EXTRACTIVE BIOCONVERSION OF POLYCAPROLACTONE BY LIPASE IN AQUEOUS TWO-PHASE SYSTEM

CHEW PEY LING

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

UNIVERSITI MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **CHEW PEY LING** (I.C/Passport No: **880726-01-5366**)

Registration/Matric No: **SGR 110091**

Name of Degree: **MASTER OF SCIENCE**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):
EXTRACTIVE BIOCONVERSION OF POLYCAPROLACTONE BY LIPASE IN AQUEOUS TWO-PHASE SYSTEM

Field of Study: **BIOTECHNOLOGY**

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: **PROFESSOR**

Witness's Signature Date
Name: **PROF. DR. MOHAMAD SUFFIAN BIN MOHAMAD ANNUAR**
Designation: **PROFESSOR**

Abstract

Aqueous two-phase system (ATPS) is a technique used for recovery and purification of biomolecules. Extractive bioconversion is a system which integrates biotransformation into ATPS to allow the *in situ* recovery of bioproducts immediately after they are formed. The extractive bioconversion of poly-ε-caprolactone (PCL) in an ATPS had not been established. In addition, the properties of the products obtained following the extractive bioconversion process are not well understood. Therefore, this study aimed to determine the ideal conditions for *Burkholderia cepacia* lipase-mediated extractive bioconversion of PCL in a polyethylene glycol (PEG)-based ATPS, as well as to examine the properties of the products obtained. A sequential optimization strategy, which comprised of full factorial design (FFD) followed by one-factor-at-a-time testing, was employed for the optimization. FFD was employed to screen for significant variables, namely pH, bioconversion temperature, molecular weight of PEG, tie-line length (TLL), volume ratio (V_R) and concentration of NaCl additive in the recovery of the hydrolyzed products and enzyme. Qualitative analysis, which included gas chromatography-tandem mass spectrometry (GC-MS/MS), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and gel permeation chromatography (GPC) were subsequently used to characterize the products recovered. Results from the FFD screening indicated that temperature, V_R and concentration of NaCl additive were the significant factors which influenced the partitioning of the hydrolyzed products into the upper phase. On the other hand, factors which could significantly influence the partitioning of lipase into the lower phase were temperature, TLL and V_R. With the exception of NaCl additive, all other significant factors were subsequently subjected to the one-factor-at-a-time optimization. It was found that the balanced tradeoff between yields of hydrolyzed PCL in the upper phase and lipase in the lower phase was observed

at a temperature of 40 ℃, TLL of 28 % (w/w), and V_R of 80:20. When these conditions were applied in a system composed of 19 % (w/w) PEG 3000 and 8.1 % (w/w) potassium phosphate at pH 7.0 without the presence of NaCl additive, 79.8 % hydrolyzed PCL was recovered from the upper phase and 42.0 % lipase was partitioned into the lower phase. GC-MS/MS analysis revealed and confirmed that the products of the extractive bioconversion were monomers and oligomers of PCL. Results of TGA analysis demonstrated that the higher the temperature of hydrolysis, the lower the onset temperature at which decomposition of PCL occurred. In addition, DSC data demonstrated a negative relationship between temperature of PCL hydrolysis and the melting as well as crystallization temperatures of the excess reactants. Besides, GPC analysis illustrated that the molecular weight of the PCL samples showed a decreasing trend with the temperature of hydrolysis, and that the higher the temperature of hydrolysis, the higher the polydispersity index. In conclusion, an optimized system for extractive bioconversion of PCL using *B. cepacia* lipase was described, which preferentially partitioned the hydrolyzed products into the upper phase and lipase into the lower phase.

Abstrak

Sistem akueous dua fasa (ATPS) adalah satu kaedah yang digunakan untuk memperoleh dan menulenkan biomolekul. Biokonversi ekstraktif merupakan satu sistem yang menggabungkan biotransformasi dengan ATPS bagi pemerolehan "*in situ*" bioproduk sebaik sahaja ia terbentuk. Kaedah biokonversi ekstraktif poli-ε-kaprolakton (PCL) di dalam sistem ATPS belum lagi dibangunkan. Selain itu, ciri-ciri produk yang diperoleh hasil daripada proses biokonversi ekstraktif tersebut adalah tidak banyak diketahui. Oleh itu, kajian ini bertujuan untuk menentukan keadaan yang paling sesuai untuk biokonversi ekstraktif PCL oleh lipase *Burkholderia cepacia* di dalam ATPS yang berdasarkan polietilena glikol (PEG), serta mengkaji ciri-ciri produk yang diperoleh. Satu strategi berturutan yang merangkumi reka bentuk faktorial penuh (FFD) dan ujian satu faktor pada satu masa telah digunakan untuk pengoptimuman. FFD digunakan untuk menentukan pembolehubah yang signifikan di antara pH, suhu biokonversi, berat molekul PEG, panjang garis tie-line (TLL), nisbah isipadu (V_R) dan kepekatan NaCl tambahan dalam pemulihan produk-produk hydrolisis dan enzim. Analisis kualitatif, termasuk kromatografi gas berseiring spektrometri massa (GC-MS/MS), analisis thermogravimetri (TGA), kalorimeter pengimbasan perubahan (DSC) dan kromatografi gel penyerapan (GPC), telah digunakan bagi menentukan ciri-ciri produk yang diperoleh. Hasil kajian dari FFD menunjukkan bahawa suhu, V_R dan kepekatan NaCl adalah faktor-faktor signifikan yang mempengaruhi pemindahan produk-produk hidrolisis ke fasa atas, manakala faktor-faktor yang mempengaruhi pemindahan lipase ke fasa bawah secara signifikan adalah suhu, TLL dan V_R. Selain daripada NaCl tambahan, pengoptimuman telah dilaksanakan pada faktor-faktor signifikan yang lain. Keseimbangan antara pemerolehan PCL terhidrolisis di fasa atas dan pemerolehan lipase di fasa bawah telah diperhatikan pada suhu 40 ℃, TLL 28 % (berat/berat) dan V_R

80:20. Apabila keadaan-keadaan ini digunakan di dalam sistem yang mengandungi 19% (berat/berat) PEG 3000 dan 8.1 % (berat/berat) potasium fosfat pada pH 7.0 tanpa kehadiran NaCl tambahan, sebanyak 79.8 % produk hidrolisis telah diperoleh daripada fasa atas dan 42.0 % lipase telah dipindahkan ke fasa bawah. Analisis menggunakan GC-MS/MS menunjukkan dan mengesahkan bahawa produk-produk biokonversi ekstraktive adalah monomer dan oligomer PCL. Hasil daripada analisis TGA pula menunjukkan bahawa semakin tinggi suhu yang digunakan untuk proses hidrolisis, semakin rendah suhu permulaan penguraian PCL. Tambahan lagi, data dari DSC menunjukkan terwujudnya hubungan negatif di antara suhu hidrolisis PCL dan suhu peleburan serta penghabluran bahan tindak balas yang berlebihan. Di samping itu, analisis GPC menunjukkan bahawa berat molekul sampel PCL menurun dengan peningkatan suhu hidrolisis, dan semakin tinggi suhu hidrolisis, semakin tinggi indeks polidispersiti. Kesimpulannya, sistem yang optimum untuk biokonversi ekstraktif PCL menggunakan lipase *B. cepacia* telah berjaya dikembangkan, di mana produk-produk hidrolisis diberi keutamaan untuk dipindahkan ke fasa atas dan lipase diberi keutamaan untuk dipindahkan ke fasa bawah.

Acknowledgements

First of all, I would like to express my greatest gratitude to my supervisor, Prof. Dr. Ling Tau Chuan, for his invaluable guidance, motivation and support throughout the period of my M. Sc. study. I also wish to express my most sincere appreciation to my co-supervisor, Prof. Dr. Mohamad Suffian Mohamad Annuar, for his priceless advice, assistance and inspirations throughout the course of this research project.

I would like to thank members of the Bioprocess and Enzyme Technology Lab, including Dr. Ahmad M. Gumel, Mohd Nur Ikhmal Salehmin, Muhd Naziz Saat, Nurul Nadiah Abdul Razak, Maryam Farhana binti Kamel Ariffin, Farhaneen Afzal binti Mazlan, Ana Muhawarah Sharip, Muhammad Haziq Aris, Khalil Munawar bin Makhdum Munawar, Lin Yu Kiat, Siti Nor Syairah binti Anis, Mimi Naterah Sharbani, Mohd Rafais Mohd Razaif, for all their facilitations while I carried out my experimental works. I am also grateful to Dr. Grrace Ng Hui Suan from UCSI University, Dr. Edward Ooi Chien Wei from Monash University, and Dr. Show Pau Loke from University of Nottingham and Mr. Tan Shing Cheng from Universiti Sains Malaysia for their generous knowledge sharing and support.

