignin-modifying enzymes
MnP	Manganese peroxidase
PDA	Potato dextrose agar
TLL	Tie-line length
V_R	Volume ratio
ATPS	Aqueous two-phase system
IL-based ATPS	Aqueous two-phase system based on ionic liquid
K_2HPO_4	Di-potassium hydrogen phosphate
KH_2PO_4	Potassium di-hydrogen phosphate
pI	Isoelectric point
K	Partition coefficient
$[C_4mim]Cl$	1-butyl-3-methylimidazolium chloride
PEG	Polyethylene glycol
EOPO	Ethylene oxide-propylene oxide
P_F	Purification factor
P_{FT}	Purification factor of top phase
H_2O_2	Hydrogen peroxide
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
U/ml	Unit/millimetre
U/mg	Unit/milligram
(w/w)	Weight per weight
Mw	Molecular weight

CHAPTER 1: INTRODUCTION

1.1. Introduction

Industrial biotechnology products which are described as products based on fermentation, enzymatic conversion and bio-based feedstock have gained increasing interest in recent years. Industrial biotechnology products represent almost high percentage of total chemicals market (Table 1.1).

Table 1.1: Segments in industrial biotechnology and their sales rate

Segment	Bio-dependent sales 2010 € billions	Assumptions	2005 basis / 2005-2010 growth
Biofuels	42	• Continuation of current strong growth in biofuels	
Plant extracts	23	• Moderate growth in hydrocolloids, strong growth in botanicals	
Pharmaceutical ingredients	20	• Increasing use of biocatalysis and higher share of biologics	
Bulk/polymers	15	• Moderate penetration of new biopolymers and biobased bulk chemicals	
Food/feed ingredients	11	• Market growth; process conversion; new biobased ingredients	
Oleochemicals	8	• Increase share of oleo vs, synthetic surfacants but decreasing prices	
Enzymes	4	• Growth with costumer markets; new applications	
Others	2	• Moderate growth in new products/services	
Total Bioproducts	125		

Source: McKinsey & Co (2008), cited in Dreyer (2008)

As it is shown in Table 1.1 enzyme is one of the growing sectors in biotechnology which has found disparate broad applications ranging from the pharmaceutical, petrochemical, food and paper. Lignin-modifying enzymes including lignin peroxidase (LiP), laccase and manganese peroxidase (MnP) are three oxidative enzymes which are commonly found extracellular in white-rot fungi. LiP has received extensive attention since it is able to degrade lignin or lignin related aromatic compounds. This capability has made LiP very useful in variety of industrial

applications such as bio-pulping, bio-bleaching and bioremediation (Roushdy *et al*., 2011).

White-rot fungi (WRF) are able to extensively degrade lignin within secreting the lignin-modifying enzymes. WRF are mostly belonging to Basidiomycota although it has been reported the ability of xylariaceae genus belongs to ascomycota in white-rot decay (Pointing, 2001). *Amauroderma rugosum* (Blume and T. Ness) Torrend belongs to order Polyporales and family Ganodermataceae was first described by Blume and Nees on 1920. It is believed that *Amauroderma rugosum* which is an indigenous species has medicinal value to treat epileptic fit which is still under discovered. Thus, the isolation of lignin peroxidase from *Amauroderma rugosum* will improve inadequate research study and discover more valuable ability in this indigenous species.

Purification and extraction of desired bio-products from the mixture of biological materials is one of the noteworthy parts of any fermentation. Typically, any bio-product often requires costly multistep processes to be purified before releasing into industrial application. These processes usually account for 30-80% of the final production cost (Ghosh 2003; Pietruszta *et al*., 2000). Despite the various application areas of enzymes, there are some drawbacks such as unavailability of low cost enzyme along with lack of suitable purification method to purify the interesting enzymes (Jing *et al*., 2007; Jing, 2010). Therefore, it is desired to propose a suitable separation and purification technique.

Conventional purification methods such as ammonium sulphate precipitation, electrophoresis, ionic and affinity chromatography have been widely used for purification of the desired bio-product. However, these methods are not considering as a favourite choice since they involve several steps, long processing time and usually represent a low yield of purification. Furthermore, using organic solvent in conventional

purification method increases the possibility of bio-molecule denaturation (Martinez-Aragón *et al*., 2009).

Due to capability of addressing challenges of conventional methods, aqueous two-phase system (ATPS) has been recognized as a suitable purification method. ATPS is based on two-phase formation due to incompatibility of two polymers or one polymers and one salt. This method has demonstrated a potential for separation of bio-products due to its simplicity, proper selectivity, high yield, low cost, short process time and relative reliability in scaling up (Daugulis *et al*., 1994; Ventura *et al*., 2009). Moreover, ATPS presents a gentle environment condition as both of phases comprise water (Baskir *et al*., 1989). However, some limitations such as poor understanding of partitioning mechanisms of bio-product between two phases and inability to predict the process outcome have impeded the ATPS widespread uses in industrial biotechnology (Benavides & Rito-Palomares, 2008).

More recently room temperature ionic liquids (ILs) have come under a harsh spotlight in the field of purification and extraction process. ILs offer special and unique properties such as chemical stability, negligible vapour pressure and non-flammability. These properties have made ILs a promising alternative media in different application processes (Earle & Seddon, 2000; Olivier-Bourbigou & Magna, 2002; Plechkova & Seddon, 2008). As pioneering work formation of aqueous two-phase system using ionic liquids (IL-based ATPS) was reported by Gutowski and his co-workers (2003). Later several researchers applied this method in analytical and separation processes (Pandey, 2006; Pei *et al*., 2011) and to investigate the mechanisms of IL-based ATP partitioning model (Dreyer & Kragl, 2008; Freire *et al*., 2009; Freire *et al*., 2010). The IL-based ATPS provides many advantages such as for instance rapid phase separation and high extraction efficiency in addition to sharing the advantages of conventional ATPS. It has been reported that ionic liquid 1-butyl-3-methylimidazolium chloride is able to form

two-phase system with the addition of di-potassium hydrogen phosphate salt (K_2HPO_4) (Ventura *et al*., 2011). Therefore, in this study, the applicability of IL-based ATPS using [C_4mim]Cl and K_2HPO_4 was investigated for primary purification of lignin peroxidase (LiP) from *Amauroderma rugosum* as well as understanding the partitioning behaviour of LiP between two phases.

1.2. Objectives

Objectives of the study were:

a) to characterise the lignin peroxidase from *Amauroderma rugosum*.

b) to investigate the application of an aqueous two-phase system based on ionic liquid (IL-ATPS) for recovery of lignin peroxidase.

CHAPTER 2: LITERATURE REVIEW

2.1. White-rot fungi

White-rot fungi (WRF) are physiological grouping of fungi with the exceptional ability to act as the natural lignin destroyer. Lignin is a complex three dimensional polymer that conveys protection and rigid structural to wood (Deacon, 1997). The name of white-rot fungi comes from the bleached appearance of wood due to removal of lignin by these fungi. WRF are mostly belonging to the Basidiomycota as one of the major divisions of fungi. However, the ability of Xylariaceae belongs to Ascomycota has been reported in white-rot decay (Pointing, 2001).

The interest in WRF arises from their ability to overcome the limitation of lignin degradation by producing one or more lignin modifying enzymes (LMEs). Degradation of lignin is considered as a limited step in carbon transmission in lignocellulosic environment. Interestingly WRF have nonspecific mechanisms of degradation of both D and L form of carbon in lignin.

Three lignin modifying enzymes (LMEs) commonly found in white-rot fungi are (1) manganese peroxidases (MnP), E.C. 1.11.1.13; (2) lignin peroxidases (LiP), E.C. 1.11.1.14, and (3) laccases (Lac), E.C. 1.10.3.2. These enzymes are indispensable for lignin degradation (Becker & Sinitsyn, 1993; Hatakka, 1994). However, in mineralisation of lignin, addition of some other enzymes including auxiliary enzymes, glucose oxidase, cellobiose dehydrogenase, and aryl alcohol oxidase is often required. The auxiliary enzymes which are unable to degrade lignin by themselves involved in hydrogen peroxidase (H_2O_2) production which is a co-substrate of LiP and MnP. The glucose oxidase, cellobiose dehydrogenase and aryl alcohol oxidase are required for linking the cellulose and hemicellulose degradation in nature with ligninolysis (Leonowicz *et al*., 1999).

With respect to the enzyme and enzyme associated process of WRF, they have been demonstrated as an attractive potential microorganism to degrade extremely diverse range of pollutants with the same mechanisms as lignin degradation (Barr & Aust, 1994). Degradation and mineralization of some pollutants such as for instance polycyclic aromatic hydrocarbon (PAHs) (Aust, 1990; Field *et al*., 1992; Acevedo *et al*., 2011), chlorophenols (Aust, 1990), nitrotoluenes (Valli *et al*., 1992), dyes (Chao & lee, 1994) and polychlorinated biphenyls (PCBs) (Boyle *et al*., 1992; Thomas *et al*., 1992; Yadav *et al*., 1995) have been reported.

2.2. *Amauroderma rugosum* (Blume and T. Nees) Torrend

Figure 2.1: The picture of *Amauroderma rugosum*

Genus *Amauroderma* fungi belongs to Polyporales order which are of particular interest since they comprise a large number of wood-decaying fungi. However, it has been reported the pathogenicity property of some Polyprales to timber such as *Antrodia vaillanti* which can cause the damage to timber (Ryvarden, 1991). Polyporales is divided into five families, i.e. Polyporaceae, Hymenochaetaceae, Ganodermataceae, Bondarzewiaceae, and Fistulinaceae (Donke, 1965). *Amauroderma*

rugosum (Figure 2.1) is belongs to Ganodermataceae family. It was first described by Blume and Nees (1920). They discussed the growing properties of *Amauroderma rugosum* in lowland and the distribution of this species in tropic. *Amauroderma rugosum* has tendency to grow in dark and usually grows well in humus soil attached to buried roots in hardwood forest. It has a round to round pileus and ravous to black in colour with white to pale brown context (Bi *et al*., 1993).

2.3. Lignin

Lignin is an aromatic chemical compound with high biological stability due to C-C and C-O-C linkages (Sivakami *et al*., 2012). 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).

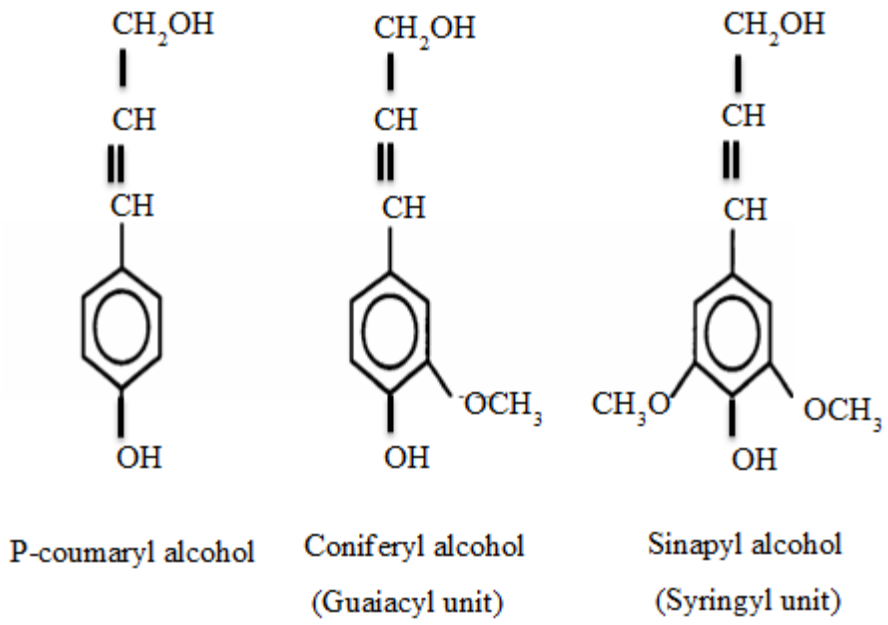

Figure 2.2**:** Structure of three phenyl propanoid precursors
Source: Ahammed, 2002

Lignin has a critical role in carbon cycle since the most renewable carbon source can be found either in lignin or in cellulose and hemicellulose (Guerra *et al*., 2004). Lignin localizes in cell wall and confers the rigidity and stability of woody plants (Crawford, 1981; Fengel & Wegener, 1983). Moreover, Lignin acts as structural impediment of wood against easily degradation of cellulose and against penetration of cell wall by distinctive enzymes (Sarkanen *et al*., 1971).

Dramatically increasing of agricultural products will inevitably end up with generating the huge amount of lignicellulosic residue. Considering environmental damage from coal burning, using lignin as an energy sources has become a very important issue in biotechnology (Roushdy *et al*., 2011). This issue has raised the considerable research efforts in order to use the lignocellulosic residue as a clean energy source. Akpan *et al*. (2004) suggested the potential use of lignicellulosic residue as enzyme substrate which can result in reduction of production cost. Naturally, the possibility of utilizing lignin compound can only be done by a few groups of microorganisms especially by whit-rot fungi.

2.4. Lignin peroxidase

2.4.1. Features of lignin peroxidase

Lignin peroxidase [EC 1.11.1.14] is an extracellular hem protein enzyme in group of oxidoreductases (Higuchi, 2004; Guillén *et al*., 2005; Hammel & Cullen, 2008). It is able to degrade lignin or lignin related aromatic compounds and oxidize a variety of reducing substrates. These features have aroused the interest of lignin peroxidase in biotechnology (Oyadomari *et al.*, 2003).

Lignin peroxidase (LiP) is an acidic enzyme with optimum activity and stability at pH 3.5 (Tien *et al*., 1986). LiP is a glycoprotein with isoelectric point of pI = 3.2-4.0 and molecular weight of 41 kDa (Kirk & Farrell, 1987) and is relatively unspecific in

reducing substrates. Therefore, it has large substrate range and is able to react with related and even unrelated lignin model compounds. Accordingly, widespread application of LiP in various industrial fields pertains to its large substrate range along with its high redox potential (Erden *et al*., 2009). Biodegradation of lignin, antagonism, and defence of fungi against antagonistic attack are some biological functions of lignin peroxidase (Score *et al*., 1997; Piontek *et al*., 2001; Trejo-Hernandez *et al*., 2001).

2.4.2. Industrial production of lignin peroxidase

White-rot fungi are able to produce lignin peroxidase in natural condition. Restrained accessibility of essential nutrients results in producing the enzymes that cleave the irregular structure of lignin and hence increase the availability of required nutrition for fungi (Hudson, 1992).

Lignin peroxidase (LiP) was first described in Basidiomycete *Phanerochaete chrysosporium* (Glenn *et al*., 1983; Tien & Kirk, 1988). Since then the presence of LiP in several others species of Basidiomycetes (Buswell *et al*., 1987; Kirk & Farrell, 1987; Pointing *et al*., 2005) and in Actinomycetes (Périé & Gold, 1991; Périé *et al*., 1996; Niladevi & Prema, 2005) was recorded. It is also reported that the bacterium *Streptomyces viridosporus* is able to produce isoenzymes of LiP, however, the LiP production in *S. viridosporus* is related to growth (primary metabolism) while its production is related to secondary metabolisms in white-rot fungi (Mester *et al*., 1996).

2.4.3. Application of lignin peroxidase

Ligninolytic enzymes produced by white-rot fungi have an increasing demand in a variety of processes. As it was mentioned above LiP as one of the ligninolytic enzymes has potential ability to degrade lignin, lignin related compounds and even unrelated molecules. This ability has generated the interest of LiP in large number of industrial applications with economic and environmental importance. Its potential

application in a variety of industrial fields such as food, pulp and paper, textile, cosmetic industrial sectors and bioremediation has been reported. These applications are summarized in Table 2.1.

Table 2.1: Lignin peroxidase application in different sectors

Sectors	Lignin Peroxidase Applications	References
Food Industry	• Source of natural aromatics • Production of vanillin	Lesage-Meessen *et al*., 1996 ;Lomascolo *et al*., 1999; Barbosa *et al*., 2008
Pulp and paper Industry	• Decolouriment of kraft Pulp • Mill effluents	Ferrer *et al*., 1991; Bajpai, 2004; Sigoillot *et al*., 2005
Textile Industry	• Textile dye degradation and bleaching	McKay, 1979; Cripps *et al*., 1990; Cooper, 1993; Riu *et al*., 1998; Wong & Yu, 1999; Abadulla *et al*., 2000; Pointing, 2001; Kasinath *et al*., 2003; Blánquez *et al*., 2004; Hou *et al*., 2004; Shin, 2004; Champagne & Ramsay, 2005; Rodríguez *et al*., 2005; Rodríguez &Toca, 2006; Kunamneni *et al*., 2008; Robles-Hernández *et al*., 2008; Gomes *et al*., 2009
Bioremediation	• Degradation of azo,heterocyclic,reactive and polymeric dyes, • Mineralization of environmental contaminants • Xenobiotic and pesticides degradation	Bumpus & 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 Applications	• Functional compounds synthesis • Cosmetics and dermatological for skin • Bio-electro-catalytic activity at atomic resolution	Christenson *et al*., 2004; Higuchi, 2004; Belinky *et al*., 2005; Barbosa *et al*., 2008

Source: Modified from Maciel *et al*., 2010

2.5. Protein purification

With regard to the conducting the most of biotechnology process in aqueous media, the desire bio-product is present in aqueous solution. Hence, the bio-product

needs to be concentrated, isolated and purified from other undesired compounds such as by-products, proteins and cells of the batch. Generally, a protein purification process involves four different sections which are named based on the main objective of each step: (1) recovery, (2) concentration, (3) purification and (4) product formulation. In each step physical and molecular characteristic of compounds are considered to separate the target bio-product from other mixtures. Figure 2.2, illustrates a schematic of purification process. As it is demonstrated in Figure 2.2 each steps consist of other defined steps in order to achieve the required purity of bio-product.

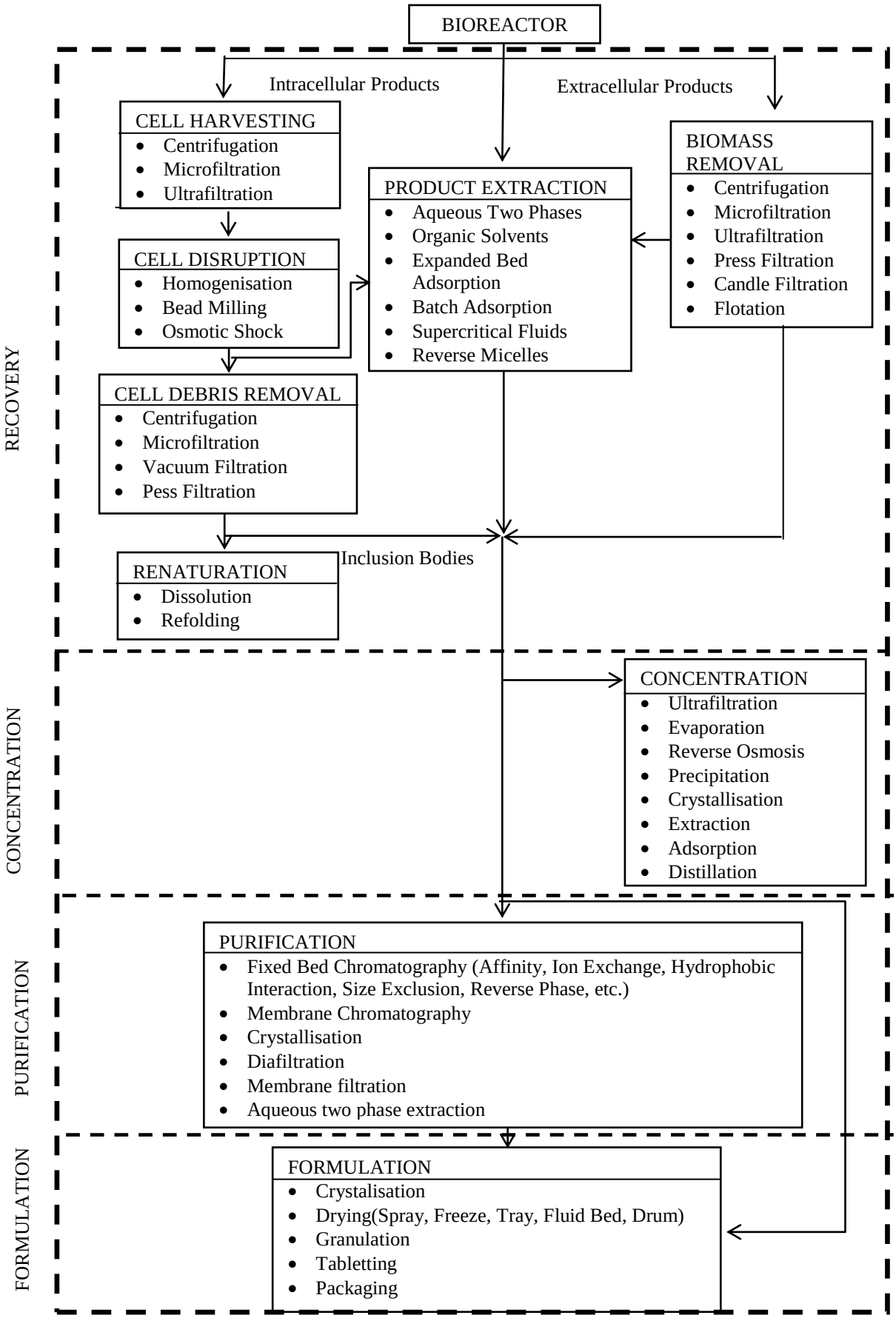

Figure 2.3: Schematic illustration of protein downstream processing

Source: Nfor *et al*., 2008

1. Recovery: separating of protein in denatured form from non-protein components is the first step of recovery. As shown in Figure 2.3 depending on the localization of target protein, well-defined steps have to be applied. Both physical methods (e. g. centrifugation, filtration) and the direct extraction of the target can be placed in this step. Using the direct extraction may result in incomplete purification of the product depending on the selectivity of the technique performed. However, in the case of presenting of the target protein as insoluble inclusion bodies, yet another method is necessary to apply in order to solubilise the protein (Cabrita & Bottomley, 2004; Choe *et al*., 2006; Clark, 1998; Freydell *et al*., 2007).