I am also indebted and appreciative to University of Malaya for providing me the SBUM fellowship during my master study, and the Postgraduate Research Grant (PPP) (PG116-2012B) for financially supporting the research work. Last but not least, I am thankful to all my family members for their continuous love and support.

Table of Contents

List of Figures

List of Tables

List of Symbols and Abbreviations

℃	Degree Celcius
AMTPS	Aqueous micellar two-phase system
ANOVA	Analysis of variance
ATPS	Aqueous two phase system
CsCl	Cesium chloride
DF	Degree of freedom
dH_2O	Distilled water
DSC	Differential scanning calorimetry
g/mol	Gram per mole
GC-MS/MS	Gas chromatograph-tandem mass spectrophometry
Gly	Glycine
GPC	Gel permeation chromatography
K	Partition coefficient
K_2HPO_4	Dipotassium hydrogen phosphate
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar concentration
mL	Milliliter
mM	Millimolar (10^{-3} M)
NaCl	Sodium chloride
PCL	Poly-ε-caprolactone
PEG	Polyethylene glycol
pI	Isoelectric point
*p*NPP	*Para*-nitrophenyl palmitate

rpm	Revolutions per minute
Ser	Serine
SS	Sum of squares
TGA	Thermogravimetric Analysis
TLL	Tie-line Length
T_m	Melting temperature
UV-Vis	Ultraviolet-visible
V_R	Volume ratio
w/v	Weight per volume
w/w	Weight per weight

List of Appendices

CHAPTER 1

INTRODUCTION

1.1 Introduction

Aqueous two-phase system (ATPS) is a technique used for recovery and purification of biological products, which is formed by the equilibration of two immiscible chemical compounds at appropriate concentrations, followed by separation of the compounds into two phases under gravity or in a centrifuge (Li *et al*., 2012; Raja *et al*., 2011). Recovery and purification are then achieved by partitioning the products of interest into one phase and the contaminants into another phase. Different types of chemical compounds can be used as the phase-forming components, which give rise to several distinct types of ATPS, of which the polymer/polymer-based system and the polymer/salt-based system are the most widely used. Compared to most conventional methods of bioproduct recovery and purification, ATPS has the advantages of being rapid, producing higher yields, maintaining the native structure of biomolecules, low cost, as well as the potential to relatively simple and practical scaling up process (Albertsson, 1986). In addition, since the phase-forming components used are typically non-toxic and recyclable, ATPS has been viewed as an environmentally benign green technology (Kalaivani & Regupathi, 2013; Li *et al*., 2012).

Coupling of ATPS with several other techniques can increase the applicability of the system. For example, integration of biotransformation into ATPS, an operation termed extractive bioconversion, allows bioproducts to be isolated *in situ* immediately after they are formed in a single step (Zijlstra *et al*., 1998). In this approach, the system parameters are adjusted to allow the bioconversion to take place in a homogeneous mixture, after which the enzymes and the unreacted substrates are partitioned to one

phase while the products formed are partitioned into another phase. Such an integrative approach not only saves the time and cost associated with the processes, but also reduces the risk of product loss during each step of the processing operations as when the steps are carried out separately. Extractive bioconversion has been used for the simultaneous production and isolation of xylo-oligosaccharides formed from xylan hydrolysis (Li *et al*., 2011), and of cyclodextrins obtained from sago starch bioconversion (Ling & Ng, 2013).

1.2 Problem statement

Poly-ε-caprolactone (PCL) is biodegradable polymer that finds applications in a diverse range of industries. PCL is readily miscible with a variety of other compounds; hence, its major use is to improve, through chemical modifications, the characteristics and/or the quality of the compounds with which it blends (Liu *et al*., 2009; Maafi *et al*., 2009). Hydrolysis of PCL, which can be achieved by using lipase as a biocatalyst, is an important step for producing shorter subunits with tailored properties. Presently, the method of extractive bioconversion of PCL with lipase in an ATPS is not well established. Hence, the development of an effective method that integrates PCL hydrolysis and isolation of the hydrolyzed products in a single system was carried out. We postulate that the combination of the two processes in a single step can allow high yield recovery of the hydrolyzed products, and at the same time reduces the time and cost associated with the operation. In addition, the characteristics of the hydrolyzed products obtained are not well understood. The present study was therefore conducted to develop a method of extractive bioconversion of PCL using *Burkholderia cepacia* lipase in an ATPS system and further, to characterize the products recovered.

1.3 Objectives

The general objective of this study was:

To study the applicability of a polyethylene glycol (PEG)-based ATPS in extractive bioconversion of poly-ε-caprolactone (PCL) using *Burkholderia cepacia* lipase, and to investigate the properties of the products obtained.

The specific objectives of this study were:

1. To study the partition behavior of the hydrolyzed PCL, as well as of lipase, in the ATPS-based extractive bioconversion system;
2. To screen for the significant factors which influence the partitioning of the hydrolyzed PCL and lipase in the above system;
3. To investigate the optimum conditions for recovery of hydrolyzed PCL and lipase in the above system;
4. To investigate the chemical and physical properties of the hydrolyzed PCL recovered from the process of extractive bioconversion.

CHAPTER 2

LITERATURE REVIEW

2.1 Aqueous two-phase system (ATPS)

Aqueous two-phase system (ATPS) is a liquid-liquid extraction method used to partition and purify biomolecules. The method was originally described by Beijerinck (1896), who found that mixtures of gelatin and agar as well as of gelatin and soluble starch separated into two distinct layers upon mixture. Albertsson (1986) later initiated a comprehensive research on the applicability of ATPS for partitioning and downstream recovery of biomolecules, by using polyethylene glycol (PEG), dextran and water, as well as PEG, potassium phosphate and water, as the phase-forming components. In fact, the components used for the formation of the phases are not limited to the above-mentioned compounds. Essentially, an ATPS is formed by having two chemically incompatible components mixed thoroughly at or above the critical concentration, during which phase separation would occur, with each phase enriched in one of the phase-forming components (Li *et al.*, 2012; Raja *et al.*, 2011). By manipulating the conditions at which phase separation occur, biomolecules of interest and other contaminants can be partitioned along with the phase-forming components into different phases, thereby achieving purification purposes.

2.1.1 Applications of ATPS

Following Albertsson's initial comprehensive work on the purification of biomolecules using the above method, a large number of extensive researches had attempted to investigate the applicability of ATPS in the recovery of various other biomolecules. ATPS had since been thought as a superior alternative for the separation and purification of biomolecules which were difficult to be recovered by using other bioseparation methods (Gupta *et al*., 1999). Over the past decades, a vast variety of biomolecules have been recovered or purified by using ATPS, including proteins (Ooi *et al*., 2009a), lactic acid (Planas *et al*., 1998), aroma compounds (Marco *et al*., 2000), viral or plasmid gene therapy vectors (Garca-Perez *et al*., 1998), recombinant proteins (Ibarra-Herrera *et al*., 2011), inclusion bodies and viral coat proteins for protein vaccines (Rito-Palomares, 2004), and nucleic acids (Ohlsson *et al*., 1978), among others. Attempts have also been made to use ATPS to recover non-biological molecules, such as metal ions (Lacerda *et al*., 2009) and small organic molecules (Rogers *et al*., 1998). Apart from the above-mentioned laboratory-scale product purification processes, ATPS has, in fact, been applied in large-scale settings as a technically- and economically-viable alternative. A notable example for large-scale application of ATPS is for the early stages of purification or partial purification of intracellular microbial enzyme, including the removal of the cell debris (Saxena *et al*., 2003).

Apart from being used for product purification, ATPS has been used to concentrate dilute products by partitioning the products into the extraction phase of smaller volume or into the ATPS interface (Antov, 2005; Ratanapongleka, 2010). In recent years, ATPSs have also been modified by integrating with several other techniques for greater applicability, such as extractive bioconversion and extractive fermentation (see section 2.1.5). These modifications allow extraction, clarification,

separation, concentration and partial purification steps, among others, to be combined into a single recovery system, thus avoiding the risk of product loss in the multiple steps of the processing operations (Becker *et al*., 2009; Gupta *et al*., 1999).

2.1.2 Formation and mechanisms of ATPS

The ATPS is formed when two chemically incompatible compounds, typically, but not limited to, two polymers or one polymer and one specific salt, are mixed thoroughly at appropriate amounts (by weight) at equilibrium and allowed to separate into two phases under gravity or in a centrifuge (Li *et al*., 2012; Raja *et al*., 2011). Thus, the system can be broadly divided into two steps, namely equilibration and phase separation steps (Hustedt *et al*., 1985). When the two phases are formed, each of the phases is enriched with one of the respective phase-forming components, and water is present in about 85-90 % in both phases (Rosa *et al*., 2010). The time needed to achieve complete separation of the phases depends on the difference in density and viscosity of the two phases (Walter *et al*., 1985). The smaller the difference in density, or the larger the difference in viscosity, the longer the time needed to achieve complete separation of the phases (Hustedt *et al*., 1985).

Despite being widely used, the complex mechanisms of ATPS partitioning are still not fully understood, although it is known that the partitioning behaviour of ATPS is affected by system components, ionic strength, concentration and molecular weight of polymer, type and concentration of salt, the presence of additives, pH, and temperature of the system (Franco *et al*., 1996; Gündüz, 2000; Zaslavsky, 1995). This is because the nature of ATPS is not only dependent on the physicochemical traits of the biomolecules such as conformation, molecular weight, size, hydrophobicity, charge, and specific binding sites, but also influenced by the van der Waals, hydrophobic and hydrogen

bonds, static effects, and electrostatic interactions between the biomaterial and the phase forming components (Albertsson, 1986; Franco *et al*., 1996; Gündüz, 2000; Huddleston *et al*., 1991b). It is the net effect of the above-mentioned parameters that together determines the phase into which the biomolecules are partitioned.

However, it is understood that a better separation of two biomolecules can be achieved when significant difference is found in their partition behaviours. Therefore, by adjusting the parameters above, biomolecules of interest could be manipulated to be partitioned into one of the phases, while undesired contaminants into another phase. However, the partitioning behaviours of biomolecules and contaminants are rather unpredictable (Goja & Hong Yang, 2013). For example, in some cases, the biomolecule to be partitioned may change its role to act as a phase-forming component when it is present in high amount (Ng *et al*., 2013; Ooi *et al*., 2009b; Show *et al*., 2012). Hence, determination of the appropriate conditions for achieving separation of biomolecules and contaminants into different phases is difficult and relies on extensive screening procedures. To minimize the experimental works and time associated with the above screening, design of experiments (DOE) methodology could be applied to statistically evaluate the impact of several candidate parameters on ATPS partitioning (Antony, 2014). Alternatively, an automated high-throughput screening approach could be used for comprehensive screening of the appropriate parameters, but this approach would incur a high cost to the overall operation and is therefore not cost-effective for small scale work (Boisclair *et al*., 2014).

Although determination of optimum conditions is tedious, the reference conditions that should be used during initial preparation of ATPS can be identified from the phase diagram (Figure 2.1). An ATPS phase diagram provides information on the

concentration of phase-forming components needed to form two immiscible phases, the subsequent concentration of phase components in each of the phases, as well as the volume ratio of phase components. A phase diagram is composed of a binodal curve and several tie-lines. The binodal curve separates the regions at which one and two immiscible phases are formed during the ATPS. On the other hand, each tie-line forms a straight line between the node on the binodal curve which represents the final concentration of the phase-forming component in the upper phase and that which represents the final concentration of the lower phase-forming component.

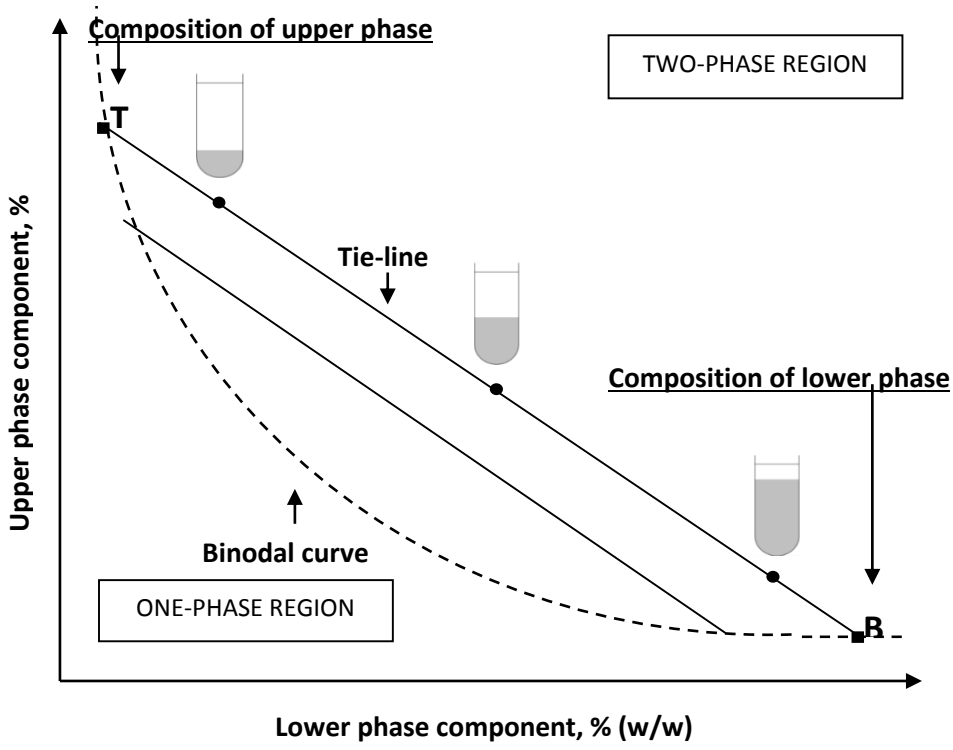


Figure 2.1: Illustration of phase diagram for an ATPS (modified from Hatti-Kaul, 2000a).

2.1.3 Advantages and disadvantages of ATPS

Apart from being able to purify target biomolecules, ATPS is commonly employed in biochemistry and biotechnology because it possesses multiple advantages. The most important advantage of ATPS compared to other well-established and highly successful techniques, such as electrophoresis and various types of chromatography, is its unique mechanism of separation, which is capable to detect any heterogeneity present in a seemingly homogeneous mixture (Walter *et al*., 1985; Zaslavsky, 1995). This allows the separation of the fundamentally heterogeneous subpopulations, facilitating the isolation of the otherwise unobtainable entities for further studies (Zaslavsky, 1995). ATPS is also easy to operate and the yield is typically high while the separation process is rapid, which allows the separation to be done at room temperature (Albertsson, 1986). The system also provides a gentle environment which allows the native structure of the biomolecules to be maintained, due to its high water content of 85-95 % and low interfacial tension of 0.0001-0.1 dyne/cm (compared to the interfacial tension of water-organic solvent systems of 1-20 dyne/cm) (Hatti-Kaul, 2001; Mattiasson, 1983; Ooi *et al*., 2009b; Ratanapongleka, 2010; Subathra *et al*., 2012). In addition, scaling up is easy and reliable, and the large loading capacity eradicates the need to perform the separations repeatedly (Castro & Aznar, 2005). It is also possible to perform continuous steady-state and automated operation, hence resulting in a lower overall energy consumption (Hatti-Kaul, 2000a). By integrating clarification, concentration and purification in a single step, ATPS also reduces overall processing time and cost (Zaslavsky, 1995). Besides, ATPS utilizes various instruments commonly employed in chemical industry, such as mixer-settlers, column contactors and centrifugal contactors, and hence, no additional, specialized instruments are necessary (Cunha & Aires-Barros, 2000; Igarashi *et al*., 2004). Finally, the overall ATPS process is environmentally

friendly, since most of the phase components are non-toxic and phase-forming components are typically recyclable (Figure 2.2).

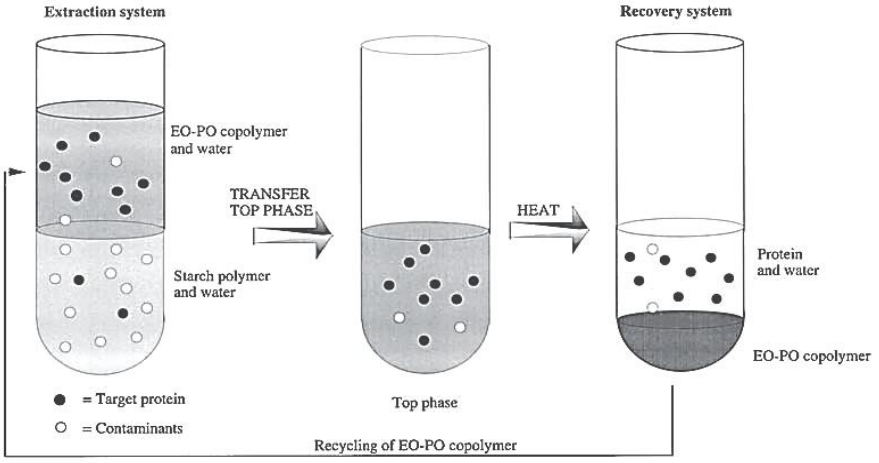


Figure 2.2: Recycling of the phase-forming components of ATPS (adapted from Gupta *et al.*, 2004)

However, the main disadvantage of the use of ATPS in recovering biomolecules arises from its incompletely understood mechanisms underlying the partitioning (Zaslavsky, 1995). Hence, screening for parameters affecting the performance of an ATPS uses a random screening approach, which is often laborious and time-consuming (Hatti-Kaul, 2000a). To minimize the number of experiments required to obtain optimized ATPS conditions, modelling experiments can be performed (Hart *et al.*, 1995; Kammoun *et al.*, 2009; Rosa *et al.*, 2007). Many prediction models have been used in such experiments to predict the partition behaviour of biomolecules in ATPS, with the Flory-Huggins theory and virial expansion model represent the fundamental viewpoints in this respect (Abbott *et al.*, 1990; Baskir *et al.*, 1989). Nonetheless, reliable prediction for the selection of optimal systems for a given specific separation has not been achieved through these models. Another disadvantage associated with the ATPS system is the difficulty to reproduce partitioning data (Walter *et al.*, 1985; Zaslavsky, 1995).