2. Concentration: the obtained target protein from the first step is almost free from particulate substrates. Based on the achieved concentration from the recovery step, more concentration methods such as ultrafiltration or extraction will be required. As long as obtained protein concentration is adequate, the solution can directly be processed in the purification step.

3. Purification: during purification step the target bio-molecule will be purified following by elimination of major and trace impurities. Typically, in purification step, chromatography procedures are applied because of their large resolving power. Nonetheless, other procedure such as aqueous two-phase system or membrane filtration is required to be used. These procedures result in greater throughputs and decrease product expenses (Przybycien *et al*., 2004; Thommes & Etzel, 2007).

4. Product formulation: the last step of protein purification process aims to obtain the protein product in the desired form. In product formulation step, some additives may also be added to enhance the stability and shelf life of the product in order to render the product in the good condition for final application (Sellers & Maa, 2005).

2.5.1. Challenges in protein purification

In all purification techniques, the level of purification has to be investigated with respect to the application of the protein product. Among all available protein products, the one which is developed for human application requires the highest purity (Headon & Walsh, 1994). It must certainly be highlighted that the purification methods ought to be developed in order to achieve the efficient economical and sufficient purity of product. There are some challenges which need to be addressed in order to achieve a commercial method (Dreyer, 2008).

a. The target product in the biological feed stock is usually very diluted.

b. Generally, there are some impurities in complex of crude feedstock which are certainly needed to be removed. At this point first the critical impurities and non-critical impurities must be distinguished. The critical impurities are needed to be completely eliminated; however, the non-critical impurities can be tolerated. As an example if the final product is an enzyme, any undesired catalysis and catalysis poisoning must be prevented by removing inhibitory elements. Additionally, presence of some impurities with similar properties to target bio-molecule may complicate the purification process.

c. Properties of crude feedstock such as molecular weight, charge, hydrophobicity, solubility, and viscosity are needed to be considered in facilitating the purification process. Unfortunately, all these mentioned properties of crude feedstock are usually poorly investigated.

d. One of the critical issues in the purification process is optimization of the best condition in which the protein product maintains its stability. Most of the protein products often require strict purity, quantity and biological activity. Protein products are sensitive to the severe pH value and are sensitive to some substrates such as surfactants and organic solvent. Therefore, the process condition optimization needs to be widely

considered. For one process or operation there might be several operating conditions among which the best condition needs to be selected.

2.6. Aqueous two-phase extraction

Liquid-liquid extraction which is also called solvent extraction is an interesting alternative method among various protein and enzyme purification methods. The mechanism is based on transferring of one compound in one phase into another phase as a result of unequal solubility in solvent. Liquid-liquid extraction result in combination of three steps of recovery, concentration and purification into a single operation (Mazzola *et al*., 2008). However, conventional liquid-liquid extraction methods consist of using organic solvent and may lead to decrease the product stability and the reliability of technique ensues (Kula *et al*., 1982). Therefore, development of an efficient separation method which can meet the requirement of product retention had aroused attention.

Aqueous two-phase system (ATPS) was first discovered in 1910 by Dutch microbiologist and then rediscovered and employed years after by Albertson (1956). Since then ATPS has been developed and widely used as a suitable separation method for extraction of cell membrane and organelles along with extraction of different kind of proteins from protein mixture (Agasoster, 1998; Gündüz 2000; Rito-Palomares, 2004; Roobol-Boza *et al*., 2004).

Generally, ATPS forms either by mixture of two incompatible polymers (polymer/polymer) (Biao *et al*., 2009) or by mixing polymer and salt (polymer/salt) (Berlo *et al*., 2000). Another two novel ATPS have been emerged which are ATPS based on using surfactant (Jiang, 2008) and ATPS based on ionic liquid/salt (Pei *et al*., 2009). The last two types have been developed in order to enhance the overall

efficiency and to increase the development of using recyclable components (Liu *et al*., 2011).

In aqueous two-phase system, mixture of phase forming compound at temperature above the cloud point results in formation of two phases. All types of aqueous two-phase system are considered to possess specific advantages over conventional purification methods. ATPS provides a large interfacial place between two phases which may lead to effective transfer of phase components. Furthermore, ATPS can minimize the protein product denaturation by giving a gentle biocompatible condition containing predominantly water (70-90%) to bio-molecule along with quick phase separation (Baskir *et al*., 1989).

2.6.1. Application of aqueous two-phase system

Generally, ATPS has been constructed for two major applications: (1) analytical application and (2) product recovery application (Dreyer, 2008)

2.6.1.1. Analytical application

The sensitivity and fast phase formation have made ATPS a promising method for analytical applications. Furthermore, it can be used for study of macromolecular and cellular structure of bio-molecules. This ability of the method can be discussed in two groups, i.e. (a) Interaction between bio-molecules and (b) studies of changes in protein surface.

a. Interaction between bio-molecules

The partitioning behaviour of bio-molecule in ATPS is affected by bio-molecule interaction due to changes of properties such as size, hydrophobicity and net surface (Middaugh & Lawson, 1980). This concept was used by Mattiasson to construct an immunoassay called ‘partition affinity ligand assay’ in ATPS (Mattiasson, 1986). The immunoassay construction was based on increase or decrease in partitioning of one of

the reactance in order to increase the concentration of another reactance. Moreover, interaction between bio-molecules can be exploited in ATPS to separate enantiomers. In the system comprising of PEG/dextran, bovine serum albumin attached selectively to L-tryptophan of the racemic mixture. Therefore, L-and D-tryptophan could be separated in ATPS (Ekberg *et al*., 1985).

b. Studies of changes in protein surface

Aqueous two phase system can be used for studying the changes of bio-molecule surface. Modification of system condition such as changing of pH and salt, resulted in investigation of bio-molecule surface charge and investigation of bio-molecule isoelectric point. Moreover, partitioning of protein in presence of hydrophobic ligand led to determination of surface hydrophobicity of protein (Dreyer, 2008).

2.6.1.2. Product recovery application

Product recovery can be considered as the main application of ATPS. The interest in product recovery using ATPS arises from versatile advantages of the system such as simplicity, reliability in scale up, providing mild biocompatible condition, high capacity, high yield and fast separation process. The usage of ATPS is not only limited to extraction of intracellular enzyme from microbial cell but also from animal tissue and plant material (Jordan & Vilter, 1991). Besides, purification of such bio-molecules which are rather difficult to purify such as DNA and amino acid were performed using ATPS (Cole 1991; Ramelmeier *et al*., 1991; Sanchez-Ferrer *et al*,. 1989; Sikdar *et al*., 1991). Another attractive application of ATPS is ‘extractive bioconversion’ which is combination of extraction and bioconversion. During the extraction bioconversion process, the product can be bio-converted in one phase and extracted in the other phase (Kuboi *et al*., 1995; Kwon *et al*., 1996; Zijlstra *et al*., 1996). Additional applications of ATPS that have been reported were: recovery of viral coat proteins from recombinant *E. coli* (Rito-Palomares & Middelberg, 2002), drowning-out crystallisation of sodium

sulphate (Taboada *et al.*, 2000), recovery of food colouring dyes from textile plant wastes (Huddleston *et al.*, 1998a) and recovery of metal ions from aqueous solutions (Rogers *et al*., 1996).

2.6.2. Limitations of aqueous two-phase system

Despite of the various advantages of ATPS, this system includes some limitations regarding the purification of the desired product. These limitations mostly arise from the poor understanding of phase equilibrium and product partitioning between two phases which restrict the exploitation of ATPS. Moreover, the limited application in ATPS process has been attribute to the required time for learning the process which is always included in the system operation and consequently retard the process commence (Benavides & Rito-Palomares, 2008). Additionally, possibility of side reaction occurrence during ATPS process and instability of some enzymes in phase forming compounds are other drawbacks of applying aqueous two phase system.

2.6.3. Different types of phase component used in ATPS

2.6.3.1. Aqueous two-phase system based of polymer/polymer

Poly ethylene glycol (PEG) and dextran are two polymers which have been widely applied in ATPS polymer/polymer type since they have stable physical and chemical structure. Moreover, these polymers do not have any toxicity toward bio-molecules. However, there are limitations of using PEG/dextran. First, dextran is an expensive polymer and cannot be a suitable selection for development of low cost purification process. Second, dextran has high viscosity (10^2-10^3 mPa·s) and its viscosity increases by increasing of concentration (Liu *et al*., 2011). For addressing these problems, researchers tried to substitute the low cost polymer with dextran. Some alternative polymers such as starch derivation (Lin *et al*., 2003), plasma substitute (Ghosh *et al*., 2004) and xanthan (Chethana *et al*., 2006) were used. Other alternative substitutes using recyclable polymer in ATPS are listed in Table 2.2. Formation of two-

phase system using recyclable polymers is controlled by physical properties of system such as temperature, pH, light and pressure. Therefore, by changing the system condition after extraction, the polymers can be recycled. Ethylene oxide-propylene oxide (EOPO) which is a thermo sensitive polymer was recycled after extraction process by changing the system condition (Show *et al*., 2012). In the study, EOPO was heated over its cloud point and the second two–phase comprising certain concentration of EOPO in bottom phase and water/lipase in top phase was formed. The concentrated EOPO in bottom phase was then recovered.

Table 2.2: Different types of polymer replaced with dextran in ATPS

Polymer	Types of polymer	Bio-molecule extracted	Polymer recycling rate (%)	References
[a]EOPO	Thermo sensitive	L-asparaginase Lipase	81.3-84.7 99	Zhu *et al*., 2007; Show *et al*., 2012
[b]HM-EOPO	Thermo sensitive	Apolipoprotein A1	----	Johansson *et al*., 1999
[c] NIPAM-VI/HM-EOPO	Thermo sensitive	BSA	53/92	Persson *et al*., 2000
[d]PNBC/PADB	pH sensitive	Lysosyme BSA	98/97	Biao *et al*., 2009
[e]PNNC/PADB	Light sensitive	BSA, L-Tyr	98/97	Wang *et al*., 2008

Source: Modified from 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]. NIPAM-VI: copolymers of 1-vinylimidazole (VI) with Nisopropylacrylamide (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, ammonium persulfate and sodium hydrogen sulfite as initiators.
[e]. PNNC: copolymer synthesized by using N-isopropyl acrylamide, N-vinyl-2 pyrrolidone, chlorophyllin sodium copper salt as monomers and2, 2' azobisisobutyronitrile as initiator.

2.6.3.2. Aqueous two-phase system based on polymer/salt

Polymer/salt system is known as a cheap type of ATPS in comparison with polymer/polymer system. PEG/phosphate is the most commonly polymer/salt system

due to the some advantages such as low viscosity (10^1~10^2 mPa·s), cost and time effectiveness, rapid phase separation and large array of applications (Rosa *et al*., 2009). The practical aqueous two-phase system using polymer/salt is divided into four main sections (Figure 2.4)

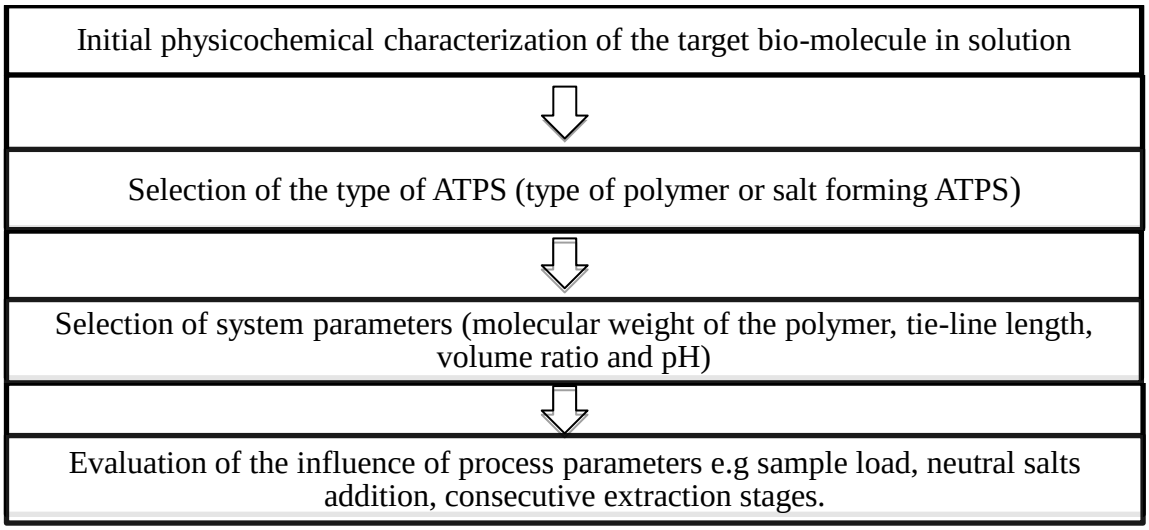

Figure 2.4: Simplified representation of practical development of ATPS based on polymer/salt

Source: Benavides & Rito-Palomares, 2008

In general, the top phase of ATPS is consisting of polymer which is enriched with target bio-molecule and the salt is concentrated at bottom phase. Theoretically, adding the suitable salt to polymer phase can be a good strategy to back extraction of the protein into salt phase and recovery of polymer (Liu *et al*., 2011). Practically, ATPS is used as a primary purification method to diminish the numerous processing streams (Dreyer, 2008). For this reason ATPS can be combined with other final purification methods such as ultrafiltration (Tanuja *et al*., 2000) and chromatography (Rojas *et al*., 2004) for complete the purification of product. However, these combinations can lead to increase the cost of purification process.

2.6.3.3. Surfactant-based aqueous two-phase system

a. Aqueous two-phase system based on single surfactant

Single surfactant above its critical point has been used to form ATPS (Tani *et al*., 1998). The single surfactants are mostly non-ionic such as polyoxyethylene alkyl ethers (PAEs) and polyoxyethylene t-octyl-p-phenyl. Aggregation of surfactant after reaching above the critical micellar concentration (CMC) forms the micellar structure. This phenomenon leads to the formation of two phases of surfactant rich phase and surfactant droplet phase. The surfactant rich phase provides the close space for hydrophobic bio-molecule and prevents denaturation of target protein due to aggregating. The target bio-molecule can be extracted in surfactant rich phase while the hydrophilic contaminants are extracted in surfactant droplet phase. Therefore, aqueous two-phase system based on single surfactant is suitable for extraction of hydrophobic bio-molecules (Xie *et al*., 2006).

b. Aqueous two-phase system based on mixed surfactants

In this type of ATPS both cationic and anionic surfactants are used to form two-phase system rather than using single non-ionic surfactant. Phase separation of mixed cationic and anion surfactant in ATPS is similar to ATPS based on single surfactant which comprises of surfactant-rich and surfactant-depleted phases (Jiang[b] *et al*., 2009). In mixed surfactants ATPS, the cationic and anionic surfactant have tendency toward precipitation if they are in excess amount. For this reason the concentration of surfactant is maintained low and the water content is high (up to 90%) in system (Lisheng, 2007). Using mixed surfactant rather than single surfactant can provide strong electrostatic interaction which can be controlled by applying ions of different salts. One difficulty of aqueous two-phase system based on mixed surfactants is that the two-phase region is too narrow to be used in bio-molecules separation. To overcome this difficulty, there are some strategies such as using short chain fatty alcohol (ethanol, n-propanol, n-butanol) and using inorganic salt (NaF, Na_2SO_4 and Na_3PO_4) to extend the two-phase region.

The other strategy is controlling physicochemical properties such as temperature or/and pH (Chen *et al*., 2003).

c. Aqueous two-phase system of surfactant/polymer

Varieties of studies have been done in order to purify the hydrophobic membrane proteins and hydrophilic proteins using non-ionic surfactant with water soluble polymer such as PEG (Sivars & Tjerneld, 2000). The phase diagram of the surfactant/polymer system is similar to polymer/polymer ATPS since the surfactant micelles are considered as second polymer. In surfactant/polymer system the membrane proteins can be partitioned into surfactant phase and the hydrophilic protein will be concentrated into polymer phase.

2.6.3.4. Aqueous two-phase system based on using ionic liquid

Ionic liquids (ILs) are novel liquid salt which comprise of large organic cations and small inorganic anions. They have a large array of unique properties such as thermal and chemical stability, negligible vapour pressure at room temperature and non-flammability. They also have adjustable chemical and physical properties by which their anions and cations can be modified based on desired reaction. With regard to lack of volatility of ILs, they have received considerable research effort since they do not release volatile organic compounds (VOSs) to atmosphere (Anonymous, 2004). All these unique features of ionic liquids have made them as an interesting alternative solvent in extraction method. The distinctive properties of ILs lead to render unlimited group of designer solvents (Candeias *et al*., 2009).

2.7. Types of aqueous two-phase system based on using ionic liquid

2.7.1. Ionic liquid-water biphasic system for extraction

The unique features of ILs have rendered them an attractive alternative solvent for liquid-liquid extraction. In particular, in ionic liquid-water biphasic system

hydrophobic ionic liquids have been substituted for volatile organic solvent. The potential formation of two-phase in the presence of ionic liquid and water (Ionic liquid-water biphasic system) was first reported by Huddleston and co-workers (1998b). They studied the partition behaviour of substituted-benzene derivation in hydrophobic ionic liquid ($[C_4mim][PF_6]$) and water. Since then, more researches have been reported for extraction of metal ions (Holbrey *et al*., 2003), amino acids (Wang *et al*., 2005) and DNA (Cheng *et al*., 2007) using ILs-water biphasic system.

With respect to the necessity of using hydrophobic ionic liquids for formation of ionic liquid-water biphasic system, however, only few researches have been reported for proteins and enzymes extraction by reason of the low solubility or insolubility of proteins in hydrophobic ILs (Fujita *et al*., 2006; Lau *et al*., 2004; Sheldon *et al*., 2002). In order to enhance the protein solubility, some approaches comprise the addition of small amount of water in ionic liquid-water biphasic system (Moniruzzaman *et al*., 2008) and modification of enzyme or protein (Ohno *et al*., 2003; Laszlo & Compton, 2001) have been reported. Nakashima *et al*. (2005) reported the enhancement of subtilisin Carlsberg solubility which is also called bacterial alkaline protease by conjugation of polyethylene glycol PM_{13} to the enzyme. Despite of successful reports of extraction of bio-molecules in IL/water biphasic system, the limitation of the system which is using only hydrophobic ionic liquids necessitated offering new and innovative separation technique.

2.7.2. Ionic liquid-based aqueous two-phase system for extraction

In order to prevail over the limitations of IL-water biphasic system, the ability of hydrophilic ionic liquid to form two-phase in the presence of inorganic salts was studied. By using hydrophilic ionic liquid rather hydrophobic in ionic liquid based-aqueous two phase system abbreviated as IL-based ATPS, the limitation of low proteins solubility was eliminated. The phase diagram of IL-based ATPS was first reported by

Gutowski *et al*. (2003). Since then this method has made many more significant progresses. The schematic illustration of ionic liquid-based aqueous two-phase system formation is shown in Figure 2.5.