Inter-assay and -laboratory irreproducibility of ATPS partitioning has been reported, which could be attributed to the use of phase-forming components made in different batches or made by different manufacturers (Walter *et al*., 1985; Zaslavsky, 1995).

2.1.4 Types of ATPS

Depending on the phase-forming components used, several types of ATPS can be formed, including a polymer/polymer-based system, a polymer/salt-based system, and an alcohol/salt-based system.

2.1.4.1 Polymer/polymer ATPS

Polymer/polymer-based systems are among the most widely employed ATPSs. The phase separation process in this type of ATPS is caused by the enthalpy of hydration, which is due to the polymer-polymer interactions in the solution, and is influenced by the molecular size and the thermodynamic properties of the polymers (Albertsson, 1986; Hatti-Kaul, 1999; Johansson *et al*., 1998). Polymer/polymer ATPS is best exemplified by a system comprising of polyethylene glycol (PEG) and dextran. In the mentioned system, a wide variety of PEG molecular weights can be chosen and used for different applications (Gupta *et al.*, 1999). Low molecular weight PEGs are typically used for purification of hydrophilic high molecular weight compounds, and *vice versa* (Cabezas, 1996).

One major reason why PEG/dextran ATPS has received much attention is that the system is easily amenable to modification or derivatization in order to make the partitioning of biomolecules more effective (Harris & Chess, 2003; Lu & Tjerneld, 1997). A PEG-dextran system is also characterized by its relatively suitability for general applicability, considering the fact that the two phases have appropriate

physicochemical properties such as viscosity and density difference (Balasubramaniam, 2003; Zaslavsky, 1995). Moreover, PEG and dextran has been shown to be non-toxic and biodegradable, hence it has been proposed to be used in large scale commercial applications, such as in the recovery of aroma compounds, natural products and therapeutic proteins in food, cosmetics and medical industries (Harris, 1992; Hatti-Kaul, 2000a; Ratanapongleka, 2010). However, since PEG has a lower critical solution temperature (temperature above which a polymer phase and a water phase is formed) of above 100 ℃, and dextran is an expensive phase-forming component, other polymers have been used as their replacements (Balasubramaniam, 2003; Saeki *et al*., 1976). Polypropylene glycol (PPG), polyvinyl alcohol (PVA), and random copolymers of ethylene oxide and propylene oxide (EOPO), for examples, have been used as the substitutes for PEG, while unfractionated dextran, Ficoll (polysucrose), methylcellulose, hydroxyethylcellulose (HEC), and Reppal PES (hydroxypropyl starch) have been used to replace dextran (Harris *et al*., 1997; Persson *et al*., 1999; Persson *et al*., 1998).

2.1.4.2 Polymer/salt ATPS

Polymer/salt-based systems are another type of commonly employed ATPS. Instead of using two polymers, polymer/salt ATPS is constructed by substituting one of the phase components with an appropriate salt. The ability of a salt in facilitating phase separation process depends on its Gibbs free energy of hydration and/or its position in Hofmeister or lyotropic series (Ananthapadmanabhan & Goddard, 1987). In fact, it is the anion of a salt which serves as the major determinant of the effectiveness of the salt in facilitating the phase separation process (Rogers *et al.*, 1996). An example of the polymer/salt ATPS is established by PEG and sodium phosphate. Following phase separation, the polymer is localized in the upper phase, while the salt is partitioned into the lower phase. Compared to the other systems, the polymer-salt ATPS has the advantages of having a

lower phase viscosity, higher selectivity, relatively lower cost, and a wider range of hydrophobicity differences between the two phases (Albertsson *et al*., 1990; Ooi *et al*., 2009a; Rito-Palomares, 2004).

However, one downside of the polymer/salt-based ATPS is that denaturation or precipitation of the target products may occur as a result of the high ionic strength conditions due to the presence of high salt concentrations (Ananthapadmanabhan & Goddard, 1987; Andersson & Hahn-Hagerdal, 1990). In addition, disposal of the salt solution appears to be environmentally unfriendly. Fortunately, the latter issue can be addressed by using either biodegradable salts which possess lower eutrophication potential (Vernau & Kula, 1990; Zafarani-Moattar *et al*., 2004), or volatile salts which can be easily recycled back into the gas phase (van Berlo *et al*., 2000).

2.1.4.3 Alcohol/salt ATPS

Water-soluble alcohols, such as methanol, ethanol, and propanol, were originally examined as the additives to increase the efficiency of ATPS. However, it was soon realized that these water-soluble alcohols can also be used to establish ATPS along with inorganic salts (Greve & Kula, 1991). Examples of alcohol/salt-based ATPS include ethanol/citrate ATPS, 1-propanol/phosphate ATPS and 2-propanol/ammonium sulphate ATPS. This type of ATPS has the advantage of being able to recover the target biomolecules easily by evaporating the alcohol, thus allowing the simple recycling of the organic solvent phase (Goja & Hong Yang, 2013). In addition, alcohol/salt-based ATPS exhibits low toxicity to the environment, and is inexpensive (Goja & Hong Yang, 2013).

However, utilization of alcohol/salt ATPS may result in the irreversible denaturation of the proteins or enzymes recovered (Li *et al*., 2010). Industrial scale bioproduct recovery using alcohol/salt ATPS is also limited, because alcohols have low flash points and high volatilities, which may pose a safety concern if used in process-scale quantities. Hence, attempts have been made to substitute alcohols with thermal-stable, non-flammable ionic liquids, such as [C4mim]Cl (1-n-butyl-3-methylimidazolium chloride) or [N_{4444}]Cl (tetrabutylammonium chloride) (Gutowski *et al.*, 2003; He *et al.*, 2005; Li *et al.*, 2005; Ruiz-Angel *et al.*, 2007). In addition, these ionic liquids have the advantages of having low viscosity, high chemical stability, amenable chemical structures and physical properties, and strong solubilizing power (Dupont *et al.*, 2002; Oppermann *et al*., 2011).

2.1.4.4 Other types of ATPS

Apart from the above-mentioned types of ATPS, several other ATPSs have also been studied and used for the recovery of various biomolecules, and these are summarized in Table 2.1.

Table 2.1: Other types of ATPSs (adapted from Ng, 2013)

Types of ATPS	Phase-forming components	Examples	Properties	References
Stimuli-responsive ATPS	pH-sensitive polymer pH-responsive copolymer Synthetic pH-sensitive polymer Light-sensitive polymer	Polydiallyaminoethanoate-dimethyl sulfoxide Eudragit S100 P_{ABC}, P_{ADB} P_{NNC}	Partitioned target product in upper phase which is attached in the form of protein-ligand-polymer complex can be easily recovered as a precipitate by altering the environmental pH or isoelectric point (*p*I). Polymer can be recovered up to 95 % by laser radiation at 488 nm after effective partitioning of target product.	Waziri *et al*., 2003 Cabral, 2007 Kamihira *et al*., 1992 Qin & Cao, 2008 Wang *et al*., 2008
Aqueous micellar two-phase system (AMTPS)	Ionic/non-ionic surfactant solution	Triton X-100, Triton X-114	Products can be recovered in coacervate phase (Sanchez-Ferrer & Garcia-Carmona, 1994; Sanchez-Ferrer *et al*., 1989; Ramelmeier *et al*., 1991) and the detergent-rich phase of AMTPS can be recycled after the temperature-induced phase separation (Minuth *et al*., 1996).	Lye *et al*., 1995 Regalado *et al*., 1996 Ooi *et al*., 2011b Saitoh *et al*., 1995 Kitajyo *et al*., 2007

Table 2.1, continued

Affinity or ligand-specific ATPS	Affinity ligand	Metal chelator, lectin, reactive dye and coenzymes bounded copolymer	Partitioning of target product into one of the phases can be improved by affinity partitioning of ligand-product complex or the addition of free ligands (Kopperschlager & Birkenmeier, 1990). Including the purification tags directly into the target product itself using small molecules such as short tryptophane-rich peptide, tyrosine and hydrophobin I (Selber *et al*, 2004; Cramer & Holstein, 2011).	Roque & Lowe, 2006 Kopperschlager, 1994 Garg *et al*., 1996 Sivars *et al*., 2000

2.1.5 Process integration with ATPS

To increase the cost and technical efficiencies of bioproduct isolation, ATPS has been integrated with other operations to achieve simultaneous processing and purification of bioproducts. Integration of ATPS with these operations can lead to new coupling techniques, such as extractive bioconversion, extractive fermentation, and cell disruption with direct product recovery (Figure 2.3).

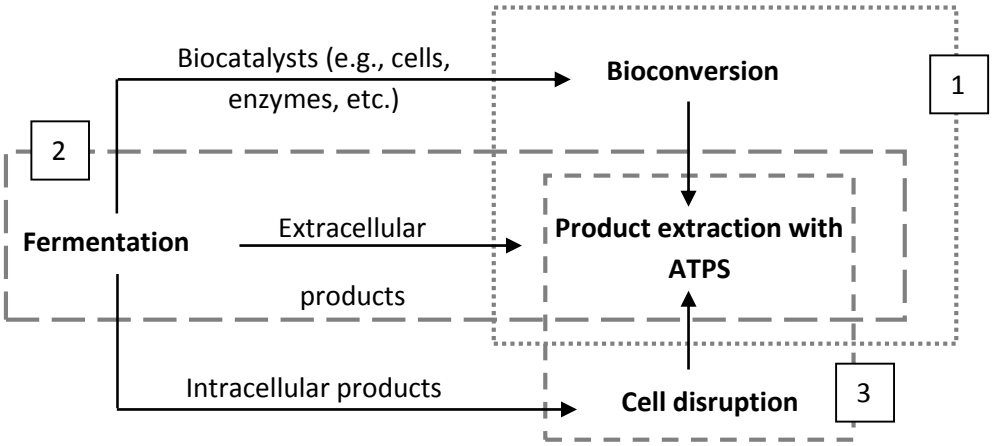


Figure 2.3: Process integration with ATPS. (1) Extractive bioconversion (.....); (2) Extractive fermentation (— — —); (3) Cell disruption with direct product recovery (- - -) (adapted from Ooi, 2011a).

2.1.5.1 Extractive bioconversion

Extractive bioconversion refers to the purification of bioproducts *in situ* immediate after biotransformation has taken place (Hua & Xu, 2011). In this integrative approach, bioconversion is completed in a homogeneous mixture containing enzymes or microorganisms, and the products formed are partitioned into one of the phases directly after separation for recovery while the enzymes, cells or unreacted substrates are partitioned into another phase. The products formed can easily be removed by evaporization, adsorption, permeabilization, membrane filtration or many other approaches, and after the products are removed, the biocatalyst-containing phase can be recycled and reused, thus making extractive bioconversion an environmentally-friendly approach (Cabral, 2007).

ATPS is well adapted for extractive bioconversion since the system provides a gentle condition for many biological enzymes and/or host cells to work. The system also does not interfere with the substrates supplied and the products generated during the bioconversion process, since it is made of very high percentages of water content. Compared to operations which involve separate steps of bioconversion and product recovery, extractive bioconversion minimizes the risk of product loss and reduces the overall time and costs required (Zijlstra, 1998). Extractive bioconversion has been used for the simultaneous production and isolation of glucose from starch (Larsson *et al.*, 1989) or cellulose (Tjerneld *et al.*, 1985) hydrolysis, of xylo-oligosaccharides formed from xylan hydrolysis (Li *et al*., 2011), and of cyclodextrins obtained from sago starch bioconversion (Ling & Ng, 2013), among other operations.

2.1.5.2 Extractive fermentation

Extractive fermentation refers to the instantaneous recovery of bioproducts formed in the fermentation broth from their enzymes or host cells (Banik & Santhiagu, 2002). ATPS provides mild environment for long-term survival of cells and/or enzymes. Similar to extractive bioconversion, the products and the enzymes or host cells must be exclusively partitioned into each distinct phase in order for efficient purification to occur. The immediate product recovery ensures that end-product inhibition in fermentation as well as loss or early degradation of labile products does not occur, which indirectly allows higher yield of products compared to other conventional methods (Banik *et al.*, 2003; Daugulis, 1994; Freeman *et al.*, 1993; Hatti-Kaul, 2000b; Rito-Palomares *et al.*, 2000). Extractive fermentation has been utilized for the recovery of acetone, lactic acid and butanol (Rito-Palomares, 2004).

2.1.5.3 Cell disruption with direct product recovery

For intracellular products, single-step recovery can be achieved by integrating cell disruption to ATPS. This reduces the number of steps required to purify products of interest from contaminants such as cell debris, hence reduces the overall cost and time. Integration of cell disruption with ATPS has been demonstrated for extraction of an intracellular enzyme (Rito-Palomares & Lyddiatt, 2002).

2.1.6 Recovery of bioproducts

After successful separation of bioproducts from their contaminants in an ATPS system, it is often needed to recover the bioproducts from the phase-forming components to obtain products of high purify for further use. The only exceptions are labile substances which would become less stable when removed from the system, as the phase-forming components provide a gentle environment, and can act as stabilizing agents, for the substances (Prasad, 2010; Sivars & Tjerneld, 2000). Several techniques can be employed for removing the phase-forming components to achieve recovery purposes. One of the commonly used techniques for this purpose includes the application of high effective gravitational force to the mixture (Asenjo & Andrews, 2012; Wijethunga & Moon, 2012). This can be achieved via centrifugation (for the recovery of large particles such as cell and cell fragments) or ultracentrifugation (for small particles such as virus particles). Cesium chloride (CsCl) density gradient centrifugation or ultracentrifugation has been found to be particularly useful for the removal of PEG from the substances of interest (Guo *et al*., 2013; Morenweiser, 2005).

In addition, ion exchange or hydroxylapatite chromatography can be used for the recovery of charged bioproducts from ATPS with uncharged phase-forming components, such as PEG/dextran system or alcohol/salt system (Gupta *et al*., 2004; Saxena *et al*., 2003). The uncharged phase-forming components can easily be washed away from the chromatographic system as they cannot bind to the ion exchanger. Similar to ion exchange or hydroxylapatite chromatography, charged bioproducts can also be purified from neutral phase-forming components by using electrophoresis (Zhai *et al*., 2001). Proteins, viruses, amino acids derivatives and DNA, for examples, have been successfully recovered from ATPSs by using chromatography and electrophoresis techniques (Saxena *et al*., 2003; Zhai *et al*., 2001a; Zhai *et al*., 2001b).

Besides, for ATPSs in which polymers are used as a phase-forming component and bioproducts of interest are partitioned into the polymer-rich phase, recovery of the bioproducts can be achieved by first adding excessive salts to the polymer-rich phase. This facilitates the migration of the desired bioproducts into the salt phase, thus being separated from the polymers (Walter *et al*., 1985; Zaslavsky, 1995). If the desired bioproducts are proteins, ammonium sulphate can be used to precipitate and recover the proteins. Alternatively, a simple dialysis can be performed to remove the salt from the bioproducts of interest, thus achieving purification purposes (Hatti-Kaul, 1999; Walter *et al*., 1985). In most cases, however, the resulting mixture contains small tolerable amounts of PEG. Nonetheless, the PEG could be removed by ultrafiltration or diafiltration (Kepka *et al*., 2004; Rathnasamy & Kumaresan, 2013; Zaslavsky, 1995).

2.2 Lipase

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are water-soluble serine hydrolases that possess a catalytic triad consisting of the consensus sequence Gly-X1-Ser-X2-Gly, where Gly=glycine, Ser=serine, X1=histidine or tyrosine and X2=any amino acid. Generally, lipases have a molecular weight of between 19 and 60 kDa, and are ubiquitous in nature (Chen & Lee, 1995; McClean & Callaghan, 2009). The serine residue in the above-mentioned consensus sequence serves as the nucleophilic member in this catalytic triad (Svendsen, 1994). Normally, the catalytic triad is fully separated from the reaction medium by a lid-like α-helical polypeptide chain in the closed and inactive conformation (Jaeger *et al.*, 1994). Under aqueous conditions where the substrate forms an emulsion at the hydrophilic-lipophilic interface, structural rearrangement occurs to the lipase to expose its active site region to the substrate. This triggers "interfacial activation" of the lipase, which is characterized by a sharp increase in the enzyme activity (Jaeger *et al*., 1999). The enzyme is then involved in catalyzing the reversible hydrolysis of triacylglycerols into glycerol and fatty acids at the water-lipid interface by acting on the carboxyl ester bonds of substrate molecules (Figure 2.4) (Svendsen, 1994). The ability of the enzyme in catalyzing the reaction at the water-lipid interface differentiates lipase from a related ester hydrolyzing enzyme, esterase.