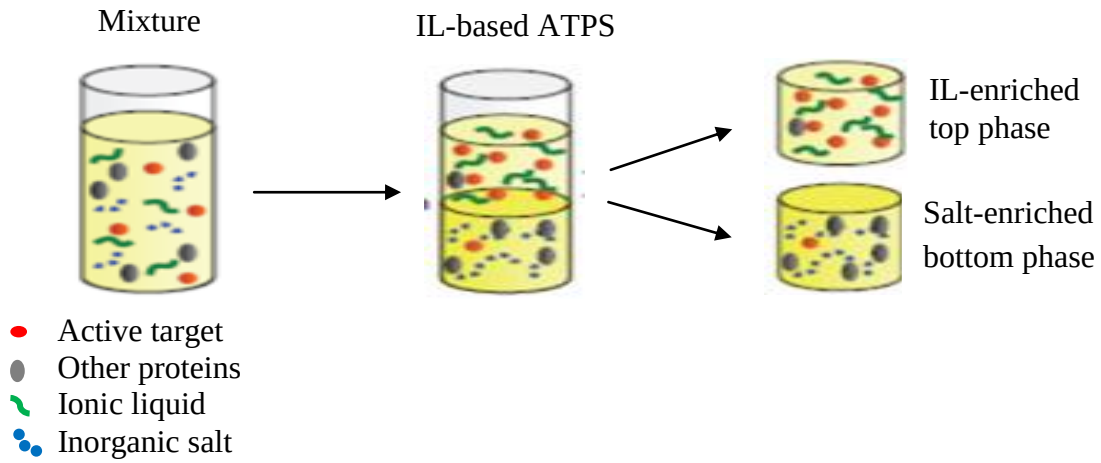

Figure 2.5: Schematic illustration of the formation of IL-based ATPS

Source: Dreyer, 2008

IL-based ATPS not only overcomes limitations of IL-water biphasic system but also confers some advantages such as rapid phase separation and low emulsion formation. Despite the increasing interest of using ionic liquid (ILs) in separation process in the last decade, some disadvantages of ILs are needed to be considered. Ionic liquids are known as green because of the lack of volatility feature; therefore, they do not release the harmful and toxic vapour into the atmosphere. On the other hand ILs are partially miscible with water. This inevitably leads to release of ILs into the aquatic environment and consequently causes the water contamination; therefore, ILs have potential toxicity toward environment (Abu-Eishah, 2011). For this reason finding an alternative way to recycle ILs is one of the important issues. Moreover, many researchers have mentioned the high cost as the main concern of using ILs in comparison with conventional alternatives solvent (Laali & Gettwert, 2001; González *et al*., 2006). For this reason ionic liquids have aroused more interest in laboratory scale rather in large-scale industrial purposes (Wagner & Hilger, 2008). Clearly, recycling of

ILs is one of the key issue not only for economical usage but also for environmental and safety issues.

2.8. Application of ionic liquid-based aqueous two-phase system

2.8.1. Extraction and separation of bio-chemicals

Many successful processes for extraction and separation of bio-chemicals using hydrophilic ILs have been reported. Some examples are summarized in Table 2.3. Since obviously high extraction efficiency of bio-chemicals was achieved, IL-based ATPS can be practically used.

Table 2.3: Separation of bio-chemicals using IL-based ATPS

Biochemical	ATPS	Extraction efficiency	References
Testosterone	$[C_4mim]Cl/K_2HPO_4$	80-90	He *et al*.,2005
Epitestosterone	$[C_4mim]Cl/K_2HPO_4$	80-90	He *et al*.,2005
Codeine	$[C_4mim]Cl/K_3PO_4$	90.0-100.2	Li *et al*.,2005
Papaverine	$[C_4mim]Cl/K_3PO_4$	99.3-102.0	Li *et al*.,2005
Bovine serum Albumin	$[C_4mim]Cl/K_2HPO_4$	90	Du *et al*.,2007
Bovine serum Albumin	$[C_nmim]Br/K_2HPO_4$ (n=4,6,8)	>70	Pei *et al*.,2009
Cytochrome C	$[C_4mim]Br/K_2HPO_4$	>90	Pei *et al*.,2009
γ-gloulins	$[C_4mim]Br/K_2HPO_4$	80	Pei *et al*.,2009
Trypsin	$[C_4mim]Br/K_2HPO_4$	>95	Pei *et al*.,2009
Horseradish peroxidase	$[C_4mim]Cl/K_2HPO_4$	80	Cao *et al*., 2008
Penicillin G	$[C_4mim]Cl/Na_2HPO_4$	93	Liu *et al*., 2006
Penicillin G	$[C_4mim][BF_4]/Na_2HPO_4$	-[a]	Jiang *et al*.,2007
L-tryptophan	ILs[b]/ K_3PO_4	-[a]	Neves *et al*., 2009;Ventura *et al*.,2009
Papaverine	$[C_4mim]Cl/K_2HPO_4$	>93	He *et al*.,2005
Morphine	$[C_4mim]Cl/K_2HPO_4$	>65	He *et al*.,2005
Lipase	$[C_nmim]Cl/K_2CO_3$ (n=2,3,4,5)	99	Deive *et al*., 2011

[a] Extraction efficiency was not given.
[b] See references for details of ILs.
Source: Modified from Li *et al*., 2010

2.8.2. Separation of small organic molecules

Bridges *et al*. (2007) reported the extraction of small molecules organic compound, i.e. methanol, propanol, butanol and pentanol in the IL-based ATPS system

comprising 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and tripotassium phosphate (K_3PO_4). In their study, the correlation between tie-line lengths (TLL) and the distribution ratio (D) of different short chain alcohols were evaluated.

2.8.3. Separation of radiological isotopes

In the study by Bridges and Rogers (2008), aqueous solutions of water-structuring kosmotropic salts and water-destructuring chaotropic ionic liquids were used to separate the radiological isotope pertechnetate (TcO^-_4) from complex salt waste. TcO_4 which has long half-lives, strong heat generation and high environmental mobility is concern to be separated from spent fuel. In the study, TcO^-_4 anion could be successfully partitioned into IL-rich phase without use of an extractant. The IL-based ATPS was comprised of hydrophilic IL [C_4mim]Cl and salt with series of different anions (PO_4^{3-}, HPO_4^{2-} or CO_3^{2-}).

2.8.4. Other applications

Two alcohol dehydrogenases, i.e. ADH from *Lactobacillus brevis and* ADH from a thermophilic organism were partially purified in aqueous two-phase system based on ionic liquid Ammoeng^{TM} 110 and salt (Dreyer & Kragl, 2008). In the study, both catalytically active bio-molecules were partitioned in top phase and the presence of ionic liquids enhanced the stability and solubility of both enzymes.

2.9. Selection of ionic liquid

In order to carry out effective enzyme extraction process using aqueous two phase system based on using ionic liquid, finding the proper ILs is the fundamental point to consider. The proper ILs can be chosen considering both phase forming ability and enzyme activity retaining characteristic. It has been reported that alkylimidazolium chloride ILs are the commonly used and inexpensive hydrophilic ILs to form two phase with K_2HPO_4 (Cao *et al*., 2008). A successful process to extract horseradish peroxidase

(HRP) was performed using aqueous biphasic systems consisting of alkylimidazolium chloride/K_2HPO_4 (Cao *et al*., 2008). In the study several imidazolium based ILs with different alkyl-chain lengths including 1-hexyl-3-methylimidazolium chloride ([C_6mim]Cl), 1-ethyl-3-methylimidazolium chloride ([C_2mim]Cl), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and 1-methyl-3-octylimidazolium chloride ([C_8mim]Cl) were studied in order to find out the proper ILs. In the study it was showed that [C_8mim]Cl which has longer alkyl-chain lengths showed better ability of phase forming; however, [C_4mim]Cl was found to be an appropriate IL for the enzyme activity maintaining. Considering both two aspects, [C_4mim]Cl was selected as the aqueous two-phase forming IL.

2.10. Phase diagram

In any aqueous two-phase system, phase diagram is required in order to quantify the phase separation process as well as displaying the miscible and immiscible parts (Koningsveld *et al*., 2001). A schematic of a phase diagram is illustrated in Figure 2.6. Phase diagram corresponds to the weight over volume of phase component. Binodal curve as one of the characteristic of phase diagram divides the region into the potential two-phase area and one phase area. All mixtures above the binodial curve give rise to phase separation while below is the area of one phase formation. In Figure 2.6, the homogenous (one phase) area is shaded.

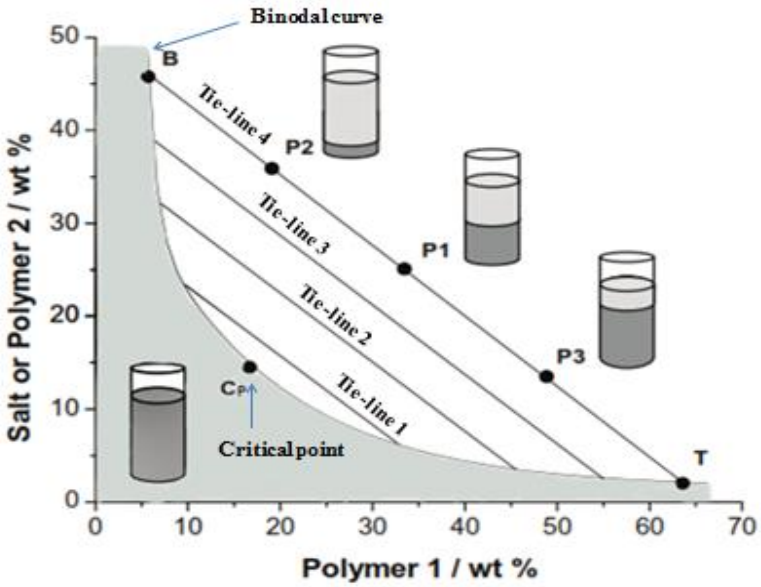

Figure 2.6: Schematic illustration of a phase diagram

The binodal curve separates one-phase region (shaded) from the two-phase region (unshaded). Source: Modified from Kual, 2000

Tie-lines which are straight lines and connect two nodes (T and B in Figure 2.6) present the concentration of compounds in top and bottom phase (Kaul, 2000). In the idealised system, the tie-lines are parallel to one another and their length reduces while the lines come close to the critical point. On the phase diagram, the point which the composition volume of both phases is almost equal is called critical point (C_P in Figure 2.6). Theoretically, every system with the same composition of top and bottom phase but with different volume ratio lies on one tie-line (Points P1, P2 and P3 in Figure 2.6). The data obtained from phase diagram are required in order to give information such as appropriate required concentration of phase compounds, the concentration of compounds in top and bottom phase and the ratio of phase volume.

CHAPTER 3: MATERIAL AND METHODS

3.1. Chemicals and media

The ionic liquid 1-butyl-3-methylimidazoilum chloride [C_4mim]Cl was obtained from Sigma-Aldrich (USA). Di-potassium hydrogen phosphate (K_2HPO_4) and potassium di-hydrogen phosphate (KH_2PO_4) were purchased from Merck (Darmstadt, Germany). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were supplied by Difco. The bovine serum albumin was purchased from Sigma-Aldrich (USA).

3.2. Fungal strain

The *Amauroderma rugosum* mycelia were obtained from Mushroom Research Centre, University of Malaya. The fungi culture was maintained on potato dextrose agar (PDA). The plates were routinely sub-cultured for 10 days at 25°C until mycelium covered the full plate. For longer storage, *Amauroderma rugosum* mycelia were maintained on PDA slant at room temperature.

3.3. Preliminary plate screening for laccase and LiP activity

Amauroderma rugosum was screened for lignin peroxidase and laccase activity on PDA. After 10 days of cultivation the mycelium plate was examined for enzymes activity. Three wells were made on PDA and labelled as a, b, c (Figure 3.1). The ethanol (95%) was added into well a as control. Syringaldazine (0.1%) was employed into well b for determination of laccase activity. Lignin proxidase activity was tested by adding mixture of 1% of pyrogalic and 0.4% of hydrogen peroxide (H_2O_2) in 1:1 ratio into well c (Abdel-Rahmeem & Shearer, 2002).

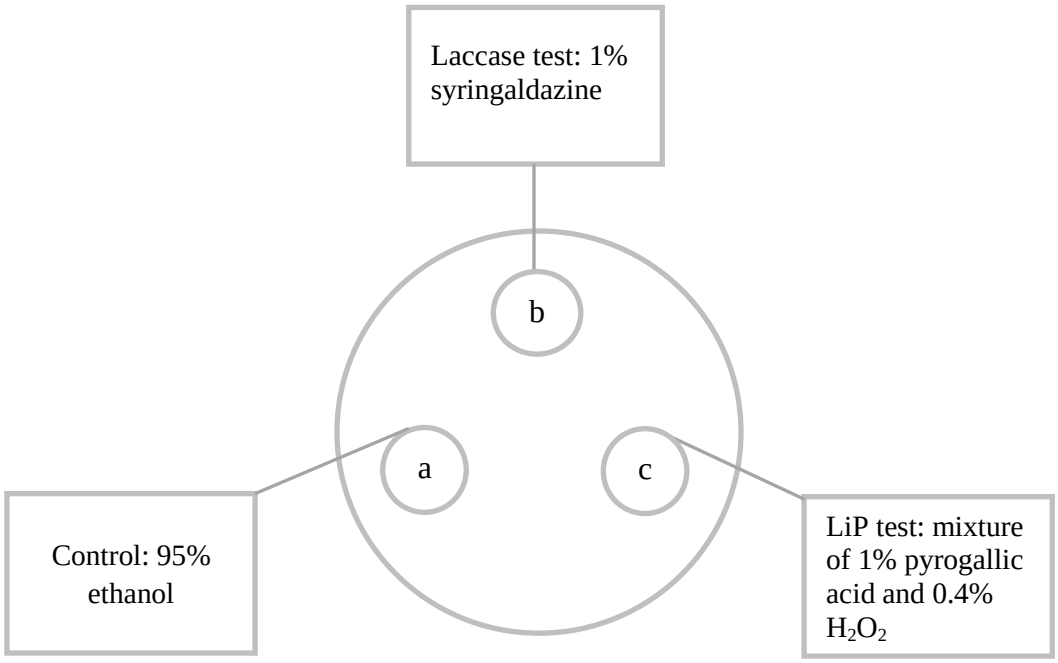

Figure 3.1**:** Preliminary plate screening for laccase and LiP produced by *Amauroderma rugosum*

3.4. Submerged fermentation

3.4.1. Preparation of mushroom mycelia for submerged fermentation

The extraction of lignin peroxidase (LiP) from *Amauroderma rugosum* was carried out using the mushroom mycelia instead of the mushroom fruiting body. It was due to the unavailability of adequate fruiting body of this indigenous species. In this study, submerged fermentation was chosen due to versatile advantages over solid culture. Liquid fermentation provides high product concentration, low fermentation period and convey possibility of process controlling. The submerged culture was carried out by inoculating of 10 day-old mycelia cultures into the flasks. The agar discs were inoculated into different cultivation media in 500ml conical flask. Ten fungal discs (2 mm^2) were transferred to each flask separately. Then the flasks were subjected to incubator shaker with a speed of 120 rpm for 14 days at 27-30°C. Triplicates were done for each experiment.

3.4.2. Selection of culture media for submerged fermentation

Different kind of substrates such as potato dextrose broth (PDB), yeast extract, glucose, sawdust, bamboo leaves and defined media were tested for optimization of LiP production. The formulation of defined media used was g/L: glucose 10; ammonium nitrate (NH_4NO_3) 2; potassium di-hydrogen phosphate (KH_2PO_4) 0.8; di-sodium hydrogen phosphate (Na_2HPO_4) 2; magnesium sulphate ($MgSO_4.7H_2O$) 0.5 and yeast extract 2 (Ding *et al*, 2012). The cultivated mycelia were harvested after 14 days using vacuum filtration through Whatman filter paper (No.1). The filtered solution was collected and used as crude enzyme for the assay procedures. Crude enzyme was tested either immediately after filtration or kept in 4°C followed by LiP and laccase activity assay.

3.5. Determination of enzyme activity

3.5.1. Laccase activity

Laccase activity was determined on spectrophotometer at=525nm at room temperature (25ºC) following the protocol described by Harkin and Obst (1973); Leonowicz and Gryzwnowicz (1981). The substrate blank consisted of 3.2 ml of 50 mM sodium citrate and 0.2 ml of 0.1 mM syringaldazine. The enzyme blank consisted of 3.2 ml of 50 mM sodium citrate and 0.2 ml of crude enzyme. The reaction mixture consisted of 3.0 ml of 50 mM sodium citrate, 0.2 ml of crude enzyme and 0.2 ml of 0.1 mM syringaldazine. The laccase activity was assayed by measuring the amount of tetramethoxy-azo-bis-mtehylenequinone resulted from laccase and syringaldazine (substrate) reaction. The reaction mixture was read 1 minute after the adding of syringaldazine substrate into the reaction mixture at=525 nm.

Calculation of laccase activity

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

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

3.5.2. Lignin peroxidase activity

Lignin peroxidase activity was determined on spectrophotometer at=310 nm at room temperature (25ºC). The enzyme activity was assayed by measuring the amount of enzyme required to convert one unit of veratryl alcohol (substrate) to veratryldehyde (product) in the presence of hydrogen peroxide (H_2O_2) at pH3.0 (Have *et al*., 1998). The test was initiated by adding 0.2 ml of hydrogen peroxide (H_2O_2) into a reaction mixture that comprises of 2.4 ml of sodium tartrate buffer, 0.2 ml of veratryl alcohol and 0.2 ml of crude enzyme and incubated for 5 minutes. The reagent blank contained 2.6 ml sodium tartrate buffer, 0.2 ml veratryl alcohol and 0.2 ml of H_2O_2. The enzyme blank comprised of 2.4 ml sodium tartrate buffer and 0.2 ml of enzyme. All the values were the means of triplicate. The reaction mixture was read 5 minutes after the adding of H_2O_2 in the reaction mixture.

Calculation of lignin peroxidase activity

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

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

3.6. Protein determination test (Bradford Assay)

The concentration of soluble protein in system was employed using crystalline bovine serum albumin (BSA) test described by Bradford (1976) (Appendix A.3). For protein concentration assay, standard curve was first drawn. About 0.2 ml of sample was mixed with 5 ml of Coomassie® Brilliant Blue G-250 reagent. After vortex, samples were incubated for 4 minutes in order to let the particles settle down. The blank was contained of 0.2 ml of distilled water mixing with 5 ml of Coomassie Brilliant Blue G-250 reagent. The test was done using spectrophotometer at=595 nm wavelength.

Calculation of soluble protein

$$\text{Soluble Protein (mg/mL)} = \left(\frac{\text{final absorbance}}{0.006}\right)\left(\frac{1}{0.2}\right)\left(\frac{1\text{mg}}{1000\mu\text{g}}\right)$$

3.7. Preparation of aqueous two-phase system

The ability of ionic liquid based aqueous two phase system (IL-based ATPS) in partitioning of lignin peroxidase (LiP) from *Amauroderma rugosum* was evaluated. The phase diagram of 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$) at≈60 wt%, and di-potassium hydrogen phosphate (K_2HPO_4) at=40 wt% was followed by the phase diagram generated by Ventura *et al*. (2011). Tie-line length (TLL) was determined using conductometer and reflectometer devices by which the concentration of salt and polymer in aqueous system were measured respectively (Jiang *et al*[a]., 2009). For determination of TLL, five different mixtures composed of K_2HPO_4, $[C_4mim]Cl$ and water with total weight of 10 g at the biphasic region were prepared in centrifuge tubes. The tubes were vigorously agitated and left at room temperature for 30 minutes. Afterwards, both phases were separated and the polymer and salt concentration were measured individually. TLL was calculated using the following equation:

$$TLL=\sqrt{\Delta P^2 + \Delta C^2} \quad (1)$$

Where ΔP is the differences of polymer concentration in top and bottom phase and ΔC illustrates the differences between salt concentration in top and bottom phase.

The aqueous two-phase system was carried out by preparation of predetermined quantities of dissolved [C_4mim]Cl and K_2HPO_4 in a 15 ml centrifugal tube and addition of 1g of crude enzyme. Gentle agitation was carried out to facilitate the two-phase formation. The tubes were then left at room temperature for 30 minutes to allow to stand and making the complete phase separation. Afterwards, the top phase and bottom phase were separated and were then assayed for LiP activity and total protein concentration.

3.8. Optimisation of aqueous two-phase system

Different parameters affecting partitioning of LiP during purification process were optimized. The tested parameters were different tie-line length (TLL), different volume ratio (V_R), different pH value and different mass of K_2HPO_4. The partitioning of LiP in different TLL was studied by selecting 5 systems from different TLL with total weight of 10 g, constant volume ratio of 1.0 and pH equal to 9.5. Along the best TLL, five systems at the constant condition of total weight of 10 g, pH equal to 9.5 but at different volume ratio were selected to evaluate the influence of V_R on LiP partitioning. The influence of pH on LiP partitioning was carried out at different pH value of 7.5, 8, 8.5, 9 and 9.5. The initial pH value of 40% di-potassium hydrogen phosphate before adding crude enzyme was 9.5. The other pH values of salt solution were adjusted by adding potassium di-hydrogen phosphate (KH_2PO_4) to di-potassium hydrogen phosphate (K_2HPO_4) as described by Lin *et al*. (2012). The influence of mass of K_2HPO_4 was studied by addition of more K_2HPO_4 with the range of 5.75-5.90 g at the fixed amount of [C_4mim]Cl.

3.9. Determination of partition coefficient (K), specific activity (SA), volume ratio (V_R), purification factor (P_F) and yield

The partition coefficient (K) of the LiP was calculated as the ratio of the LiP concentration in top and bottom phases (Equation 2):

$$K = \frac{C_T}{C_B} \quad (2)$$

Where C_T and C_B are the LiP activity in U/mL in top phase and in bottom phase respectively.