Under micro- or non-aqueous conditions, however, lipases are capable of reversing hydrolysis (Figure 2.4), as well as catalyzing synthesis reactions such as transesterification or interesterification (Houde *et al*., 2004; Jaeger *et al*., 1999). Transesterification is the process by which two acyl moieties are exchanged between two acylglycerols, and lipase-mediated transesterification can be exemplified by glycerolysis (Figure 2.5) and alcoholysis (Figure 2.6) (Paiva *et al.*, 2002; Sharma *et al.*, 2001). On the other hand, interesterification refers to the process by which the positions

of acyl groups are exchanged within a triglyceride or among triglyceride molecules, and acidolysis (Figure 2.7) and ester exchange (Figure 2.8) represent the classic examples of interesterification reactions that are catalysed by lipases (Amir *et al*., 2012; Ramjit *et al*., 1976).

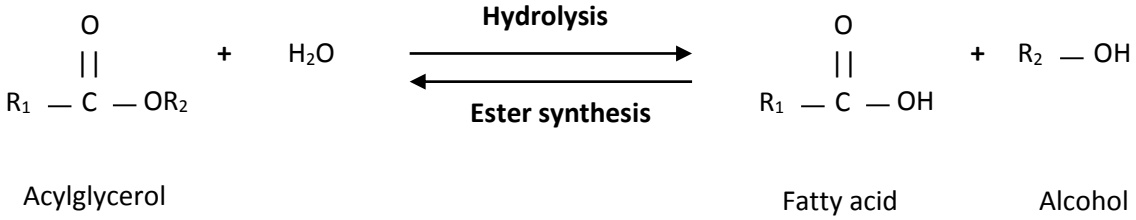


Figure 2.4: Simple hydrolysis and ester synthesis reactions catalyzed by lipase. Under aqueous conditions, lipase catalyzes the hydrolysis of carboxyl ester bonds to release fatty acids and organic alcohols. However, under water-restricted environments, lipases can reverse the process of hydrolysis and catalyze the esterification reaction (modified from Saxena *et al.*, 1999).

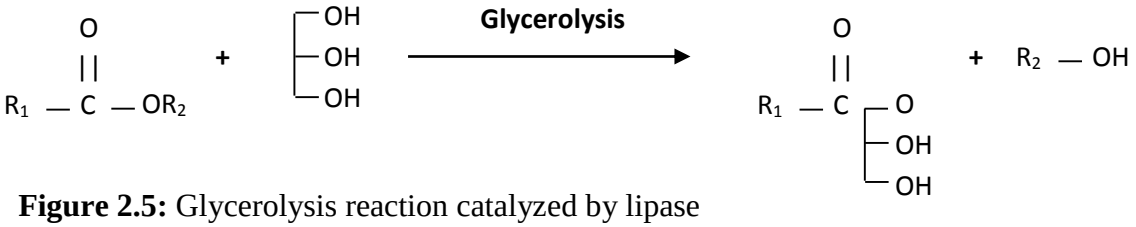


Figure 2.5: Glycerolysis reaction catalyzed by lipase

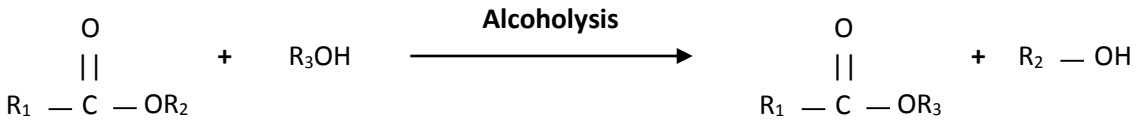


Figure 2.6: Alcoholysis reaction catalyzed by lipase

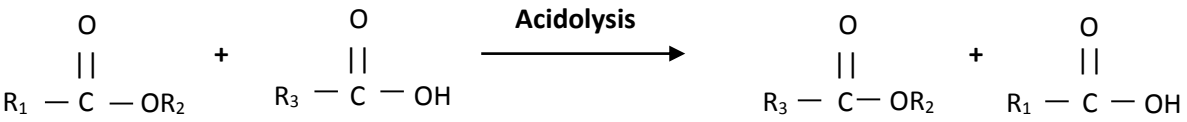


Figure 2.7: Acidolysis reaction catalyzed by lipase

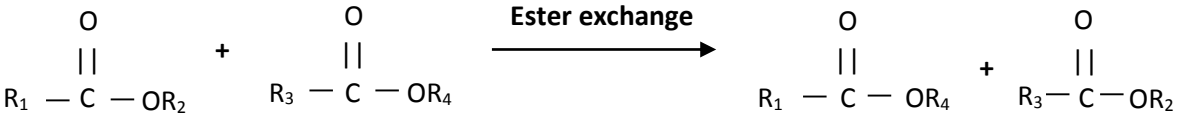


Figure 2.8: Ester exchange reaction catalyzed by lipase

2.2.1 Factors which influence lipase activity

The catalytic activity of lipases can be influenced by the nature of the substrates (Gupta, 2000; James *et al*., 2013). For example, some lipases showed high selectivity towards long and medium chain fatty acids, and exhibit low activity towards fatty acids with short chains (Alhir *et al*., 1990). Lipases from *Staphylococcus xylosus* and *Staphylococcus warneri*, for instance, demonstrated increased esterification rate with increasing fatty acid chain length (Gumel *et al*., 2011). However, each enzyme is unique in its specificity and it is therefore not accurate to generalize the activity of lipase based on the fatty acid chain length (Gumel *et al*., 2011). Lipases from some organisms (e.g. lipolase 100T from *Thermomyces lanuginosus*) favor short chain fatty acids, while the activity of lipases from other organisms (e.g. *Candida Antarctica*) appears to be unaffected by fatty acid chain length (Ferrer *et al*., 2005).

In addition, it has been observed that the catalytic activity of lipases in non-aqueous conditions tends to decrease over time, probably due to the generation of water molecule during the reaction, or due to prolonged exposure of the enzyme's hydration

layer to organic solvent, which leads to the changes in the three-dimensional conformation of the enzyme (Castillo *et al*., 2006; Idris, & Bukhari, 2011; Pereira *et al.*, 2003; Singh *et al.*, 2013; Sinha & Khare, 2014). Such a structural alteration leads to changes in enantio-, stereo- and regio-selectivities of the enzyme, and typically limits the overall rate of product formation in the enzymatic reaction, necessitating various manipulations such as immobilization, microwave irradiation or enzyme equilibration to improve the activity of the enzyme (Carlsson *et al*., 2012; Yang *et al*., 2013; Yu *et al.*, 2011).

2.2.2 Bacterial lipase

Lipases are abundant in nature as they are produced by many plants, animals and microorganisms (Chen and Lee, 1995). Among these, lipases produced by microorganisms are of the greatest importance to industrial and biotechnical applications due to their stabilities in various organic solvents, pH, and temperature, high chemo-, regio-, and enantioselectivity, ability to function without cofactors, ability to perform novel reactions in aqueous and non-aqueous media, low cost, and specificity to a broad range of substrates (Ghosh *et al*., 1996; Gupta *et al*., 2004; Jaeger *et al*., 1999; Jaeger & Reetz, 1998; Rathi *et al*., 2001). Bacteria lipases have received greater practical attention compared to lipases from other microorganisms (e.g. yeasts), because bacteria lipases typically have a shorter generation time, higher activity, and more well-characterized method for preparation and genetic manipulation (Frost & Moss, 1987; Hasan *et al.*, 2006). The lipase generation time, however, varies according to the bacterial species, but it can be as short as less than 24 hours for fast growing bacteria.

Most, if not all, bacterial lipases are extracellular enzymes, which are released into the culture broth during the cultivation period, specifically at the late logarithmic or

stationary phase (Aravindan *et al*., 2007). Bacterial lipases are principally produced by submerged cultures and solid state fermentations supplied with carbon sources, nitrogen and metal ions as nutrients (Petre, 2011). An inducer is needed for stimulating the production of lipase, and oil is typically used as an inducer, although hydrolysable esters, triglycerides, free fatty acids, glycerol and bile salts have also been used (Aravindan *et al*., 2007). An example of a bacterial lipase is the alkaline lipase produced by *Burkholderia* sp. ST8, a Gram-negative bacterium. The lipases are highly resistant to heat inactivation, with a half-life of more than 12 hours at 90-100^{o}C (Bisht *et al.*, 2013). *Burkholderia* lipases are also stable over alkaline pH range of 6-12, and remain enzymatically active in the presence of several different organic solvents and metal ions at temperatures that range over 30-60^{o}C (Bradoo *et al.*, 2002; Rathi *et al.*, 2000; Rathi *et al.*, 2001).

2.2.3 Applications of lipases

According to the Euromonitor International Company Report in December 2011, the global market of lipase has been constantly increasing since the past decade. Lipase is now recognized as one of the most important enzymes for biotechnological applications, as it could be used as a sustainable replacement for the chemically driven synthesis of various products (Hasan *et al.*, 2006). Specifically, lipases find their applications in food industry (Table 2.2) as well as in the processing of detergents and degreasing formulation, leather, oleo-chemical, textiles, synthesis of fine chemicals, paper manufacture and production of cosmetics (Quyen *et al.*, 1999). Lipases have also been widely used in the making of biodegradable polymers, biodiesel, pharmaceuticals, and agrochemicals, among others (Hasan *et al.*, 2006). In addition, since lipases are very versatile enzymes capable of catalyzing reactions involving a broad range of substrates, there is a growing demand for the use of lipase in new areas and applications (Hasan *et*

al., 2006). In most industries, bacterial lipases are used due to their various advantages described in the preceding section. An overview of the use of bacterial lipases in industrial applications is shown in Table 2.3.

Table 2.2: Lipase applications in food industry (adapted from Ooi, 2010)

Food industry	**Action**	**Product of application**
Fat and oils	Transesterification and hydrolysis	Margarine and fatty acids
Beverages	Improved aroma	Alcoholic beverages
Bakery foods	Flavour improvement	Shelf-life propagation
Dairy foods	Hydrolysis	Flavouring agent in milk, cheese and butter.

Table 2.3: An overview of the use of bacterial lipases in industrial applications (adapted from Ooi, 2010)

Industry/field	Applications	Lipase source
Food processing	Flavor development and improvement; fat removal; shelf-life prolongation; quality improvement	*Burkholderia cepacia; Pseudomonas sp.; Staphylococcus warneri*; *Staphylococcus xylosus*
Pharmaceutical and pesticide	Production of chiral intermediates or optically active compound for the synthesis of drugs of pharmaceutical interest, fungicides insecticides and herbicides; digestive aids	*Bacillus subtilis; Chromobacterium viscosum; Pseudomonas cepacia*; *Pseudomonas fluorescens*; *Serratia marcescens*; *Achromobacter*; *Arthrobacter sp.*
Waste/effluent management	Wastewater treatment (breakdown of fats, oils and grease)	*Acinetobacter calcoaceticus*; *Pseudomonas aeruginosa*
Biodiesel production	Conversion of oil to methyl- or other short-chain alcohol esters	*Pseudomonas cepacia*; *Pseudomonas fluorescens*
Detergent formulation	Additive in detergent (removal of oil/ fatty deposits from fabrics)	*Pseudomonas alkaligenes*; *Pseudomonas mendocina*; *Chromobacterium*
Paper manufacturing	Quality improvement (enzymatic pitch removal)	*Pseudomonas sp.*
Cosmetic and perfume	Preparation of skin cleansers; pearling agents; moisturizers; fragrance ingredient	*Pseudomonas cepacia*

2.3 Poly-ε-caprolactone

2.3.1 General properties of poly-ε-caprolactone

Poly-ε-caprolactone (PCL) is a synthetic hydrophobic semicrystalline polymer which belongs to the family of aliphatic polyester. The polymer has a low melting point of approximately 59-64 ℃ and a glass transition temperature of about -60 ℃, owing to the semicrystalline nature of the molecule which facilitates its formability at low temperatures (Celik *et al*., 2009). Generally, the crystallinity of PCL is negatively influenced by its molecular weight, and the number average molecular weight of PCL ranges between 3000 and 80,000 (Celik *et al*., 2009). The chemical structure of PCL is shown in Figure 2.9. At room temperature, PCL is highly soluble in a wide range of organic solvents, including chloroform, 2-nitropropane, cyclohexanone, toluene, benzene, carbon tetrachloride, and dichloromethane (Labet, & Thielemans, 2009). It is, however, moderately soluble in acetone, acetonitrile, dimethylformamide, ethyl acetate and 2-butanone, and insoluble in alcohol, diethyl ether and petroleum ether (Labet, & Thielemans, 2009). PCL possesses a high decomposition temperature of 350 ℃, a tensile modulus of 0.45 GPa and a melt viscosity of 443 Pa s at 150 ℃ (Gnanasekaran *et al*., 2009).

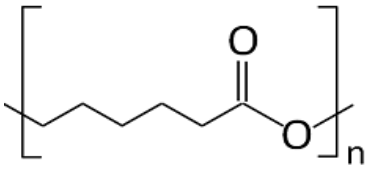


Figure 2.9: Chemical structure of poly-ε-caprolactone (Celik *et al*., 2009)

2.3.2 Synthesis of PCL

PCL does not occur naturally and can only be produced synthetically. There are two major pathways by which PCL could be synthesized, namely the polycondensation pathway and ring-opening polymerization pathway (Ebnesajjad, 2013). Polycondensation represents the conventional method for synthesizing polyesters, and production of PCL can be achieved via polycondensation of a hydroxycarboxylic acid, 6-hydroxyhexanoic acid (Ebnesajjad, 2013). However, ring-opening polymerization of ε-caprolactone has emerged as the preferred pathway for producing PCL, as polymers produced through this pathway tend to be of higher superiority, with little, if any, side product generated (Kfoury *et al*., 2013). Ring-opening polymerization of ε-caprolactone is facilitated by the presence of catalysts. A great variety of enzymatic, organic and metal catalysts for production of PCL through this pathway have been described (reviewed in Labet and Thielemans, 2009), and each catalytic system can result in distinct structures and properties of the resulting PCL. Apart from that, production of PCL via a free radical ring-opening polymerization pathway has also been described, by using 2-methylene-1-3-dioxepane as the substrate (Bailey *et al*., 1982).

2.3.3 Degradation of PCL

PCL is biodegradable by various microorganisms which possess appropriate hydrolytic enzymes such as lipases and cutinases (Abdel-Motaal *et al*., 2013). The polymer is not biodegradable in the body of higher organisms, but it can be eliminated through bioresorption by first undergoing hydrolytic degradation of its ester linkages, which can proceed through either surface erosion or bulk degradation pathways (Woodruff & Hutmacher, 2010). This hydrolytic degradation process takes 0-12 months and produces low molecular weight polymers (M_w < 3000) of higher crystallinity, which can be intracellularly degraded in phagosomes (Pitt *et al*., 1981; Sun *et al*., 2006; Woodward *et*

al., 1985). The entire process of PCL degradation in the human body can take several years, depending on the initial molecular weight of the homopolymer (Middleton & Tipton, 2000). The slow degradation rate of PCL makes it an attractive material to be used in biomedical applications that need to remain active or intact for a long term, such as several drug delivery devices and suture materials.

2.3.4 Applications of poly-ε-caprolactone

In addition to the physical and chemical properties described above, PCL possesses several advantages over other biopolymers which make it suitable to be used in various biomedical applications. Firstly, PCL has high biocompatibility with the human tissues (Woodruff & Hutmacher, 2010). Sutures made from PCL, for example, trigger relatively milder inflammatory responses compared to those made from other aliphatic polyesters. In addition, the remarkable ability of PCL to blend with many other polymers can be exploited to enhance the biocompatibility of the molecule and to improve the characteristics of the resulting products. For instance, blending of PCL with cellulose propionate, cellulose acetate butyrate, polylactic acid or other polymers can improve the resistance of the resulting biomedical applications from stress cracking, control the rate of drug release from microcapsules produced, and manipulate the degradation rate of the materials derived from the polymer (Ali *et al*., 2014; Giunchedi *et al*., 1994). Besides, the superior rheological properties and viscoelasticity of PCL allows it to be shaped into a large range of scaffolds, enabling manufacturing of products with tailored properties which are suitable for specific applications (Woodruff & Hutmacher, 2010).

Given these strengths of PCL and its relatively inexpensive cost of production compared to other biopolymers, PCL has found its use in many biomedical applications.