The specific activity (SA) is the enzyme activity (U/ml) in the phase sample divided by the protein concentration (µg/ml) and was expressed U/µg of protein (Equation 3):

$$SA = \frac{\text{Enzyme activity}}{\text{Protein concentration}} \quad (3)$$

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 4):

$$V_R = \frac{V_T}{V_B} \quad (4)$$

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

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

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

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

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

3.10. Characterization of protein

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular mass of crude enzyme and recovered enzyme from top phase in ATPS. The sample was pre-treated with SDS and β-mercaptoethanol. The SDS-PAGE was carried out by adding 18 µl of crude enzyme to 4 µl of loading dye to determine the molecular mass of crude enzyme. To determine the molecular mass of recovered enzyme from top phase 20 µl of the enzyme from top phase and 5 µl of loading dye were added. In SDS-PAGE method, the enzyme samples were separated in an acrylamide gel consisting of 12% resolving gel and 5% stacking gel described by Laemmli and Favrel (1973) (Appendix A.4). The electrophoresis was run at 100V for 120 minutes. Then, the gel was stained using Coomassie® Brilliant Blue R-250 solution consisted of Coomassie® Brilliant Blue, methanol, acetic acid and distilled water. It was then de-stained using the same buffer solution without Coomassie® Brilliant Blue. Lignin peroxidase activity was visualized in native-PAGE using the same protocol as described above only with exclusion of SDS and β-mercaptoethanol in order to retention of protein structure. The bands from native-PAGE were excised to analyse for lignin peroxidase activity test (Have *et al*., 1998).

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Preliminary plate screening for laccase and LiP activity

Amauroderma rugosum was able to produce lignin peroxidase as indicated by the formation of golden brown colour (Figure 4.1(c)). Formation of pink colour (Figure 4.1(b)) in plate indicated the presence of laccase. The results from Table 4.1 show the growing trend of lignin peroxidase activity from 10^{th} to 14^{th} days. During that particular time the golden brown colour intensity and stability were significant; however the enzyme was lost after 14^{th} day. From the results, the laccase activity was observed at 10^{th}, 11^{th} and 12^{th} days, however, on 13^{th} and 14^{th} days no laccase activity was observed.

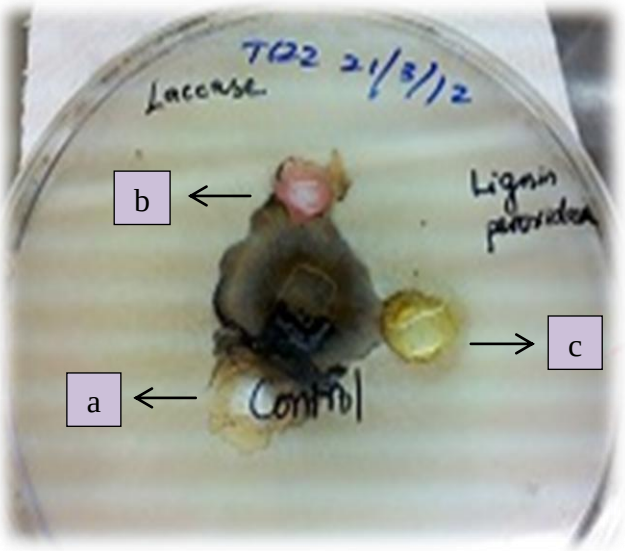

Figure 4.1: Screening for laccase and LiP activity determination on-agar plate

(a) Control containing ethanol 95%. (b) Formation of pink colour illustrated the presence of laccase. (c) Formation of golden brown colour illustrated the presence of lignin peroxidase.

Table 4.1: Preliminary plate screening to determine the laccase and LiP activity produced by *Amauroderma rugosum*

	10^{th} Day	11^{th} Day	12^{th} Day	13^{th} Day	14^{th} Day
Control (95% ethanol)	-	-	-	-	-
Laccase	++	+++	+++	-	-
Lignin peroxidase	+++	+++	++++	++++	++++

Note: (+) indicates low colour changes, (++) indicates intermediate, (+++) indicates high, (++++) indicates very high and (-) indicates no colour changes.

Preliminary plate screening is an effective, simple and economical way to assess the reliability of research being performed. Formation of golden brown and pink colour ensured the production of lignin peroxidase and laccase from *Amauroderma rugosum* respectively. From the results (Table 4.1) the highest laccase activity was on 11^{th} to 12^{th} days following by deterioration of laccase production on 13^{th} and 14^{th} days. The highest lignin peroxidase activity was detected on 12^{th} to 14^{th} days of subculture. Therefore, the submerged fermentation was carried out at the selected days at which the LiP showed the highest colour intensity and stability to ensure high enzymes productivity during submerged fermentation. The lignin peroxidase is taking a major role in growth and development of *Amauroderma rugosum* and masking the production of laccase. Since laccase is not needed in the later development of fungus. For this reason the production of this laccase deteriorated after 12^{th} day of subculture.

4.2. Optimisation of culture media for submerged fermentation

The effect of different substrates with different level of carbon and nitrogen on LiP and laccase productivity from *Amauroderma rugosum* was studied. Table 4.2 illustrated the enzyme production for each liquid media. The results from the Table 4.2 indicated that none of the media produced laccase although laccase activity was detected during preliminary screening. Almost all selected media produced lignin peroxidase. The *Amauroderma rugosum* cultivation liquid media comprised of potato dextrose broth, 0.5% yeast and 1% sawdust gave the highest lignin peroxidase activity

of 39.03±3.33 U/ml. Defined media also showed the high range activity of 36.46±2.93 U/ml.

Table 4.2: Optimization of laccase and LiP productivity from *Amauroderma rugosum* under different media

Medium	Laccase activity (U/ml)	Lignin peroxidase activity (U/ml)
Potato Dextrose Broth (PDB) + 1% sawdust	-	$14.68 \pm 2.94^{(b)}$
Potato Dextrose Broth (PDB) +1% sawdust + 0.5 % yeast	-	$39.03 \pm 3.33^{(a)}$
Potato Dextrose Broth (PDB)	-	$3.78 \pm 1.1^{(c)}$
Glucose 1% + 1% sawdust	-	$4.42 \pm 1.92^{(c)}$
Glucose 2 % + 1% sawdust	-	$5.7 \pm 1.1^{(c)}$
Defined media	-	$36.46 \pm 2.93^{(a)}$

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.

In all media tested, when both of carbon and nitrogen concentration increased, the LiP productivity increased. According to Rogalski *et al*. (2001) production of ligninolytic enzyme such as laccase, lignin peroxidase, and manganese peroxidase by white-rot fungi is dependent on growth condition such as the media selected and their carbon and nitrogen level. It has been reported that the highest production of ligninolytic enzyme in *Pleurotus saju-caju* was observed in the medium supplemented with complex polysaccharides such as olive mill wastewater (OMW) or wheat straw compared to utilizing monosaccharides or disaccharides as carbon source. The growth and the activity of LiP and MnP were almost similar using either wheat straw or OMW as substrates. However, the production of laccase enzyme was higher using OMW rather than using wheat straw as a carbon source (Massadeh *et al*., 2010). *Amauroderma rugosum* showed no laccase production under different selected media whereas in plate screening test the production of laccase was detected. One of the explanations can be laccase in Amauroderma species prefer to grow in a set environment for example solid state fermentation. Moreover It might be due to the LiP in *Amauroderma rugosum* is

taking the major role in growth and development. However, further experiments need to be carried out to investigate thoroughly.

The medium contained of PDB, 1% sawdust and 0.5% yeast gave the highest LiP production of 39.03±3.33 U/ml. It was due to the media supplemented with natural nitrogen source in the form of yeast and the non-limiting nitrogen and carbon sources in the form of sawdust. Kapich *et al.* (2004) reported that lignocellulosic substrates such as wheat straw increased the production of LiP by *P. chrysosporium*. The lignocellulosic substrates provide the non-limiting nitrogen and carbon sources. Moreover, it was found that *Amauroderma rugosum* grows on hardwood forest which can be in the category of secondary decomposer. The secondary decomposers are dependent on primary fungi species to break down the substrates for growth (Elevitch, 2004). Thus, *Amauroderma rugosum* produced higher LiP by utilizing the soft rubber sawdust which contains low lignin content. Chae *et al*. (2001) stated that yeast extract is enriched with peptides, all kinds of amino acids, nucleotides and the soluble components of yeast cells so that it can be advantageously used over ordinary nitrogen sources. Defined media was a good candidate for LiP production with activity of 36.46±2.93 U/ml; however, the results were unstable from batch to batch which might be due to the lack of continuous supply of nitrogen source. For this reason the medium consisting of potato dextrose broth +1% sawdust + 0.5 % yeast was chosen to carry out for further experiments.

4.3. Phase diagram

Phase diagram is required not only for design of aqueous two–phase system but also for prediction of protein partitioning. It is also essential in order to evaluate whether the ionic liquid is applicable for ATPS. The phase diagram of $[C_4mim]Cl$ and K_2HPO_4 is shown in Figure 4.2 which was followed by the phase diagram generated by Ventura *et al*. (2011)

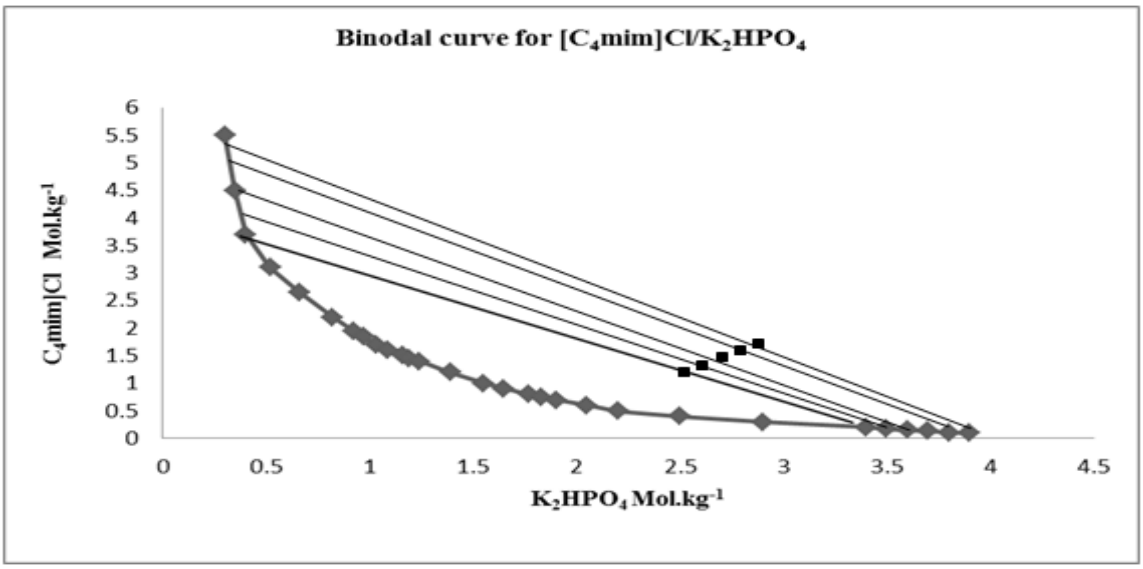

Figure 4.2: Phase diagram of the ionic liquid $[C_4mim]Cl$ and the inorganic salt K_2HPO_4

The $[C_4mim]Cl$ was plotted on ordinate and the K_2HPO_4 was plotted on the abscissa. The composition of ATPS with volume ratio of approximately 1 is denoted as ▪ in each TLL.

The curve line which donated the area at which two phases exist is called binodial curve (Figure 4.2). All mixtures above the binodial curve give rise to two-phase separation while below the binodial curve is the area of one phase formation. The tie-line length shows the equilibrium composition for two phases. From the phase diagram, five systems with total weight of 10 g, the constant volume ratio of 1.0 and pH equal to 9.5 were selected in order to evaluate the recovery of LiP (Table 4.3).

Table 4.3: Five different systems with 1 to 1 ratio and tie-line length for evaluate partition behaviour of LiP from *Amauroderma rugosum*

System No	% $[C_4mim]Cl$ (w/w)	% K_2HPO_4 (w/w)	% Tie-lie Length (w/w)
1.	12	23	60.82
2.	13	23.5	77.60
3.	14	24	82.51
4.	15	25	87.57
5.	15.5	25.5	89.09

4.4. Optimization of aqueous two-phase system

Different types of ATPS have been implemented in order to extraction and purification of enzyme produced by several mushroom species (Naganagouda & Mulimani, 2008; Mayolo-Deloisa *et al*., 2009; Ratanapongleka & Phetsom, 2011). To our knowledge, this was the first report of lignin peroxidase purification from *Amauroderma rugosum* using IL-based ATPS. In a study by He *et al*. 2005 the ability and suitability of various salts were investigated for formation of IL-based ATPS. Among all salts, K_2HPO_4 was the best candidate to be applied in the system with ionic liquid $[C_4mim]Cl$ due to its high solubility with water as well as its strong ability of two-phase formation. According to Pei *et al*. 2009, bio-molecule partitioning in IL-based ATPS is affected by several factors such as type of ionic liquid, alkyl chain length of ionic liquid, type of inorganic salt, temperature and pH. In this study, several parameters such as the influence of TLL length, the influence of the V_R, the influence of pH and addition of more K_2HPO_4 were studied in order to optimize the ATPS and obtain the best recovery of LiP. In all system studied, LiP was selectively partitioned to the IL-rich top phase.

4.4.1. Influence of tie-line length on lignin peroxidase partitioning

The influence of TLL on purification factor of top phase (P_{FT}) and Log K was studied in all systems with a constant volume ratio of 1 and initial pH value of 9.5. To avoid any potential concentration effects, the initial value of 1 was selected for V_R

(Table 4.4). The results indicated the increase of P_{FT} and Log K at the TLL from 60.8 to 87.57 % (w/w), however, the P_{FT} and Log K slightly decreased at TLL 82.51%. System 5 showed negative result on P_{FT} and Log K with increasing TLL. The maximum purification factor of LiP obtained was 3.12±0.90 at TLL 87.57% (w/w) with a Log K value of 0.88±0.19.

Table 4.4: Influence of TLL on LiP partitioning

System No	V_R	TLL(%w/w)	P_{FT}	Log k
1.	1	60.82	$1.44\pm0.48^{(b)}$	$0.51\pm0.05^{(b)}$
2.	1	77.60	$1.45\pm0.57^{(b)}$	$0.64\pm0.04^{(b)}$
3.	1	82.51	$1.30\pm0.19^{(b)}$	$0.60\pm0.02^{(b)}$
4.	1	87.57	$3.12\pm0.9^{(a)}$	$0.88\pm0.19^{(a)}$
5.	1	89.09	$3.05\pm0.79^{(a)}$	$0.60\pm0.12^{(b)}$

Note: Purification factor of top phase and Log K with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Uses Harmonic Mean Sample Size=3.00.

Increasing of TLL caused reduction of free volume available in the bottom phase which led the LiP to partition in IL-rich top phase. Moreover, the salt concentration at the bottom phase increased with increasing the TLL followed by raising the hydrophobicity in bottom phase. Berggren *et al*. 1995 reported that by raising the hydrophobicity in bottom phase, more salt ions will be available at the bottom phase to dehydrate the protein and constitute a double layer of ionic group. Therefore, the salt can react with other opposite charge groups in protein. Formation of a double layer of ionic group causes protein dehydration and formation of protein hydrophobic surface ensue (Bonomo *et al.,* 2006). This may lead to promote polymer-protein interaction and driving most of the target protein to the top phase (Vojdani, 1996). However, the P_{FT} and Log K slightly decreased at TLL 89.09 % (w/w). This can be explained that increasing of free volume in top phase in that particular TLL led to increasing of contaminants occurrence and consequently caused the P_{FT} and Log K reduction.

At TLL 87.57% (w/w), the system composition consisting of 15% [C_4mim]Cl, 25% K_2HPO_4 achieved a fine balance between the salting out ability of K_2HPO_4 and hydrophobicity of [C_4mim]Cl. Therefore, partitioning of LiP at particular TLL led to obtain the highest value of Log k and P_{FT}. As a result TLL of 87.57 % (w/w) was selected for further study.

4.4.2. Influence of volume ratio on lignin peroxidase partitioning

According to Kual (2000), along one tie line systems have same final concentration but different volume ratio and total phase composition. The effect of V_R changing upon lignin peroxidase partitioning was studied. In this case TLL and pH value were kept constant at 87.57 % (w/w) and 9.5 respectively and V_R range was varied. The selected volume ratios were ranged from 0.45 to 2.9.

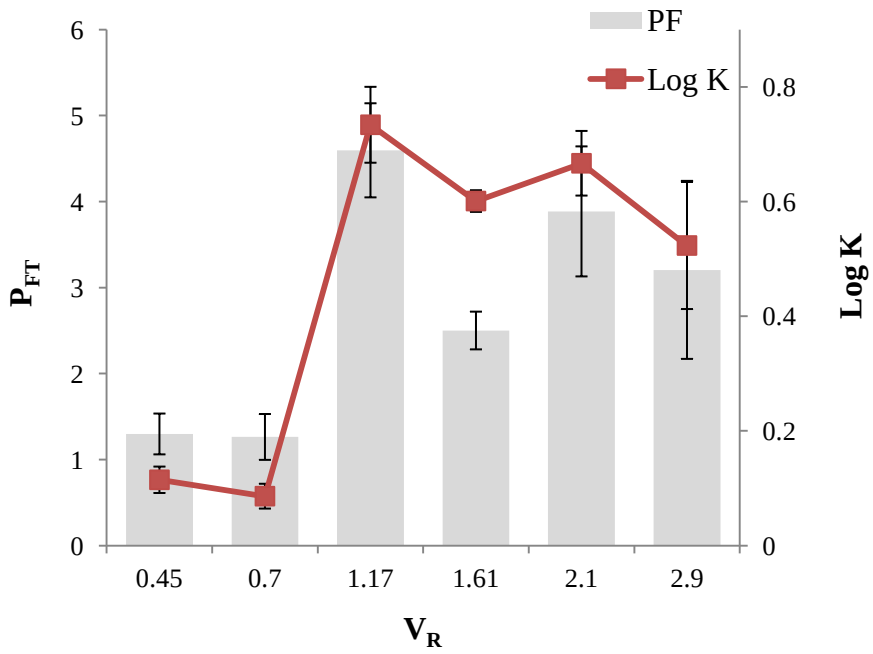

Figure 4.3: Influence of V_R on LiP partitioning

The V_R ranging from 0.45 to 2.9 along TLL 87.57% (w/w) were evaluated to investigate partitioning behaviour of LiP.
Note: Purification factor of top phase and Log K with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Uses Harmonic Mean Sample Size=3.0

As shown in Figure 4.3, the purification factor of top phase (P_{FT}) and Log K increased markedly when volume ratio changed from 0.7 to 1.17. Optimum purification factor of 4.60 ±0.95 and Log K of 0.73±0.11 were obtained at a volume ratio of 1.17.

At an extremely low volume ratio from 0.45 to 0.7, low value of P_{FT} (1.30±0.41 and 1.26±0.46) and Log K (0.11±0.03 and 0.09 ±0.04) were obtained. Protein precipitation in interface caused reduction of purification factor of top phase at low range of V_R. Moreover, Ooi *et al*. 2009 reported that decrease in volume ratio causes reduction of free volume and consequently leads to the losses of enzyme remained in top phase. Therefore, enzyme partitioning has limited at the top phase which may lead to moving of enzyme from top phase to intermediate or bottom phase.

At volume ratio of 2.9, the P_{FT} value decreased. At high volume ratio, the free available volume in top phase was too high which could cause the diluting of the enzyme and consequently P_{FT} reduction. At volume ratio of 1.17, the free volume for enzyme partitioning at fine balance between system compositions thus gave highest P_{FT} and Log K.

4.4.3. Influence of pH on lignin peroxidase partitioning

Influence of pH on lignin peroxidase partitioning in IL-based ATPS were studied. TLL and V_R values were kept constant at 87.57% and 1.17 respectively and the pH was varied ranging from 7.5 to 9.5.

Since $[C_4mim]Cl/K_2HPO_4$ system could not form two phases in the selected concentration at pH below 7.5, the pH value to study was started from 7.5. The failure of two-phase formation at pH below 7.5 was due to changes of liquid phase composition by replacement of K_2HPO_4 salt by KH_2PO_4 which lead to produce a single phase (Ruiz-Angel *et al*., 2007). In order to avoid enzyme denaturation and conformation, the extreme pH value (pH> 9.5) was not selected for further study.

As illustrated in Figure 4.4, P_{FT} and Log K increased when pH increased from 7.5 to 8. Then, decreased in P_{FT} ranging from 8.5 to 9.5 and fluctuated Log K were observed. The optimum value of LiP top purification factor was 8.59±0.53 with Log K of 0.83±0.05 at pH 8.

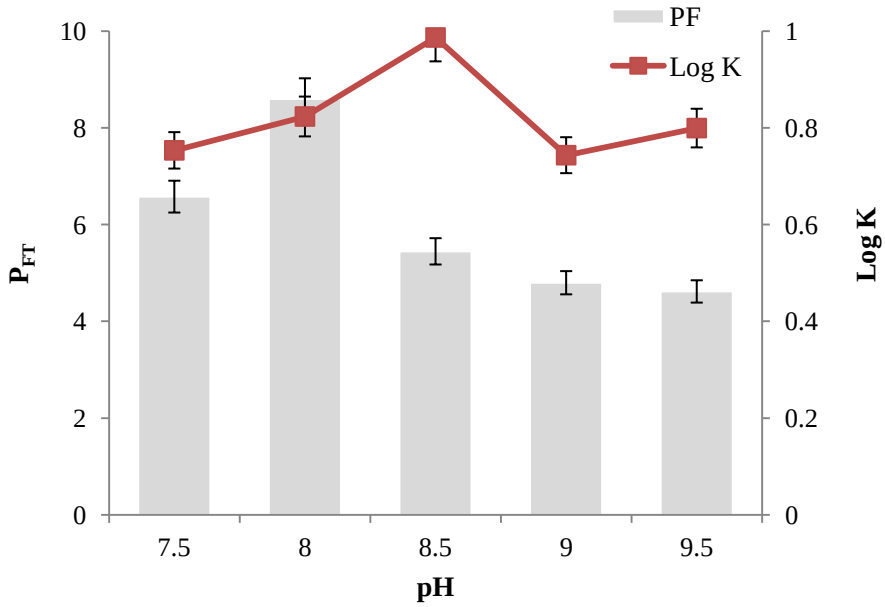

Figure 4.4: Influence of pH on LiP partitioning

The pH of ATPS was varied between 7.5 and 9.5 in order to optimization of [C_4mim] Cl/K_2HPO_4 system at a TLL of 87.57% (w/w) and volume ratio of 1.17.
Note: Purification factor of top phase with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Uses Harmonic Mean Sample Size=3.00.

Isoelectric point of protein (pI) is a parameter to determine the dependency of purification factor and partition coefficient on pH (Pei *et al.*, 2009). Therefore, electrostatic interactions between charged group of protein and ionic group of IL have a decisive role in the correlation of P_F and Log K together with pH. Lignin peroxidase has been reported to have isoelectric point of 3.2- 4 (Gold *et al.*, 1989; Kirk and Farrell, 1987; Renganathan *et al.*, 1985; Leisola *et al.*, 1987). It is reported that when the pH value of experiment condition is higher than the isoelectric point (pI) of protein, the net charge of protein will be negative, whereas top phase of system containing polymer will be more positive (Naganaanda & Mulimani, 2008). Therefore, when pH increased from 7.5 to 8, attraction of negatively charged LiP by positively charged of $[Bmim]^+$ in the

top phase and at the same time repulsion of LiP from $[HPO_4]^{-2}$ of the bottom phase led to transfer of LiP into top phase. Hence, an increase of P_{FT} and Log K was resulted.

However, the purification factor value decreased ranging from pH 8.5 to pH 9.5. This might be due to the lower solubility and surface properties changes in extreme pH value. In extreme pH value the enzyme will gently loose its activity which consequently causes reduction of P_{FT} and Log K. Besides, higher pH would increase the affinity of contaminants toward top phase.

With regard to the impact of pH on lignin peroxidase partitioning in IL-based ATPS, a closer look has to be taken at interaction between the ionic liquid and the charged protein. It has been reported that protein are dissolved in top IL-rich phase (Pei *et al*., 2009). Presence of water exhibits direct influence on disruption of hydrogen bonds between cations and anions of IL. This leads to increase the number of free anion in IL-rich phase followed by an increase of the ionic mobility (Cammarata *et al*., 2001). Therefore, interactions between ionic liquid and charged group in protein play a key role in driving the lignin peroxidase to partition into IL-rich top phase.

4.4.4. Influence of mass of di-potassium hydrogen phosphate (K_2HPO_4) on lignin peroxidase partitioning

The effect of addition of more K_2HPO_4 with the range of 5.75-5.90 g at the fixed amount of $[C_4mim]Cl$ is shown in Figure 4.5. The results showed 80-91% of recovery yield was obtained for all systems in this study. It was observed that by adding more K_2HPO_4 up to 5.85 g, the P_{FT} increased to 10.76±1.61. However, when the salt mass increased to 5.9 g, the P_{FT} decreased to 8.7±2.45 with concurrent decreasing of Log K to 0.56±0.05. The highest purification factor of 10.76±1.61 and Log K of 0.98±0.02 with the highest yield of 91.80%±0.3 were obtained when 5.85 g of K_2HPO_4 was added to the system.

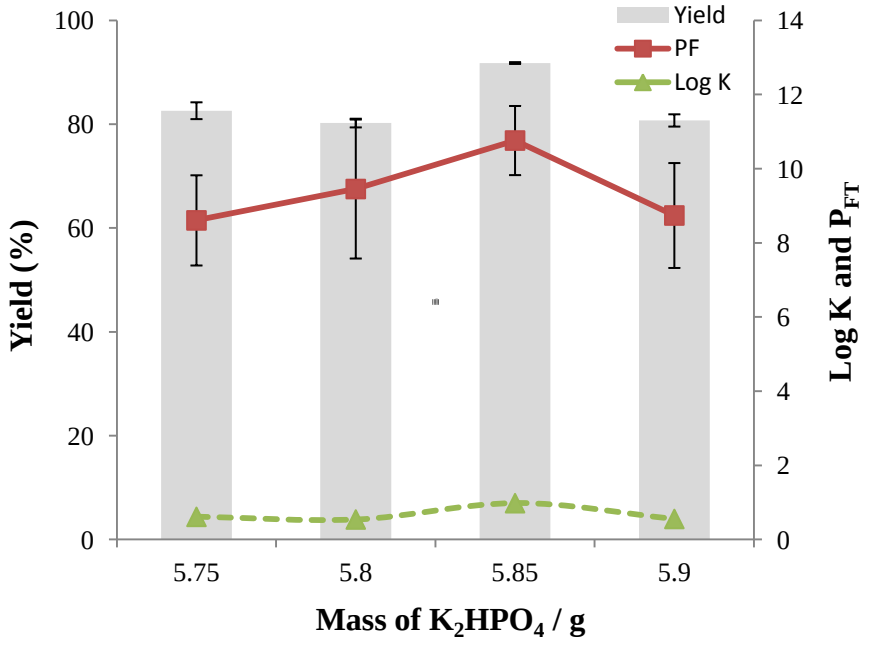

Figure 4.5: Influence of mass of K_2HPO_4 on LiP partitioning

All the experiments were performed in the system with TLL 87.57% (w/w) V_R at 1.17 and pH 8.
Note: Log K and yield with different letter(s) were significantly different by Turkey's HSD ($p<0.05$). Uses Harmonic Mean Sample Size=3.00.

Di-potassium hydrogen phosphate (K_2HPO_4) which is known as a kosmotropic (water structuring) salt has a strong ability of salting out effect (Arakawa & Timasheff, 1982). The Addition of more K_2HPO_4 in the system increased the hydrophobicity of bottom phase. Thus, the bottom phase became more kosmotropic and more structured (Lu *et al*., 1994). This led to decrease the solubility of protein in the bottom phase due to competition of salt with protein for water molecules. Therefore, the protein transferred from bottom phase to top phase (Du *et al*., 2007).

The salting-out mechanism implies the structure-making anion (HPO_4^{-2}) of K_2HPO_4 is a high valence anion and is able to hydrate more water molecules (Pei *et al*., 2009). The preference of cation $[C_4mim]^+$ of IL for transfer to the top phase can be given by solvophobic interaction (Gutowski *et al*., 2003). Solvophobic interaction is about salting out of hydrophilic ionic liquids with big cations by a salt containing more kosmortopic anion. According to Gutowski *et al*. (2003), kosmotrophic salts increase

the dielectric constant of aqueous phase; therefore, cation with low dielectric will partition to top phase with concurrent transfer of anion.

4.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Native-PAGE analysis for the purified lignin peroxidase

In order to analyse the purity of LiP from top phase, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was implemented using the method described by Laemmli and Favrel (1973) (Figure 4.6). The crude enzyme showed multiple impurities bands (lane 1) which presented the contaminant proteins in the crude enzyme. Three bands were obtained in the sample from the top phase at molecular weight of 38 kDa; 45-46 kDa and 66 kDa (Figure 4.6 a-c). Native-PAGE was used to study the purified lignin peroxidase activity using method described by Have *et al*. (1998). Three bands (lane 4) estimated at molecular weight of 38 kDa, 45-46 kDa and 66 kDa were shown in native-PAGE analysis (Figure 4.6 d-f); however, the band at molecular weight of 66 kDa (Figure 4.6 f) represented a clump. The three obtained bands were subjected to lignin peroxidase activity test. All three bands showed positive lignin peroxidase activity of 10.82 U/ml, 12.36 U/ml and 6.4 U/ml for bands at molecular weight of 38 kDa, 45-46 kDa and 66 kDa respectively (Table 4.5)

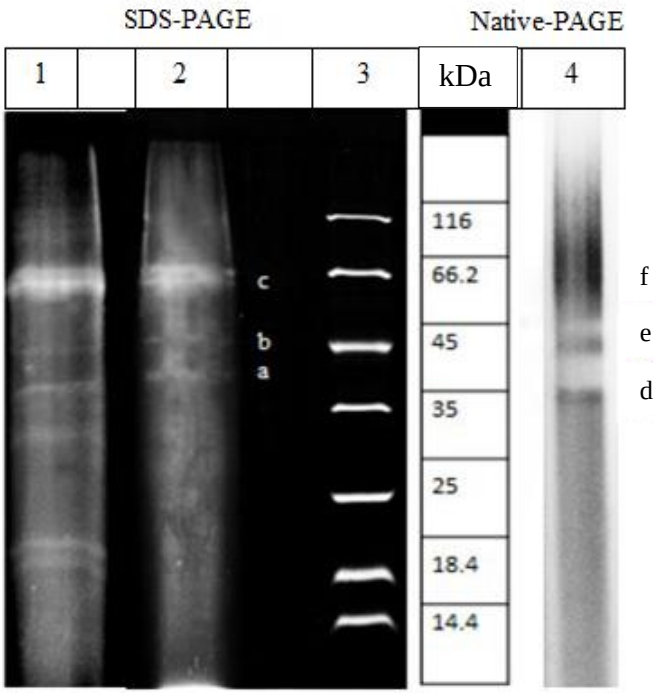

Figure 4.6: SDS-PAGE and Native-PAGE analysis on the recovery of LiP of top phase sample

The purity of lignin peroxidase partitioned in top phase was evaluated by 12% SDS-PAGE analysis. The molecular weight of leader was between 14.4 and 116 kDa. The gels are arranged as follow: (l) crude enzyme, (2) Lip in top phase of IL-based ATPS, (3) protein standard, (4) LiP in Native-PAGE. * (letters a, b and c are referred to LiP with different molecular weight. a: 38 kDa; b: 45-46 kDa; c: 66 kDa in SDS-PAGE. letters d, e and f are referred to LiP with different molecular weight. d: 38 kDa; e: 45-46 kDa; f: 66 kDa in Native-PAGE).

Table 4.5: The LiP test for three excised bands in Native-PAGE

Band No	a	b	c
M_W of band	38 kDa	45-46 kDa	66 kDa
Lignin peroxidase activity (U/ml)	10.82	12.36	6.4

In both SDS-PAGE and native-PAGE, brown colour bands were observed which affected the resolution of the enzyme bands. Vares *et al*. (1995) stated that lignin peroxidase enzyme often represent the coloured compounds in SDS-PAGE and can be only removed using ultrafiltration method.

It was reported that lignin peroxidase produced by *Phlebia radiate* has two bands at molecular weight of 44 kDa and 45-46 kDa (Vares *et al*., 1995). The molecular weight between 38-47 kDa of LiP from white-rot fungi has also been reported by the

other work (Fakoussa & Hofrichter, 1999). Two lignin peroxidase bands obtained in this study at molecular weight of 38 kDa and 45-46 kDa were in the range reported; however, molecular weight of 66 kDa has never been reported for lignin peroxidase in fungi. It was only reported from the purified lignin peroxidase at molecular weight of 66 kDa from the bacteria *Kocuria rosea* MTCC 1532 (Parshetti *et al*., 2012).

In Native-PAGE analysis, all three bands showed positive lignin peroxidase activity. However, enzyme with molecular weight of 66 kDa showed lowest LiP activity (6.4 U/ml) compared to the other two enzymes with molecular weight of 38 kDa (10.82 U/ml) and 45-46 kDa (12.36 U/ml). Although molecular weight of 66 kDa showed lower activity, it still yet to be concluded that the molecular weight of 66 kDa could be isomers of the LiP since it showed the LiP activity in native-PAGE. Arakawa *et al.* (2006) reported that steps of purification of enzyme may cause stress such as shear strain and adsorption. Moreover, the specific structure of lignin peroxidase in which carbohydrate residues are attached to the active site of enzyme may act as an artefact or may play some determining rules in the structure of lignin peroxidase (Schoemaker and Piontek, 1996).

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The aim of this study was to characterise the lignin peroxidase from *Amauroderma rugosum* and study of IL-based ATPS applicability for primary purification of lignin peroxidase. The study depicted that the white-rot fungi *Amauroderma rugosum* has the ability to produce lignin peroxidase. Production of LiP from *Amauroderma rugosum* was optimized with regard to the enhancement of enzyme activity. The optimum enzyme activity of 39.03±3.33 was obtained in the medium comprising PDB, 0.5% yeast and 1% sawdust with the shaking speed of 120 rpm at 27-30°C for 14 days. Then, the cultivated enzyme was subjected for ATPS purification in order to investigate the application of aqueous two-phase system based on ionic liquid for recovery of lignin peroxidase. In IL-based ATPS, LiP was selectively partitioned in the top phase. After optimization of several parameters in terms of tie-line length, volume ratio, pH and addition of more K_2HPO_4, it was found that $[C_4mim]Cl/K_2HPO_4$ system gave the best results at TLL 87.57% (w/w), volume ratio at 1.17 pH 8 and with addition of more K_2HPO_4 into the system up to 5.85 g. The optimum purification factor of top phase obtained from the optimization was 10.76±1.61, with Log K of 0.98±0.02 and optimum yield of 91.80%±0.3. In SDS-PAGE, analysis three bands at molecular weight of 38 kDa, 45-46 kDa and 66 kDa were observed. From native-PAGE analysis it was shown that all three obtained bands represented lignin peroxidase activity.

It can be deduced that extraction of enzyme using IL-based ATPS offers advantages over conventional aqueous two-phase system. It was found that $[C_4mim]Cl$ combined with K_2HPO_4 represented an interesting purification method since the high purification factors of 10.76±1.61 and yield of 91.80%±0.3 was obtained in the study.

5.2. Recommendations for future study

Despite the widespread uses of enzymes in biotechnology, little researches have been conducted to large-scale production of enzymes. Difficulties of set up an appropriate large-scale apparatus have impeded the industrial scale production of enzymes. Laboratory scale of lignin peroxidase production for research purposes from *Phanerochaete chrysosporium* and *Phlebia radiate* have been reported and no study has been done to date producing LiP in large-scale. It is important to study and optimize the appropriate condition for large-scale enzyme production. *Amauroderma rugosum* represented a high titre of LiP production; therefore, more study can be conducted to optimize conditions for large-scale production of LiP from this species. Further study can be conducted to settle the ILs-based ATPS system in industrial scale rather in laboratory scale.

Considering that ILs-based aqueous two-phase system renders advantages of both aqueous two-phase system and unique properties of ILs; however, recycling of ILs should be more consider in terms of economic and environmental issue. The general point is that IL-based ATPS would have more interest if the ILs could be recycled from phase component for reuse. The limitation of IL recycling in this study was separation of lignin peroxidase from $[C_4mim]Cl$ phase. Liu *et al*. (2011) reported that separation of target protein from polymer phase is the main bottlenecks of ATPS. In the study reported by Wu *et al*. (2008) ionic liquid 1-butyl-3-methylimidazolium tetrafluoraborate ($[C_4mim]BF_4$) could be successfully recycled from two-phase system composed of IL, saccharides and water using ethyl acetate. In other study $[C_4mim]BF_4$ which is hydrophilic ionic liquid was recycled from aqueous two-phase system using method based on hydrophobic and hydrophilic ionic liquid (Jiang *et al*., 2007). This method was used for penicillin separation from mixture of components in fermentation broth and also for ionic liquid recovery from aqueous solution. To date, there has been no report

on recycling of 1-butyl-3-methylimidazolium chloride from mixture of enzyme and salt in aqueous solution.

Besides, studies can also target the purified lignin peroxidase from IL-based ATPS to be subjected to some biological tests and also to melanin degradation. It is reported that lignin peroxidase from *Phanerochaete chrysosporium* can decolorize melanin (Woo *et al*., 2004).

REFERENCES

Abadulla, E., Tzanov, T., Costa, S., Robra, K-H., Cavaco-Paulo, A., & Gübitz, G. M. (2000). Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Applied & Environmental Microbiology*, **66**(8): 3357-3362.

Abdel-Raheem, A., & Shearer, C. A. (2002). Extracellular enzyme production by freshwater ascomycetes. *Fungal Diversity*, **11**:1-19.

Abu-Eishah, S. I. (2011). Ionic liquids recycling for reuse. *Ionic Liquids-Classes & Properties*, 239-272.

Abraham, W. R., Nogales, B., Golyshin, P. N., Pieper, D. H., & Timmis, K.N. (2002). Polychlorinated-biphenyl-degrading microbial communities in soils and sediments. *Current Opinion in Microbiology*, **5**(3): 246-253.

Acevedo, F., Pizzul, L., Castillo, M. P., Cuevas, R., & Diez, M. C. (2011). Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus Anthracophyllum discolor. *Journal of Hazardous Materials*, ***185***(1): 212.

Agasoster, T. (1998). Aqueous two-phase partitioning sample preparation prior to liquid chromatography of hydrophilic drugs in blood. *Journal of Chromatography B: Biomedical Sciences & Applications*, **716**(1): 293-298.

Ahammed, S. (2002). *Production and Characterization of lignin peroxidases from Mangrove Ascomvcetes* (Doctoral dissertation). Cochin University of Science and Technology, India.

Akpan, I., Uraih, N., Obuekwe, C. O., & Ikenebomeh, M. J. (2004). Production of ethanol from cassava waste. *Acta Biotech-nologica*, **8**(1): 39-45.

Albertsson, P. A. (1956). Chromatography and partition of cells and cell fragments. *Nature*, **177**: 771-774.

Anonymous. (2004). Accelerating Ionic Liquid Commercialization– Research Needs to Advance New Technology, Prepared by BCS Incorporated, Approved and Issued by the Chemical Industry Vision2020 Technology Partnership. June 2004, Available from http://www.docstoc.com/docs/93827746/Accelerating-Ionic-Liquid-Commercialization-Research-Needs-to

Arakawa, T., & Timasheff, S. N. (1982). Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry*, **21**(25): 6545-6552.

Arakawa,T., Philo, J-S., Ejima, D., Tsumoto, K., & Arisaka, F. (2006). Aggregation analysis of therapeutic proteins, part 1: General aspects and techniques for assessment. *BioProcess International*, **4**(10): 42-43.

Aust, S. D. (1990). Degradation of environmental pollutants by Phanerochaete chrysosporium. *Microbial Ecology*, **20**(1): 197-209.

Bajpai, P. (2004). Biological bleaching of chemical pulps. *Critical Reviews in Biotechnology*, **24**(1): 58.

Barbosa, E. S., Perrone, D., Vendramini, A. L. A., & Leite, S. G. F. (2008).Vanillin production by *Phanerochaete chrysosporium* grown on green coconut agro-industrial husk in solid state fermentation. *BioResources*, **3**(4): 1042-1050.

Barr, D. P., & Aust, S. D. (1994). Mechanisms white rot fungi use to degrade pollutants. *Environmental Science & Technology*, **28**(2): 78A-87A.

Baskir, J. N., Hatton, T. A., & Suter, U. W. (1989). Protein partitioning in two-phase aqueous polymer systems. *Biotechnology & bioengineering*, **34**(4): 541-558.

Becker, H. G., & Sinitsyn, A. P. (1993). Mn-peroxidase from Pleurotus ostreatus: the action on the lignin. *Biotechnology Letters*, **15**(3): 289-294.

Belinky, P., Lasser, H., & Dosoretz, C. (2005). U.S. Patent No.7422734 B2. U.S. R.B.T. (Rakuto Bio Technologies) Ltd.

Benavides, J., & Rito-Palomares, M. (2008). Practical experiences from the development of aqueous two-phase processes for the recovery of high value biological products. *Journal of Chemical Technology & Biotechnology*, **83**(2): 133-142.

Berggren, K., Johansson, H. O., & Yjerneld, F. (1995). Effects of salts and the surface hydrophobicity of proteins on partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide-propylene oxide copolymers. *Journal of Chromatography A*, **718**(1): 67-79.

Berlo, M. V., Ottens, M., Luyben, K. C. A., & van der Wielen, L. A. (2000). Feasible boundaries of aqueous two-phase systems with NH3 and CO2 as recyclable volatile salts. *Biotechnology & Bioengineering*, **70**(1): 65-71.

Biao, N., Junfen, W., & Xuejun, C. (2009). Preparation and recycling of aqueous two-phase systems with pH-sensitive amphiphilic terpolymer PADB. *Biotechnology Progress*, **25**(3):820-824.

Bi, Z. S., Zheng, G. Y., & Li, T. H. (1993). *The macrofungus flora of China's Guangdong province*. Hong Kong: The Chinese University Press.

Blánquez, P. C., Casas, N., Font, X., GabarrelL, X., Sarrá, M., Caminal, G., & Vicent, T. (2004). Mechanism of textile metal dye biotransformation by *Trametes versicolor*. *Water Research*, **38**(8): 2166-2172.

Blume & Nees, T. (1920). Les Polyporacees du Bresil. I. Polyporacees stipites: *Amauroderma rugosum*. Retrieved from http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&MycoBankNr_=107717

Bonomo, R. C. F., Minim, L. A., Coimbra, J. S. R., Fontan, R. C. I., Mendes da Silva, L. H., & Minim, V. P. R. (2006). Hydrophobic interaction adsorption of whey proteins: effect of temperature and salt concentration and thermodynamic analysis. *Journal of Chromatography B*, **844**: 6-14.

Boyle, A. W., Silvin, C. J., Hassett, J. P., Nakas, J. P., & Tanenbaum, S. W. (1992). Bacterial PCB biodegradation. *Biodegradation*, **3**(2-3): 285-298.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Analytical Biochemistry*, **72**: 248-254.

Bridges, N. J., Gutowski, K. E., & Rogers, R. D. (2007). Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt–salt ABS). *Green Chemistry*, **9**(2): 177-183.

Bridges, N. J., & Rogers, R. D. (2008). Can kosmotropic salt/chaotropic ionic liquid (salt/salt aqueous biphasic systems) be used to remove pertechnetate from complex salt waste? *Separation Science & Technology*, **43**(5): 1083-1090.

Bumpus, J. A., & Aust, S. D. (1987). Biodegradation of DDT [1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane] by the white-rot fungus *Phanerochaete chrysosporium*. *Applied & Environmental Microbiology*, **53**(9): 2001-2008.

Buswell, J. A., Odier, E., & Kirk, T. K. (1987). Lignin biodegradation. *Critical Reviews in Biotechnology*, **6**(1): 1-60.

Cabrita, L. D., & Bottomley, S. P. (2004). Protein expression and refolding-a practical guide to getting the most out of inclusion bodies. *Biotechnology Annual Review*, **10**: 31-50.

Cammarata, L., Kazarian, S. G., Salter, P. A., & Welton, T. (2001). Molecular states of water in room temperature ionic liquids. *Physical Chemistry Chemical Physics*, **3**(23): 5192-5200.

Candeias, N. R., Branco, L. C., P. Gois, P. M., Afonso, C. A., & Trindade, A. F. (2009). More Sustainable Approaches for the Synthesis of N-Based Heterocycles. *Chemical Reviews*, **109**(6): 2703-2802.

Cao, Q., Quan, L., He, C., Li, N., Li, K., & Liu, F. (2008). Partition of horseradish peroxidase with maintained activity in aqueous biphasic system based on ionic liquid. *Talanta*, **77**(1): 160-165.

Chae, H. J., Joo, H., & In, M. J. (2001). Utilization of brewer's yeast cells for the production of the food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, **76**(3): 253-258.

Champagne, P. P., & Ramsay, J. A. (2005). Contribution of manganese peroxidase and laccase to dye decoloration by *Trametes versicolor*. *Applied Microbiology & Biotechnology*, **69**(3): 276-285.

Chao, W. L., & Lee, S. L. (1994). Decoloration of azo dyes by three white rot fungi: influence of carbon source. *World Journal of Microbiology & Biotechnology*, **10**(5): 556-559.

Chen, L., Xiao, J. X., & Ma, J. M. (2003). Aqueous two-phase system of catanionic surfactant mixtures formed by adding salts. *Acta P*hysicochimica *Sinica*, **19**(7): 577-579.

Cheng, D. H., Chen, X. W., Wang, J. H., & Fang, Z. L. (2007). An Abnormal Resonance Light Scattering Arising from Ionic-Liquid/DNA/Ethidium Interactions. *Chemistry-A European Journal*, **13**(17): 4833-4839.

Chethana, S., Rastogi, N. K., & Raghavarao, K. S. M. S. (2006). New aqueous two phase system comprising polyethylene glycol and xanthan. *Biotechnology Letters*, **28**(1): 25-28.

Choe, W. S., Nian, R., & Lai, W. B. (2006). Recent advances in biomolecular process intensification. *Chemical Engineering Science*, **61**(3): 886-906.

Christenson, A., Dimcheva, N., Ferapontova, E. E., Gorton, L., Ruzgas, T., Stoica, L., Shleev, S., Yaropolov, A. I., Haltrich, D., Thorneley, R. N. F., & Aust, S. D. (2004). Direct electron transfer between ligninolytic redox enzymes and electrodes. *Electroanalysis*, **16**(13-14): 1074-1092.

Clark, E. D. B. (1998). Refolding of recombinant proteins. *Current Opinion in Biotechnology*, **9**(2): 157-163.

Cole, K. D. (1991). Purification of Plasmid and High Molecular Mass DNA Using Peg-Salt 2-Phase Extraction. *Biotechniques*, **11**(1): 18, 20, 22-4.

Cooper, P. (1993). Removing colour from dye house wastewaters-a critical review of technology available. *Journal of the Society of Dyers and Colourists*, **109**(3): 97-100.

Crawford, R. L. (1981). *Lignin biodegradation & transformation* (p.154). New York: Wiley.

Cripps, C., Bumpus, J. A., & Aust, S. D. (1990). Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. *Applied & Environmental Microbiology*, **56**(4): 1114-1118.

Daugulis, A. J., Axford, D. B., Ciszek, B., & Malinowski, J. J. (1994). Continuous fermentation of high-strength glucose feeds to ethanol. *Biotechnology Letters*, **16**(6): 637-642.

Deacon, J. W. (1997). Modern mycology. Oxford: Blackwell Science

Deive, F. J., Rodríguez, A., Pereiro, A. B., Araújo, J. M. M., Longo, M. A., Coelho, M. A. Z., Canongia Lopes, J. N., Esperança, J. M. S. S., Rebelo, L. P. N., & Marrucho, I. M. (2011). Ionic liquid-based aqueous biphasic system for lipase extraction. *Green Chemistry*, **13**(2): 390-396.

Ding, Z. Y., Peng, Lin., Zhang, L., Gu, Z, H., Shi, G. Y., & Zhang, K. C. (2012). Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. *African Journal of Microbiology Research*, **6**(6): 1147-1157.

Donk, M. A. (1965). The mycological publications of K. B. Boedijin. *Persoonia*, **3**(3): 325-330.

Dreyer, S. E. (2008). *Aqueous two-phase extraction of proteins and enzymes using tetraalkylammonium-based ionic liquids* (Doctoral dissertation). Retrieved from http://d-nb.info/993350089/34.

Dreyer, S., j., & Kragl, U. (2008). Ionic liquids for aqueous two-phase extraction and stabilization of enzymes. *Biotechnology & Bioengineering*, **99**(6): 1416-1424.

Du, Z., Yu, Y. L., & Wang, J. H. (2007). Extraction of Proteins from Biological Fluids by Use of an Ionic Liquid/Aqueous Two-Phase System. *Chemistry-A European Journal*, **13**(7): 2130-2137.

Earle, M. J., & Seddon, K. R. (2000). Ionic liquids. Green solvents for the future. *Pure and Applied Chemistry*, **72**(7): 1391-1398.

Ekberg, B., Sellergren, B., & Albertsson, P. A. (1985). Direct Chiral Resolution in an Aqueous 2-Phase System Using the Countercurrent Distribution Principle. *Journal of Chromatography*, **333**(1): 211-214.

Elevitch, C. R., (Ed.). (2004). *The Overstory Book: Cultivating Connections with Trees*. (2nd ed.) (pp. 111). Holualoa, HI: Permanent Agriculture Resources.

Erden, E., Ucar, M. C., Gezer, T., & Pazarlioglu, N. K. (2009). Screening for ligninolytic enzymes from autochthonous fungi and applications for decolorization of Remazole Marine Blue. *Brazilian Journal of Microbiology*, **40**(2): 346-353.

Fakoussa, R. M., & Hofrichter, M. (1999). Biotechnology and microbiology of coal degradation. *Applied Microbiology & Biotechnology*, **52**: 25-40.

Fengel, D., & Wegener, G. (Eds.). (1983). *Wood: Chemistry, Ultrastructure, Reactions* (p. 132). Berlin: Walter de Gruyter.

Ferrer, I, Dezotti, M., & Durán, N. (1991). Decolorization of Kraft effluent by free and immobilized lignin peroxidases and horseradish peroxidase. *Biotechnology Letters*, **13**(8): 577-582.

Field, J. A., De Jong, E., Costa, G. F., & De Bont, J. A. (1992). Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. *Applied & Environmental Microbiology*, **58**(7): 2219-2226

Freire, M. G., Carvalho, P. J., Silva, A. M. S., Santos, L. M. N. B. F., Rebelo, L. P. N., Marrucho, I. M., & Coutinho, J. A. (2009). Ion specific effects on the mutual solubilities of water and hydrophobic ionic liquids. *The Journal of Physical Chemistry B*, **113**(1): 202-211.

Freire, M. G., Neves, C. M., Silva, A. M., Santos, L. M., Marrucho, I. M., Rebelo, L. P. N., Shah, J. K., Maginn, E. J., & Coutinho, J. A. P. (2010). 1H NMR and molecular dynamics evidence for an unexpected interaction on the origin of salting-in/salting-out phenomena. *The Journal of Physical Chemistry B*, **114**(5): 2004-2014.

Freydell, E. J., Ottens, M., Eppink, M., van Dedem, G., & van der Wielen, L. (2007). Efficient solubilization of inclusion bodies. *Biotechnology Journal*, **2**(6): 678-684.

Fujita, K., Forsyth, M., MacFarlane, D. R., Reid, R. W., & Elliott, G. D. (2006). Unexpected improvement in stability and utility of cytochrome c by solution in biocompatible ionic liquids. *Biotechnology & Bioengineering*, **94**(6): 1209-1213.

Ghosh, R. (2003). *Protein Bioseparation: An Overview*. In *Protein Bioseparation Using Ultrafiltration: Theory, Applications & New Developments* (pp.6). London: Imperial College Press.

Ghosh, S., Vijayalakshmi, R., & Swaminathan, T. (2004). Evaluation of an alternative source of dextran as a phase forming polymer for aqueous two-phase extractive system. *Biochemical Engineering Journal*, **21**(3): 241-252.

Glenn, J. K., Morgan, M.A., Mayfield, M. B., Kuwahara, M., & Gold, M. H. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochemistry Biophysics Research Community*, **114**(3): 1077-83.

Gold, M., Wariishi, H., & Valli, K. (1989). Extracellular peroxidases involved in lignin degradation by the white rot basidiomycete Phanerochaete chrysosporium. In Whitaker, J. R., & Sonnet, P. E, *Biocatalysis in Agricultural Biotechnology* (pp. 120-140). Washington, DC : American Chemical Society

Gomes, E., Aguiar, A. P., Carvalho, C. C., Bonfá, M. R. B., Da Silva, R., & Boscolo, M. (2009). Ligninases production by Basidiomycetes strains on lignocellulosic agricultural residues and their application in the decolorization of synthetic dyes. *Brazilian Journal of Microbiology*, **40**(1): 31-39.

González, E. J., Alonso, L., & Domínguez, Á. (2006). Physical properties of binary mixtures of the ionic liquid 1-methyl-3-octylimidazolium chloride with methanol, ethanol, and 1-propanol at T=(298.15, 313.15, and 328.15) K and at P= 0.1 MPa. *Journal of Chemical & Engineering Data*, **51**(4): 1446-1452.

Guerra, A., Mendonça, R., Ferraz, A., Lu, F., & Ralph, J. (2004). Structural characterization of lignin during Pinus taeda wood treatment with Ceriporiopsis subvermispora. *Applied & Environmental Microbiology*, **70**(7): 4073-4078.

Guillén, F., Martínez, M. J., & Gutiérrez, A. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*, **8**: 195-204.

Gündüz, U. (2000). Partitioning of bovine serum albumin in an aqueous two-phase system: optimization of partition coefficient. *Journal of Chromatography B: Biomedical Sciences & Applications*, **743**(1-2): 259-262.

Gutowski, K. E., Broker, G. A., Willauer, H D., Huddleston, J. G., Swatloski, R. P., Holbrey, J. D., & Rogers, R. D. (2003). Controlling the aqueous miscibility of ionic liquids: Aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *Journal of the American Chemical Society*, **125**(22): 6632-6633.

Hammel, K. E., & Cullen, D. (2008). Role of fungal peroxidases in biological ligninolysis. *Current Opinion in Plant Biology*, **11**(3): 349-355.

Harkin, J. M., & Obst, J. R. (1973). Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*, **29**(4): 381-387.

Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *FEMS Microbiology Reviews*, **13**(2): 125-135.

Have, R. T., Hartmans, S., Teunissen, P. J. M., & Field, J. A. (1998). Purification and characterization of two lignin peroxidase isozymes produced by Bjerkandera sp. Strain. *FEBS Letters*, **422**(3): 391-394.

He, C., Li, S., Liu, H., Li, K., & Liu, F. (2005). Extraction of testosterone and epitestosterone in human urine using aqueous two-phase systems of ionic liquid and salt. *Journal of Chromatography, A*, **1082**(2): 143-149.

Headon, D. R., & Walsh, G. (1994). The Industrial Production of Enzymes. *Biotechnology Advances*, **12**(4): 635-646.

Higuchi, T. (2004). Microbial degradation of lignin: role of lignin peroxidase, manganese peroxidase, and laccase. *Proceedings of the Japan Academy. Series B Physical & Biological Sciences*, **80**(5): 204-214.

Holbrey, J. D., Visser, A. E., Spear, S. K., Reichert, W. M., Swatloski, R. P., Broker, G. A., & Rogers, R. D. (**2003**). Mercury (II) partitioning from aqueous solutions with a new, hydrophobic ethylene-glycol functionalized bisimidazolium ionic liquid. *Green Chemistry*, **5**(2):129-135.

Hou, H., Zhou, J., Wang, J., Du, C., & Yan, B. (2004). Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochemistry*, **39**(11): 1415-1419.

Huddleston, J. G., Willauer, H. D., Boaz, K. R., & Rogers, R. D. (1998a). Separation and recovery of food coloring dyes using aqueous biphasic extraction chromatographic resins. *Journal of Chromatography B-AnalyticalTechnologies in the Biomedical & Life Sciences*, **711**(1-2): 237-244.

Huddleston, J. G, & Rogers, R. (1998b). Room temperature ionic liquids as novel media for 'clean'liquid–liquid extraction. *Chemical Communications*, (16): 1765-1766.

Hudson, H. J. (1992). *Fungal biology*. London: Edward Arnold.

Jiang[a], B., Li, Z. G., Dai, J. Y., Zhang, D. J., & Xiu, Z. L. (2009). Aqueous two-phase extraction of 2, 3-butanediol from fermentation broths using an ethanol/phosphate system. *Process Biochemistry*, **44**(1): 112-117.

Jiang, R., Huang, Y. X., Zhao, J. X., & Huang, C. C. (2008). Transformation of Vesicles in Aqueous Two-Phase System of an Anionic Gemini Surfactant and a Cationic Conventional Surfactant Mixture. *Chinese Journal of Chemistry*, **26**(4): 635-639.

Jiang[b], R., Huang, Y. X., Zhao, J. X., & Huang, C. C. (2009). Aqueous two-phase system of an anionic gemini surfactant and a cationic conventional surfactant mixture. *Fluid Phase Equilibria*, **277**(2): 114-120.

Jiang, Y., Xia, H., Guo, C., Mahmood, I., & Liu, H. (2007). Phenomena and mechanism for separation and recovery of penicillin in ionic liquids aqueous solution. *Industrial & Engineering Chemistry Research*, **46**(19): 6303-6312.

Jing, D. B. (2010). Improving the simultaneous production of laccase and lignin peroxidase from *Streptomyces lavendulae* by medium optimization. *Bioresource Technology*, **101**: 7592-7597.

Jing, D. B., Li, P. J., Xiong, X. Z., & Wang, L. H. (2007). Optimization of cellulase complex formulation for peashrub biomass hydrolysis. *Applied Microbiology & Biotechnology*, **75**: 793-800.

Johansson, H. O., Persson, J., & Tjerneld, F. (1999). Thermoseparating water/polymer system: A novel one-polymer aqueous two-phase system for protein purification. *Biotechnology & Bioengineering*, **66**(4): 247-257.

Jordan, P., & Vilter, H. (1991). Extraction of proteins from material rich in anionic mucilages: Partition and fractionation of vanadate-dependent bromoperoxidases from the brown algae *Laminaria digitata* and *L.saccharina* in aqueous polymer two-phase systems. *Biochimical Biophysical Acta(BBA)-General Subjects*, **1073**(1): 98-106.

Kasinath, A., Novotny, C., Svobodova, K., Patel, K. C., & Saseck, V. (2003). Decolorization of synthetic dyes by *Irpex lacteus* in liquid cultures and packed-bed bioreactor. *Enzyme & Microbial Technology*, **32**(1): 167-173.

Kapich, A. N., Prior, B. A., Botha, A., Galkin, S., Lundell, T., & Hatakka, A. (2004). Effect oflignocellulose-containing substrates onproduction of ligninolytic peroxidases in submerged cultures of *Phanerochaete chrysosporium* ME-446. *Enzyme and Microbial Technology*, **34**:187-195.

Kaul, A. (2000). The Phase Diagram. In Hatti-Kaul, R. (Eds.). *Aqueous Two-phase Systems: Methods and Protocols* (pp. 11-21). Totowa, New Jersey, (NJ): Humana Press

Kirk, T. K., & Farrell, R. L. (1987). Enzymatic" combustion": the microbial degradation of lignin. *Annual Reviews in Microbiology*, **41**(1): 465-501.

Koningsveld, R., Stockmayer, W. H., & Nies, E. (2001). *Polymer phase diagrams: a textbook*. Oxford: Oxford University Press.

Kuboi, R., Umakoshi, H., & Komasawa, I. (1995). Extractive Cultivation of Escherichia-Coli Using Poly (Ethylene Glycol) Phosphate Aqueous 2-Phase Systems to Produce Intracellular Beta-Galactosidase. *Biotechnology Progress*, **11**(2): 202-207.

Kula, M. R., Kroner, K. H., & Hustedt, H. (1982). Purification of enzymes by liquid-liquid extraction. *Advances in Biochemical Engineering*, **24**: 73-118.

Kunamneni, A., Plou, F.J., Ballesteros, A., & Alcalde, M. (2008). Laccases and their applications: A Patent Review. *Recent Patents on Biotechnology*, **2**: 10-24.

Kwon, Y. J., Kaul, R., & Mattiasson ,B. (1996). Extractive lactic acid fermentation in poly(ethyleneimine)-based aqueous two-phase system. *Biotechnology and Bioengineering*, **50**(3): 280-290.

Laali, K. K., & Gettwert, V. J. (2001). Fluorodediazoniation in ionic liquid solvents: new life for the Balz–Schiemann reaction. *Journal of Fluorine Chemistry*, **107**(1): 31-34.

Laemmli, U. K., & Favre, M. (1973). Maturation of the head of bacteriophage T4. I. DNA packaging events. *Journal of Molecular Biol*ogy, **80**:575-599.

Laszlo, J. A., & Compton, D. L. (2001). α-Chymotrypsin catalysis in imidazolium-based ionic liquids. *Biotechnology & Bioengineering*, **75**(2): 181-186.

Lau, R. M., Sorgedrager, M. J., Carrea, G., van Rantwijk, F., Secundo, F., & Sheldon, R. A. (2004). Dissolution of Candida antarctica lipase B in ionic liquids: effects on structure and activity. *Green Chemistry*, **6**(9):483-487.

Leisola, M. S., Kozulic, B., Meussdoerffer, F., & Fiechter, A. (1987). Homology among multiple extracellular peroxidases from Phanerochaete chrysosporium. *Journal of Biological Chemistry*, **262**(1): 419-424.

Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtaś-Wasilewska, M., Cho, N. S., Hofrichter, M., & Rogalski, J. (1999). Biodegradation of lignin by white rot fungi. *Fungal Genetics & Biology*, **27**(2): 175-185.

Leonowizc, A., & Grzywnowicz, K. (1981). Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme & Microbial Technology*, **3**: 55-58.

Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J. F., Ceccaldi, B. C., Brunerie, P., & Asther, M. (1996). A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger*and *Pycnoporus cinnabarinus*. *Journal of Biotechnology*, **50**(2-3): 107-113.

Li, S., He, C., Liu, H., Li, K., & Liu, F. (2005). Ionic liquid-based aqueous two-phase system, a sample pretreatment procedure prior to high-performance liquid chromatography of opium alkaloids. *Journal of Chromatography B*, **826**(1): 58-62.

Li, Z., Pei, Y., Wang, H., Fan, J., & Wang, J. (2010). Ionic liquid-based aqueous two-phase systems and their applications in green separation processes. *TrAC Trends in Analytical Chemistry*, **29**(11): 1336-1346.

Lin, D. Q., Wu, Y. T., Mei, L. H., Zhu, Z. Q., & Yao, S. J. (2003). Modeling the protein partitioning in aqueous polymer two-phase systems: influence of polymer concentration and molecular weight. *Chemical Engineering Science*, **58**(13): 2963-2972.

Lin, Y. K., Ooi, C. W., Ramanan, R. N., Ariff, A., & Ling, T.C. (2012). Recovery of Human Interferon Alpha-2b from Recombinant Escherichia coli by Aqueous Two-Phase System. *Separation Science & Technology*, **47**(7): 1023-1030.

Lisheng, N. Y. H. (2007) Mixed Cationic and Anionic Surfactant Systems. *Progress in Chemistry*, **9**.

Liu, Q., Yu, J., Li, W., Hu, X., Xia, H., Liu, H., & Yang, P. (2006). Partitioning behavior of penicillin G in aqueous two phase system formed by ionic liquids and phosphate. *Separation Science & Technology*, **41**(12): 2849-2858.

Liu, Y., Yu, Y. L., Chen, M. Z., & Xiao, X. (2011). Advances in aqueous two-phase systems and applications in protein separation and purification. *Canadian Journal on Chemical Engineering & Technology*, **2**(2): 1-7.

Lomascolo, A., Stentelaire, C., Asther, M., & Lesage-Meessen, L. (1999). Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. *Trends in Biotechnology*, **17**(7): 282-289.

Lu, M., Albertsson, P. Å., Johansson, G., & Tjerneld, F. (1994). Partitioning of proteins and thylakoid membrane vesicles in aqueous two-phase systems with hydrophobically modified dextran. *Journal of Chromatography A*, **668**(1): 215-228.

Maciel, M. J. M., Silva, A. S., & Riberio, H. C. T. R. (2010). Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: A review. *Electronic Journal of Biotechnology*, **13**(6): 14-15.

Martinez-Aragon, M., Burghoff, S., Goetheer, E. L. V., & de Haan, A. B. (2009). Guidelines for solvent selection for carrier mediated extraction of proteins. *Separation & Purification Technology*, **65**(1): 65-72.

Massadeh, M. I., Fraij, A., & Fandi, K. (2010). Effect of carbon sources on the extracellular lignocellulolytic enzymatic system of Pleurotus *sajor-caju*. *Jordan Journal Biological Science*, **3**(2): 51-54.

Mattiasson, B. (1986). Application of aqueous two-phase systems in biotechnology. *Trends Biotechnology*, **1**:16-20.

Mayolo-Deloisa, K., Trejo-Hernandez, M.D.R., & Rito-Palomares, M. (2009). Recovery of laccase from the residual compost of *Agaricus bisporus* in aqueous two-phase systems. *Process Biochemistry*, **44**: 435-439.

Mazzola, P. G., Lopes, A. M., Hasmann, F. A., Jozala, A. F., Penna, T. C. V., Magalhaes, P. O., Rangel-Yagui, C. O., & Pessoa, A. (2008). Liquid-liquid extraction of biomolecules: an overview and update of the main techniques. *Journal of Chemical Technology & Biotechnology*, **83**:143-157.

McKay, G. (1979). Waste color removal from textile effluents. *Journal American Dyestuff Reporter*, **86**(4): 29-36.

Mester, T., Pena, M., & Field, J. A. (1996). Nutrient regulation of extracellular peroxidases in the white rot fungus, Bjerkandera sp. strain BOS55. *Applied Microbiology & Biotechnology*, **44**(6): 778-784.

Middaugh, C. R., & Lawson, E. Q. (1980). Analysis of Protein Association by Partitioning in Aqueous 2-Phase Polymer Systems - Applications to the Tetramer-Dimer Dissociation of Hemoglobin. *Analytical Biochemistry*, **105**(2):364-368.

Moniruzzaman, M., Kamiya, N., Nakashima, K., & Goto, M. (2008). Water-in-ionic liquid microemulsions as a new medium for enzymatic reactions. *Green Chemistry*, **10**(5):497-500.

Naganagouda, K., & Mulimani, V. H. (2008). Aqueous two-phase extraction (ATPE): An attractive and economically viable technology for downstream processing of Aspergillus oryzae α-galactosidase. *Process Biochemistry*, **43**(11): 1293-1299.

Nakashima, K., Maruyama, T., Kamiya, N., & Goto, M. (2005). Comb-shaped poly (ethylene glycol)-modified subtilisin Carlsberg is soluble and highly active in ionic liquids. *Chemical Communications*, (34): 4297-4299.

Neves, C. M., Ventura, S. P., Freire, M. G., Marrucho, I. M., & Coutinho, J. A. (2009). Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems. *The Journal of Physical Chemistry B*, **113**(15): 5194-5199.

Nfor, B. K., Ahamed, T., van Dedem, G. W., van der Wielen, L. A., van de Sandt, E. J., Eppink, M. H., & Ottens, M. (2008). Design strategies for integrated protein purification processes: challenges, progress and outlook. *Journal of Chemical Technology & Biotechnology*, **83**(2): 124-132.

Niladevi, K. N., & Prema, P. (2005). Mangrove actinomycetes as the source of ligninolytic enzymes. *Actinomycetologica*, **19**(2): 40-47.

Ohno, H., Suzuki, C., Fukumoto, K., Yoshizawa, M., & Fujita, K. (2003). Electron transfer process of poly (ethylene oxide)-modified cytochrome c in imidazolium type ionic liquid. *Chemistry Letters*, **32**(5): 450-451.

Ohtsubo, Y., Kudo, T., Tsuda, M., & Nagata, Y. (2004). Strategies for bioremediation of polychlorinated biphenyls. *Applied Microbiology & Biotechnology*, **65**(3): 250-258.

Olivier-Bourbigou, H., & Magna, L. (2002). Ionic liquids: perspectives for organic and catalytic reactions. *Journal of Molecular Catalysis a-Chemical*, **182**(1):419-437.

Ooi, C. W., Tey, B. T., Hii, S. L., Ariff, A., Wu, H. S., Lan, J. C. W., Juang, R. S., Kamal, S. M. M., & Ling, T. C. (2009). Direct purification of *Burkholderia Pseudomallei* lipase from fermentation broth using aqueous two-phase systems. *Biotechnology and Bioprocess Engineering*, **14**(6): 811-818.

Oyadomari, M., Shinohara, H., Johjima, T., Wariishi, H., & Tanaka, H. (2003). Electrochemical characterization of lignin peroxidase from the white-rot basidiomycete Phanerochaete chrysosporium. *Journal of Molecular Catalysis B: Enzymatic*, **21**(4): 291-297.

Pandey, S. (2006). Analytical applications of room-temperature ionic liquids: A review of recent efforts. *Analytica Chimica Acta*, **556**(1): 38-45.

Parshetti, G. K., Parshetti, S., Kalyani, D. C., Doong, R-a., & Govindwar, A. P. (2012) Industrial dye decolourizing lignin peroxidase from *Kocuria rosea* MTTC 1523. *Annals of Microbiology*, **62**(1): 217-223.

Pei, Y. C., Wang, J. J., Xuan, X. P., Fan, J., & Fan, M. (2007). Factors affecting ionic liquids based removal of anionic dyes from water. *Environmental Science & Technology*, **41**(14): 5090-5095.

Pei, Y., Wang, J., Wu, K., Xuan, X., & Lu, X. (2009). Ionic liquid-based aqueous two-phase extraction of selected proteins. *Separation & Purification Technology*, **64**(3): 288-295.

Périé, F. H., & Gold, M. H. (1991). Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. *Applied and Environmental Microbiology*, **57**(8): 2240-2245.

Périé, F. H., Sheng, D., & Gold, M. H. (1996). Purification and characterization of two manganese peroxidase isozymes from the white-rot basidiomycete *Dichomitus squalens*. *Biochimica et Biophysica Acta*, **1297**(2): 139.

Persson, J., Johansson, H. O., Galaev, I., Mattiasson, B., & Tjerneld, F. (2000). Aqueous polymer two-phase systems formed by new thermoseparating polymers. *Bioseparation*, **9**(2): 105-116.

Pietruszka, N., Galaev, I. Y., Kumar, A., Brzozowski, Z. K., & Mattiasson, B. (2000). New polymers forming aqueous two-phase polymer systems. *Biotechnology Progress*, **16**(3): 408-415.

Piontek, K., Smith, A. T., & Blodig, W. (2001). Lignin peroxidase structure and function. *Biochemical Society Transactions*, **29**(Pt 2): 111-6.

Plechkova, N. V., & Seddon, K. R. (2008). Applications of ionic liquids in the chemical industry. *Chemical Society Reviews*, **37**(1): 123-150.

Pointing, S. B. (2001). Feasibility of bioremediation by white-rot fungi. *Applied Microbiology & Biotechnology*, **57**(1-2): 20-33.

Pointing, S. B., Pelling, A. L., Smith, G. J., Hyde, K. D., & Reddy, C. A. (2005). Screening of basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific sequences. *Mycological Research*, **109**(1): 115-124.

Przybycien, T. M., Pujar, N. S., & Steele, L. M. (2004). Alternative bioseparation operations: life beyond packed-bed chromatography. *Current Opinion in Biotechnology*, **15**(5): 469-478.

Ramelmeier, R. A., Terstappen, G. C., & Kula, M. R. (1991). The partitioning of cholesterol oxidase in Triton X-114- based aqueous two-phase systems. *Bioseparation*, **2**(5): 315-24.

Ratanapongleka, K., & Phetsom, J. (2011). Extraction in Two-Phase Systems and some properties of Laccase from *Lentinus polychrous. World Academy of Science, Engineering & Technology*, **57**: 812-815.

Renganathan, V., Miki, K., & Gold, M. H. (1985). Multiple molecular forms of diarylpropane oxygenase, an H2O2-requiring, lignin-degrading enzyme from Phanerochaete chrysosporium. *Archives of Biochemistry & Biophysics*, **241**(1): 304-14.

Rito-Palomares M. (2004). Practical application of aqueous two-phase partition to process development for the recovery of biological products. *Journal of Chromatography B-Analytical Technologies in the Biomedical & Life Sciences*, **807**(1): 3-11.

Rito-Palomares M., & Middelberg, A. P. J. (2002). Aqueous two-phase systems for the recovery of a recombinant viral coat protein from *Escherichia coli*. *Journal of Chemical Technology & Biotechnology*, **77**(9): 1025-1029.

Riu, J., Schönsee, I., & Barceló, D. (1998). Determination of sulfonated azo dyes in groundwater and industrial effluents by automated solid-phase extraction followed by capillary electrophoresis-mass spectrometry. *Journal of Mass Spectrometry*, **33**(7): 653-663.

Robles-Hernández, L., Gonzales-Franco, A. C., Crawford, D. L., & Chun, W. W. C. (2008). Review of environmental organopollutants degradation by white-rot basidiomycete mushrooms. *Tecnociencia Chihuahua*, **2**(1): 32-39.

Rodríguez, S., & Toca, J. L. (2006). Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances*, **24**(5): 500-513.

Rodríguez, S., Sanromán, M., & Gübitz, G. M. (2005). Influence of redox mediators and metal ions on synthetic acid dye decolourization by crude laccase from *Trametes hirsuta*. *Chemosphere*, **58**(4): 417-422.

Rogalski, J., Fiedurek, J., & Leonowicz, A. (2001). Production of lignolytic and feed-back type enzymes by White rot fungi on different media. *Enzyme Microbiology Technology*, **52**: 149-160.

Rojas, E. E. G., dos Reis Coimbra, J. S., Minim, L. A., Zuniga, A. D. G., Saraiva, S. H., & Minim, V. P. R. (2004). Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems. *Process Biochemistry*, **39**(11): 1751-1759.

Rogers, R. D., Bond, A. H., Bauer, C. B., Zhang, J. H., & Griffin, S.T. (1996). Metal ion separations in polyethylene glycolbased aqueous biphasic systems: Correlation of partitioning behavior with available thermodynamic hydration data. *Journal of Chromatography B-Biomedical Applications*, **680**(1-2): 221-229.

Roobol-Bóza, M., Dolby, V., Doverskog, M., Barrefelt, A., Lindqvist, F., Oppermann, U. C., Alstine, K. K. V., & Tjerneld, F. (2004). Membrane protein isolation by in situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems: Purification of the human type 1 11β-hydroxysteroid dehydrogenase. *Journal of Chromatography A*, **1043**(2): 217-223.

Rosa, P. A. J., Azevedo, A. M., Sommerfeld, S., Mutter, M., Aires-Barros, M. R., & Bäcker, W. (2009). Application of aqueous two-phase systems to antibody purification: a multi-stage approach. *Journal of Biotechnology*, **139**(4): 306-313.

Roushdy, M. M., Abdel-Shakour, E. H., & El-Agamy, E. I. (2011). Biotechnological Approach for Lignin Peroxidase (LiP) Production from Agricultural Wastes (Rice Husk) by *Cunninghamella elegans*. *Journal of American Science*, **7**(5).

Ruiz-Angel, M. J., Pino, V., Carda-Broch, S., & Berthod, A. (2007). Solvent systems for countercurrent chromatography: An aqueous two phase liquid system based on a room temperature ionic liquid. *Journal of Chromatography A*, **1151**(1): 65-73.

Ryvarden, L. (1991). *Genera of Polypores: Nomenclature and Taxonomy*. Oslo, Norway: Fungiflora.

Sanchez-Ferrer, A., Bru, R., & Garcia-Carmona, F. (1989). Novel Procedure for Extraction of a Latent Grape Polyphenoloxidase Using Temperature-Induced Phase-Separation in Triton X-114. *Plant Physiology,* **91**(4): 1481-1487.

Sarkanen, K. V. & Ludwig, C. H. (Eds.). (1971). *Lignin: Occurrence, Formation, Structure & Reactions*. New York. NY: Wiley-Interscience.

Schoemaker, H. E., & Piontek, K. (1996). On the interaction of lignin peroxidase with lignin. *Pure & Applied Chemistry*, **68**: 2089–2096.

Score, A. J., Palfreyman, J. W., & White, N. A. (1997). Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *International Biodeterioration & Biodegradation*, **39**(2): 225-233.

Sellers, S. P., & Maa, Y-F. (2005). Principles of biopharmaceutical protein formulation - An overview. *Methods in Molecular Biology*, **308**: 243-263.

Sheldon, R., Lau, R. M., Sorgedrager, M. J., van Rantwijk, F., & Seddon, K. R. (2002). Biocatalysis in ionic liquids. *Green Chemistry*, **4**(2): 147-151.

Shin, K. S. (2004). The role of enzymes produced by white-rot fungus *Irpex lacteus* in the decolorization of the textile industry effluent. *The Journal of Microbiology*, **42**(1): 37-41.

Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K., & Ling, T. C. (2012). Primary recovery of lipase derived from *Burkholderia cenocepacia* strain ST8 and recycling of phase components in an aqueous two-phase system. *Biochemical Engineering Journal*, **60**: 74-80.

Sigoillot, C., Camarero, S., Vidal, T., Record, E., Asther, M., Perez-Boada, M., Martinez. M. J., Sigoillot, J. C., Asther, M., Colom, J. F., & Martinez, A. T. (2005). Comparison of different fungal enzymes for bleaching high-quality paper pulps. *Journal of Biotechnology*, **115**(4): 333-343.

Sikdar, S. K., Cole, K. D., Stewart, R. M., Szlag, C., Todd, P., & Cabezas, H. (1991). Aqueous 2-Phase Extraction in Bioseparations - an Assessment. *Bio-Technology*, **9**(3): 252-256.

Sivakami, V., Ramachandran, B., Srivathsan, J., Kesavaperumal, G., Smily, B., & Kumar, D. M. (2012). Production and optimization of laccase and lignin peroxidase by newly isolated *Pleurotus ostreatus* LIG 19. *Journal of Microbiology & Biotechnology Research*, **2**(6): 875-881.

Sivars, U., & Tjerneld, F. (2000). Mechanisms of phase behaviour and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins. *Biochimica et Biophysica Acta (BBA)-General Subjects*, **1474**(2): 133-146.

Taboada, M. E., Graber, T. A., Asenjo, J. A., & Andrews, B. A. (2000). Drowning-out crystallisation of sodium sulphate using aqueous two-phase systems. *Journal of Chromatography B-Analytical Technologies in the Biomedical & Life Sciences*, **743**(1-2): 101-105.

Tani, H., Kamidate, T., & Watanabe, H. (1998) Aqueous micellar two-phase systems for protein separation. *Analytical Science*, **14**(5): 875-888.

Tanuja, S., Srinivas, N. D., Gowthaman, M. K., & Raghavarao, K. S. M. S. (2000). Aqueous two-phase extraction coupled with ultrafiltration for purification of amyloglucosidase. *Bioprocess Engineering*, **23**(1): 63-68.

Thomas, D. R., Carswell, K. S., & Georgiou, G. (1992). Mineralization of biphenyl and PCBs by the white rot fungus Phanerochaete chrysosporium. *Biotechnology & Bioengineering*, **40**(11): 1395-1402.

Thommes, J., & Etzel, M. (2007). Alternatives to chromatographic separations. *Biotechnology Progress*, **23**(1): 42-45.

Tien, M., & Kirk, T. K. (1988). Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods in Enzymology*, **161**: 238-249.

Tien, M., Kirk, T. K., Bull, C., & Fee, J. A. (1986). Steady-state and transient-state kinetic studies on the oxidation of 3, 4-dimethoxybenzyl alcohol catalyzed by the ligninase of Phanerocheate chrysosporium Burds. *Journal of Biological Chemistry*, **261**(4): 1687-1693.

Trejo-Hernandez, M. R., Lopez-Munguia, A., & Quintero Ramirez, R. (2001). Residual compost of *Agaricus bisporus* as a source of crude laccase for enzymic oxidation of phenolic compounds. *Process Biochemistry*, **36**(7): 635-639.

Valli, K. H. A. D. A. R., Brock, B. J., Joshi, D. K., & Gold, M. H. (1992). Degradation of 2, 4-dinitrotoluene by the lignin-degrading fungus Phanerochaete chrysosporium. *Applied & Environmental Microbiology*, **58**(1): 221-228.

Vares, T., Kalsi, M., & Hatakka, A. (1995). Lignin peroxidases, manganese peroxidases, and other lignolytic enzymes produced by *Phlebia radiate* during solid-state fermentation of wheat straw. *Applied Environmental Microbiology*, **61**(10): 3515.

Ventura, S. P., Neves, C. M., Freire, M. G., Marrucho, I. M., Oliveira, J., & Coutinho, J. A. (2009). Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems. *The Journal of Physical Chemistry B*, **113**(27): 9304-9310.

Ventura, S. P., Sousa, S. G., Serafim, L. S., Lima, Á. S., Freire, M. G., & Coutinho, J. A. (2011). Ionic liquid based aqueous biphasic systems with controlled pH: the

ionic liquid cation effect. *Journal of Chemical & Engineering Data*, **56**(11): 4253-4260.

Vojdani, F. (1996). *Solubility*. In: Hall, G. M. (ed.). Methods of testing protein functionality. United Kingdom, London: Blackie Academic and Professional, pp. 11-60.

Wagner, M., & Hilgers, C. (2008). Quality aspects and other questions related to commercial ionic liquid production. In Wasserscheid, P., & Welton, T (Eds.), *Ionic Liquids in Synthesis* (2nd ed., Vol. 1, pp. 26-45). Weinheim: Wiley-VCH.

Wang, J., Pei, Y., Zhao, Y., & Hu, Z. (2005). Recovery of amino acids by imidazolium based ionic liquids from aqueous media. *Green Chemistry*, **7**(4): 196-202.

Wang, W., Wan, J., Ning, B., Xia, J., & Cao, X. (2008). Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. *Journal of Chromatography A*, **1205**(1): 171-176.

Wen, X., Jia, Y., & Li, J. (2009). Degradation of tetracycline and oxytetracycline by crude lignin peroxidase prepared from *Phanerochaete chrysosporium*-a white rot fungus. *Chemosphere*, **75**(8): 1003-1007.

Wong, Y., & Yu, J. (1999). Laccase-catalysed decolorization of synthetic dyes. *Water Research*, **30**(16): 3512-3520.

Woo, S. H., Cho, J. S., Lee, B. S., & Kim, E. K. (2004). Decolorization of melanin by lignin peroxidase from *phanerochaete chrysosporium*. *Biotechnology & Bioprocess Engineering*, **9**: 256-260.

Wu, B., Zhang, Y., & Wang, H. (2008). Phase behavior for ternary systems composed of ionic liquid+ saccharides+ water. *The Journal of Physical Chemistry B*, **112**(20): 6426-6429.

Xie, H. G., Wang, Y. J., & Sun, M. (2006). Modeling of the partitioning of membrane protein and phase equilibria for Triton X-100–salt aqueous two-phase systems using a modified generalized multicomponent osmotic virial equation. *Process Biochemistry*, **41**(3): 689-696.

Yadav, J. S., Quensen, J. F., Tiedje, J. M., & Reddy, C. A. (1995). Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus Phanerochaete chrysosporium as evidenced by congener-specific analysis. *Applied & Environmental Microbiology*, **61(**7): 2560-2565.

Zhu, J. H., Yan, X. L., Chen, H. J., & Wang, Z. H. (2007). In situ extraction of intracellular L-asparaginase using thermoseparating aqueous two-phase systems. *Journal of Chromatography A*, **1147**(1): 127-134.

Zijlstra, G. M., deGooijer, C. D., vanderPol, L. A., & Tramper, J. (1996). Design of aqueous two-phase systems supporting animal cell growth: A first step toward extractive bioconversions. *Enzyme & Microbial Technology*, **19**(1): 2-8.

APPENDICES

Appendix A: Analytical technique

A.1: Determination of laccase activity

Reagent

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

Procedure

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

Table A. 1: Assay mixture for determination of laccase activity

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

Calculation of unit of laccase activity

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

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

A.2: Determination of lignin peroxidase activity

Reagent

Hundred mM Sodium Tartrate buffer, pH 3.0; Thirty mM veratryl alcohol (VA); zero point five mM H_2O_2. (Have *et al.*, 1997)

Procedure for preparation of veratraldehyde (VAD) standard plot

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 adjusted to 3.0mLwith buffer and the test tubes were mixed with a vortex. Finally, 0.5mL of 0.5 mM hydrogen peroxide was added and then the reaction was read at wavelength λ = 310nm.

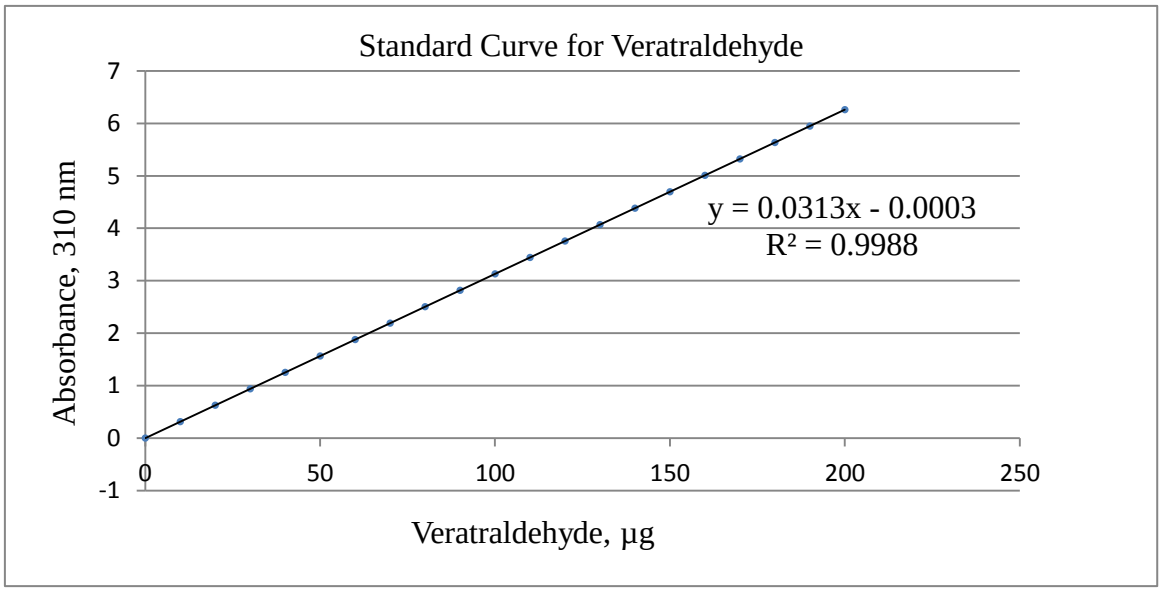

Figure A. 1: Standard curve for veratraldehyde

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

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

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

Procedure

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 λ =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. 2: Assay mixtures for determination of LiP 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.3: Determination of soluble protein concentration

Reagent

Hundred milligrams (100mg) of Coomasive Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. Hundred millilitres (100ml) of 85% (w/v) phosphoric acid was

added to the solution. The solution was toped to a final volume of I L using distilled water (Bradford, 1976).

Procedure for preparation of protein calibration lot

The Bovine Serum Albumin (BSA) solution was made from crystalline BSA with the different protein concentration ranging from 10 to 100µg in a volume ranging from 0.1mL to 1.0mL. The prepared BSA solutions were pipetted into ten different test tubes. Distilled water was then added to each tube to make the final volume of 1.0mL. The blank contained of only 1.0ml of distilled water was prepared separately. Then, 5mL of the Coomasie Brilliant Blue reagent was added in each tubes and the reagent was mixed thoroughly using the vortex at wavelength λ=595nm using spectrophotometer. The tubes left for 2 minutes to let the particles to settle down before reading. The whole process shall not exceed more than 1 hour. The weight of protein was measured against corresponding absorbance resulting in a standard curve.

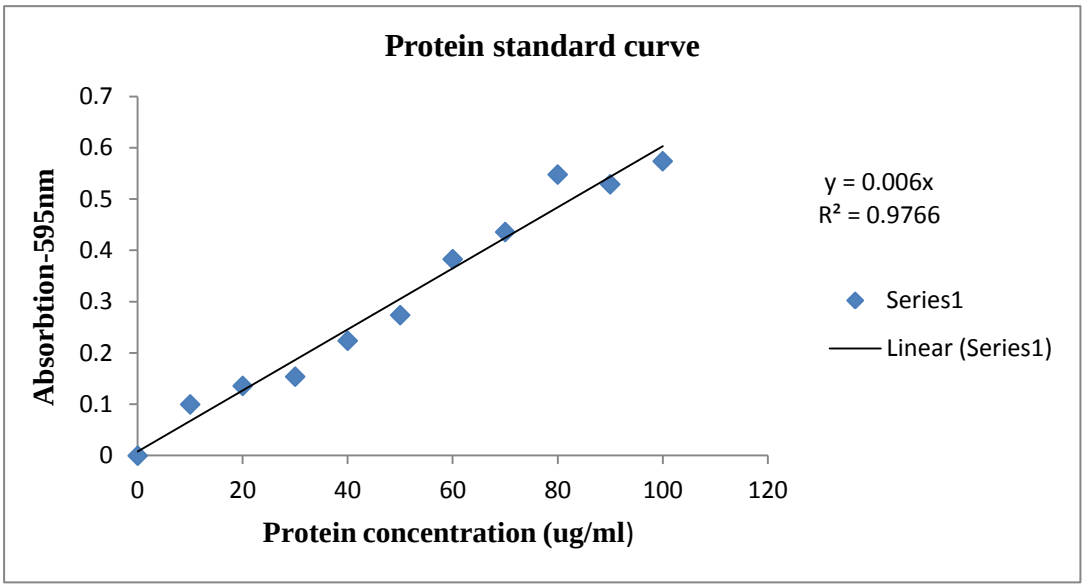

Figure A. 2: Protein standard curve
Note: The linear correlation and its R^2 are depicted in the Figure.

Procedure for determination of soluble protein concentration

The enzyme sample in a volume of 0.2mL was pipette into a test tube and about 5mL of Coomasive Brilliant Blue reagent was added later. The solution in the test tube was mixed using the vortex mixer and the absorbance was read at wavelength λ=595nm. The blank was consisted of 0.2 mL of distilled water with 5 mL of coomassie Briljant Blue reagent. The total soluble protein was then calculated using the formulated formula obtained from the graph.

$$\text{Soluble Protein (mg/mL)} = \left(\frac{\text{Final absorbance}}{0.006}\right)\left(\frac{1}{0.2}\right)\left(\frac{1\text{mg}}{1000\mu\text{g}}\right)$$

A4: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

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

12% resolving gel (~12 mL) for 2 gels

Table A. 3: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis protocol

	Resolving gel	Stacking gel
Distilled water (ddH_2O)	5.22 mL	3.59 mL
Resolving gel (1.5M Tris-HCL, pH8.8)	3.00 mL	-
Stacking gel (0.5M Tris HCL, pH6.8)	-	1.50 mL
10% Sodium Dodecyl Sulphate (SDS)	0.12 mL	0.06 mL
40% Bis-acrylamide	3.60 mL	0.75 mL
10% Ammonium Persulphate	50 µL	20 µL
TEMED	10 µL	10 µL

Preparation of resolving gel buffer (1.5 M of Tris-HCL; pH 8.8)

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

Preparation of stacking gel buffer (0.5M 0f Tris-HCL; pH 6.8)

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

Preparation of 10% of sodium do-decylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS powder (10g) was dissolved in100 mL of distilled water.

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

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

SDS-PAGE electrophoresis running buffer (Tank buffer, 10 x conc.)

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

SDS-PAGE Coomasie staining solution

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

SDS-PAGE Coomasie destaining solution

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

Appendix B: SPSS statistical analysis

Table B. 1: SPSS Data analysis for media optimization

Oneway

Descriptives

LiP

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
1*	3	14.6743	2.93732	1.69586	7.3776	21.9710	12.11	17.88
2	3	39.0273	3.32958	1.92233	30.7562	47.2985	37.11	42.87
3	3	3.7803	1.10967	.64067	1.0238	6.5369	2.50	4.42
4	3	4.4213	1.92250	1.10996	-.3544	9.1971	2.50	6.34
5	3	5.7030	1.11024	.64100	2.9450	8.4610	4.42	6.34
6	3	36.4640	2.93645	1.69536	29.1695	43.7585	33.26	39.03
Total	18	17.3451	15.44829	3.64120	9.6628	25.0273	2.50	42.87

ANOVA

LiP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3988.049	5	797.610	138.728	.000
Within Groups	68.993	12	5.749		
Total	4057.043	17			

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

Homogeneous Subsets

LiP

	Meida	N	Subset for alpha = 0.05		
			1	2	3
	3	3	3.7803		
	4	3	4.4213		
	5	3	5.7030		
Tukey HSD[a]	1	3		14.6743	
	6	3			36.4640
	2	3			39.0273
	Sig.		.915	1.000	.775
	3	3	3.7803		
	4	3	4.4213		
	5	3	5.7030		
Duncan[a]	1	3		14.6743	
	6	3			36.4640
	2	3			39.0273
	Sig.		.369	1.000	.215

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

* The types of media are shown by numbers as following:
  1) Potato Dextrose Broth (PDB) + 1% sawdust
  2) Potato Dextrose Broth (PDB) + 1% sawdust + 0.5% yeast
  3) Potato Dextrose Broth (PDB)
  4) Glucose 1% + 1% sawdust
  5) Glucose 2% + 1% sawdust
  6) Defined media

Table B. 2: SPSS data analysis for influence of TLL on P_{FT} and Log K

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
P_{FT}_TLL	60.82	3	1.4427	.48268	.27868	.2436	2.6417	1.16	2.00
	77.60	3	1.4497	.57274	.33067	.0269	2.8724	.79	1.85
	82.51	3	1.3023	.18947	.10939	.8317	1.7730	1.19	1.52
	87.57	3	3.1230	.89924	.51918	.8892	5.3568	2.42	4.14
	89.09	3	3.0457	.78585	.45371	1.0935	4.9978	2.41	3.93
	Total	15	2.0727	1.01176	.26124	1.5124	2.6330	.79	4.14
LogK_TLL	60.82	3	.5140	.05370	.03101	.3806	.6474	.48	.58
	77.60	3	.6390	.03759	.02170	.5456	.7324	.60	.68
	82.51	3	.5957	.02483	.01433	.5340	.6573	.57	.62
	87.57	3	.8827	.19200	.11085	.4057	1.3596	.71	1.09
	89.09	3	.5947	.11985	.06920	.2969	.8924	.52	.73
	Total	15	.6452	.15771	.04072	.5579	.7325	.48	1.09

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
P_{FT}_TLL	Between Groups	10.285	4	2.571	6.355	.008
	Within Groups	4.046	10	.405		
	Total	14.331	14			
LogK_TLL	Between Groups	.236	4	.059	5.253	.015
	Within Groups	.112	10	.011		
	Total	.348	14			

For One Way Anova, the p-value of influence of TLL on both P_{FT} and Log K were less than 0.05, which indicated the significant differences between the groups.

Homogeneous Subsets

P_{FT}_TLL

	TLL	N	Subset for alpha = 0.05	
			1	2
Tukey B[a]	82.51	3	1.3023	
	60.82	3	1.4427	
	77.60	3	1.4497	
	89.09	3		3.0457
	87.57	3		3.1230
Duncan[a]	82.51	3	1.3023	
	60.82	3	1.4427	
	77.60	3	1.4497	
	89.09	3		3.0457
	87.57	3		3.1230
	Sig.		.792	.885

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Homogeneous Subsets

LogK_TLL

	TLL	N	Subset for alpha = 0.05	
			1	2
Tukey B[a]	60.82	3	.5140	
	89.09	3	.5947	
	82.51	3	.5957	
	77.60	3	.6390	
	87.57	3		.8827
Duncan[a]	60.82	3	.5140	
	89.09	3	.5947	
	82.51	3	.5957	
	77.60	3	.6390	
	87.57	3		.8827
	Sig.		.207	1.000

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

Table B. 3: SPSS data analysis for influence of V_R on P_{FT} and Log K

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
P_{FT}_V_R	.45	3	1.2960	.41129	.23746	.2743	2.3177	1.00	1.77
	.70	3	1.2617	.46180	.26662	.1145	2.4088	.88	1.77
	1.17	3	4.5950	.94775	.54718	2.2407	6.9493	3.71	5.60
	1.61	3	2.4987	.38011	.21946	1.5544	3.4429	2.26	2.94
	2.10	3	3.8843	1.30986	.75625	.6305	7.1382	2.89	5.37
	2.90	3	3.2043	1.79280	1.03507	-1.2492	7.6579	1.92	5.25
	Total	18	2.7900	1.54444	.36403	2.0220	3.5580	.88	5.60
LogK_V_R	.45	3	.1140	.04004	.02312	.0145	.2135	.07	.14
	.70	3	.0853	.03765	.02174	-.0082	.1789	.04	.11
	1.17	3	.7333	.11451	.06611	.4489	1.0178	.62	.85
	1.61	3	.6003	.03308	.01910	.5182	.6825	.57	.63
	2.10	3	.6663	.09815	.05667	.4225	.9102	.55	.72
	2.90	3	.5227	.10644	.06146	.2582	.7871	.41	.62
	Total	18	.4537	.27425	.06464	.3173	.5900	.04	.85

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
P_{FT}_ V_R	Between Groups	27.840	5	5.568	5.257	.009
	Within Groups	12.710	12	1.059		
	Total	40.550	17			
LogK_ V_R	Between Groups	1.202	5	.240	37.777	.000
	Within Groups	.076	12	.006		
	Total	1.279	17			

For One Way Anova, the p-values of influence of V_R on both P_{FT} and Log K were less than 0.05, which showed that there were significant differences between the groups.

Homogeneous Subsets

P_{FT}_V_R

	Vr	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	.70	3	1.2617		
	.45	3	1.2960		
	1.61	3	2.4987	2.4987	
	2.90	3	3.2043	3.2043	
	2.10	3	3.8843	3.8843	
	1.17	3		4.5950	
Duncan[a]	.70	3	1.2617		
	.45	3	1.2960		
	1.61	3	2.4987	2.4987	
	2.90	3	3.2043	3.2043	3.2043
	2.10	3		3.8843	3.8843
	1.17	3			4.5950
	Sig.		.053	.142	.141

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

LogK_V_R

	Vr	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	.70	3	.0853		
	.45	3	.1140		
	2.90	3		.5227	
	1.61	3		.6003	.6003
	2.10	3		.6663	.6663
	1.17	3			.7333
Duncan[a]	.70	3	.0853		
	.45	3	.1140		
	2.90	3		.5227	
	1.61	3		.6003	.6003
	2.10	3		.6663	.6663
	1.17	3			.7333
	Sig.		.668	.057	.075

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

Table B. 4: SPSS data analysis for influence of pH on P_{FT} and Log K

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
P_{FT}_pH	7.50	3	6.5720	.37068	.21401	5.6512	7.4928	6.35	7.00
	8.00	3	8.5913	.52744	.30452	7.2811	9.9016	8.02	9.05
	8.50	3	5.4427	.85212	.49197	3.3259	7.5594	4.89	6.42
	9.00	3	4.9010	.03538	.02043	4.8131	4.9889	4.87	4.94
	9.50	3	4.5950	.94775	.54718	2.2407	6.9493	3.71	5.60
	Total	15	6.0204	1.59643	.41220	5.1363	6.9045	3.71	9.05
LogK_pH	7.50	3	.7593	.04563	.02635	.6460	.8727	.71	.80
	8.00	3	.8270	.05246	.03029	.6967	.9573	.77	.88
	8.50	3	.9457	.07605	.04391	.7567	1.1346	.89	1.03
	9.00	3	.7010	.16289	.09404	.2964	1.1056	.59	.89
	9.50	3	.7333	.11451	.06611	.4489	1.0178	.62	.85
	Total	15	.7933	.12346	.03188	.7249	.8616	.59	1.03

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
P_{FT}_pH	Between Groups	31.598	4	7.899	19.350	.000
	Within Groups	4.082	10	.408		
	Total	35.680	14			
LogK_pH	Between Groups	.113	4	.028	2.807	.085
	Within Groups	.101	10	.010		
	Total	.213	14			

For One Way Anova, the p-values of influence of pH on P_{FT} were less than 0.05, which showed that there were significant differences between the groups.

Homogeneous Subsets

P_{FT}_pH

	pH	N	Subset for alpha = 0.05		
			1	2	3
Tukey B[a]	9.50	3	4.5950		
	9.00	3	4.9010		
	8.50	3	5.4427	5.4427	
	7.50	3		6.5720	
	8.00	3			8.5913
	Sig.		.152	.056	1.000
Duncan[a]	9.50	3	4.5950		
	9.00	3	4.9010		
	8.50	3	5.4427	5.4427	
	7.50	3		6.5720	
	8.00	3			8.5913
	Sig.		.152	.056	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Homogeneous Subsets

LogK_pH

	pH	N	Subset for alpha = 0.05	
			1	2
Tukey B[a]	9.00	3	.7010	
	9.50	3	.7333	
	7.50	3	.7593	
	8.00	3	.8270	
	8.50	3	.9457	
Duncan[a]	9.00	3	.7010	
	9.50	3	.7333	
	7.50	3	.7593	.7593
	8.00	3	.8270	.8270
	8.50	3		.9457
	Sig.		.182	.054

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

Table B. 5: SPSS data analysis for influence of mass of K_2HPO_4 on $P_{FT,}$ Log K and yield

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
$P_{FT}\_K_2$	5.75	3	8.6070	2.10680	1.21636	3.3734	13.8406	6.99	10.99
	5.80	3	9.4520	3.24524	1.87364	1.3904	17.5136	6.23	12.72
	5.85	3	10.7603	1.61429	.93201	6.7502	14.7705	9.38	12.53
	5.90	3	8.7387	2.45049	1.41479	2.6513	14.8260	5.95	10.54
	Total	12	9.3895	2.25467	.65087	7.9569	10.8221	5.95	12.72
$LogK\_K_2$	5.75	3	.6120	.08833	.05100	.3926	.8314	.56	.71
	5.80	3	.5403	.03943	.02276	.4424	.6383	.50	.58
	5.85	3	.9810	.01732	.01000	.9380	1.0240	.96	.99
	5.90	3	.5553	.05645	.03259	.4151	.6956	.49	.60
	Total	12	.6722	.19442	.05612	.5486	.7957	.49	.99
$Yeild\_K_2$	5.75	3	82.6100	2.80592	1.62000	75.6397	89.5803	80.99	85.85
	5.80	3	80.2300	1.45146	.83800	76.6244	83.8356	78.66	81.52
	5.85	3	91.7967	.30022	.17333	91.0509	92.5425	91.45	91.97
	5.90	3	80.7350	2.04699	1.18183	75.6500	85.8200	78.46	82.43
	Total	12	83.8429	5.14343	1.48478	80.5749	87.1109	78.46	91.97

For One Way Anova, the p-values of influence of mass of K_2HPO_4 on Log K and yield were less than 0.05, which showed that there were significant differences between the groups.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Pft_K_2	Between Groups	8.757	3	2.919	.495	.696
	Within Groups	47.162	8	5.895		
	Total	55.919	11			
$LogK\_K_2$	Between Groups	.390	3	.130	40.496	.000
	Within Groups	.026	8	.003		
	Total	.416	11			
$Yeild\_K_2$	Between Groups	262.484	3	87.495	24.542	.000
	Within Groups	28.521	8	3.565		
	Total	291.004	11			

Homogeneous Subsets

P_{FT}_K_2

	Mass of K_2HPO_4	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	5.75	3	8.6070
	5.90	3	8.7387
	5.80	3	9.4520
	5.85	3	10.7603
	Sig.		.707
Duncan[a]	5.75	3	8.6070
	5.90	3	8.7387
	5.80	3	9.4520
	5.85	3	10.7603
	Sig.		.336

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

LogK_K_2

	Mass of K_2HPO_4	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	5.80	3	.5403	
	5.90	3	.5553	
	5.75	3	.6120	
	5.85	3		.9810
	Sig.		.455	1.000
Duncan[a]	5.80	3	.5403	
	5.90	3	.5553	
	5.75	3	.6120	
	5.85	3		.9810
	Sig.		.176	1.000

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

Yeild_K_2

	Mass of K_2HPO_4	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	5.80	3	80.2300	
	5.90	3	80.7350	
	5.75	3	82.6100	
	5.85	3		91.7967
	Sig.		.458	1.000
Duncan[a]	5.80	3	80.2300	
	5.90	3	80.7350	
	5.75	3	82.6100	
	5.85	3		91.7967
	Sig.		.177	1.000

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