In fact, PCL has been approved for use for medical purposes by the Food and Drug Administration (FDA) since the 1970s and as such, it can be found in many common medical devices. For example, PCL has been used to produce microspheres and nanospheres for delivering therapeutic drugs to specific target sites in the body (Kumar & Sawant, 2013; Shady*et al*., 2013; Singh *et al*., 2013; Wu *et al*., 2011). PCL has also been used to produce sutures and utilized as antiseptic-releasing film for dressing cutaneous wounds (Ng *et al*., 2007). In addition, the use of PCL or its copolymers as materials for fixation of bone fracture, scaffolds for tissue engineered skin (Ng *et al*., 2001), conduit for axonal regeneration (Koshimune, 2003) and scaffolds for supporting fibroblasts and osteoblasts growth (Hutmacher, 2001; Rai, 2004), among others, have been reported.

Besides biomedical applications, PCL has also been extensively used in developing various novel materials. The most common use of PCL is in the chemical modifications of polyurethane, resins, or polyesters, which not only result in the improved processing characteristics of the products, but also confer the water-, oil-, solvent-, or chlorine-resistance properties to the modified products (Kariduraganavar *et al*., 2014). Considering the low melting point of PCL, it can also be added to polyvinyl chloride (PVC) as a polymeric plasticizer to enhance composting of PVC as a means of disposal (Kariduraganavar *et al*., 2014).

2.4 Current state of knowledge on lipase-mediated extractive bioconversion of PCL using ATPS

Although there is already a large body of database which provides many relevant insights on partitioning of products using ATPS, only few studies to date have focused directly on extractive bioconversion in ATPS. Biotransformation and conventional method of multistep product recovery have become the key barriers to the economical and high yield production of the desired products, especially in a large-scale operation. Therefore, ATPS, which combines biotransformation and recovery processes in a single system, has been introduced for cost- and time-effective product recovery. Since PCL has shown a variety of applications in different fields, including biomedical and chemical fields, the study of extractive bioconversion of PCL in ATPS is of particular interest. However, this area of research has not been explored. Thus, the core of this research was to study the applicability of ATPS in extractive bioconversion of poly-ε-caprolactone (PCL) using *Burkholderia cepacia* lipase. Although the advantages of ATPS have been shown in a number of researches, the mechanisms of ATPS are still not completely understood. Therefore, it is expected that the findings in this study will serve as a reference in the database of extractive bioconversion in ATPS and aid in understanding the mechanisms of ATPS in the future.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Reagents

All the reagents used in this study were of analytical grade. The list of reagents used along with their manufacturers is shown in Table 3.1.

3.1.2 Equipment

The list of equipment used and their functions in this study are shown in Table 3.2.

Table 3.1: List of reagents used and their descriptions

Reagent	**Manufacturer**
Poly-ε-caprolactone	Sigma-Aldrich, USA
Poly(ethylene glycol) 3000	Sigma-Aldrich, USA
Poly(ethylene glycol) 6000	Sigma-Aldrich, USA
Poly(ethylene glycol) 8000	Sigma-Aldrich, USA
Dipotassium hydrogen phosphate	Systerm ChemPur, Malaysia
Potassium dihydrogen phosphate	Systerm ChemAR, Malaysia
4-Nitrophenyl palmitate	Sigma-Aldrich, USA
4-Nitrophenol	Sigma-Aldrich, USA
Amano Lipase PS, from *Burkholderia* cepacia	Sigma-Aldrich, USA
Sodium deoxycholate	Sigma-Aldrich, USA
Triton X-100	Sigma Chemical Company, USA
Acacia gum	Systerm Biochemistry, Malaysia
Chloroform	Merck, Germany
Tetrahydrofuran	Merck, Germany
Dichloromethane	Merck, Germany
2-Propanol	Merck, Germany
n-Hexane	Merck, Germany

Table 3.2: List of equipment used and their descriptions

Equipment	Function	Manufacturer
Shaking incubator	To agitate the reaction samples under controlled temperature, rpm and time duration during bioconversion	Daihan Labtech, Korea
Density meter	To measure the density of solvents and samples	Anton Paar, Austria
Magnetic stirrer hotplate	To stir and mix solutions at an elevated temperature	Heidolph, Germany
Analytical balance	To accurately measure masses of reagents (e.g. phase-forming components)	Sartorius, Germany
Conductivity meter	To measure the electrical conductivity in a solution	Mettler Toledo, Switzerland
Water purification system	To obtain deionized and ultrapure water	Heal Force, Shanghai
Spectrophotometer	To measure the amount of light of a specified wavelength which passes through a medium for quantifying the concentrations of compounds in solution	Jasco, Japan
Autoclave	To sterilize apparatus	Tomy, Japan
Centrifuge	To spin the ATPS mixture to achieve phase separation	Hettich, Germany
Vortex mixer	To thoroughly mix the contents of samples	Gemmy Industrial Corp., Taiwan
pH meter	To measure the pH of the ATPS	Thermo Scientific, USA
Pocket refractometer	To measure the index of refraction of phase forming components	Atago, Japan
GC-MS System	To perform GC-MS/MS for identification of samples	Agilent, USA

Table 3.2, continued

Differential scanning calorimeter (DSC)	To study the effects of temperature on samples	Perkin-Elmer Inc., USA
Thermogravimetric analyzer	To study the effects of temperature on the physical and chemical changes of samples	Perkin-Elmer Inc., USA
Gel permeation chromatography	To determine the average molecular weight and distribution of samples	Waters corporation, USA

3.2 Methods

3.2.1 Phase diagram

Phase diagrams of PEG/salt ATPS were modified from Ooi *et al*. (2009b). Phase systems were prepared from stock solutions of PEG-3000 (50 % w/w), PEG-6000 (50% w/w), PEG-8000 (50 % w/w), and sodium phosphate salt solution (40 % w/w; pH 6, pH 7 and pH 8, respectively). Phase diagrams of ATPS were determined by turbidometric titration method described by Albertsson (1986). Mixtures of standard solutions of PEG as well as salt of known concentrations were titrated by adding diluents until the formation of only one phase was observed, as indicated by the change of the solution from turbid to clear. The changes in solution turbidity were utilized for measurement of mixture immiscibility. The weight of distilled water added was noted for determination of the points in the binodal curves. A series of systems with different weight compositions were made to generate series points for binodal curves. Tie-line length (TLL) was described as the compositions of the two phases which were in equilibrium and was determined as in Equation 3.1:

$$TLL = \sqrt{\Delta P^2 + \Delta C^2} \qquad \text{(Equation 3.1)}$$

where ΔP and ΔC are the differences between the polymer and potassium phosphate concentrations in the top and lower phases, respectively.

The concentrations of the polymer and salt were respectively determined by measuring the refractive index and the conductivity (Albertsson, 1986; Regupathi *et al*., 2009). A standard salt conductivity curve with a range of salt concentrations (Appendix A) was plotted and utilized to determine the salt concentration in the particular phase-forming mixture. The concentration of PEG was subsequently estimated by deducting the refractive index value (Appendix A) of the salts (Ooi *et al*., 2009a). The binodal curve was then plotted for various polymer and salt concentrations.

3.2.2 Extractive bioconversion

Extractive bioconversion was carried out as described in previous literatures, with slight modification (Ng *et al.*, 2013; Ooi *et al.*, 2009a). ATPSs were prepared from 50 % (w/w) PEG stock solutions with different molecular weights (3000, 6000 and 8000) and 40 % (w/w) potassium phosphate stock solutions. The ATPSs were prepared by first adding appropriate concentrations of PEG stock solution and potassium phosphate solution at a defined pH in shake flask. Each ATPS was subsequently added with appropriate amounts of distilled water, 3 % (w/w) of PCL and 30 mg of *Burkhoderia sp*. lipase in order to obtain an ATPS with a final mass of 10 g. Systems with all of the above components except the commercial lipase as well as systems with all of the above components, together with deactivated lipase, were used as controls. The ATPSs were then incubated in an incubator shaker at a specified temperature for 72 hours. All experiments were conducted in triplicates. Subsequently, the systems were centrifuged at 5,000 × *g* for 5 min to allow complete phase separation. The top and lower phases were taken out separately for analyses of hydrolyzed PCL and enzyme activity.

3.2.3 Quantification of PCL concentration

The mass concentration of the hydrolyzed PCL was determined by using a density meter in accordance to manufacturer's instructions. A total of 5 mL of sample was transferred into the density meter gently in order to prevent bubble formation in the column of density meter. After a constant reading was taken, the column of density meter was washed and rinsed with distilled water before the next sample was tested. The initial mass concentrations of the top and lower phases were also measured at the beginning of the experiment as the blank references.

3.2.4 Lipase activity assay

The lipase activity was measured by using colorimetric method (Arnau *et al*., 2010). Firstly, 100 µl Triton X-100 was added to 5 ml 4-nitrophenyl palmitate (pNPP) and heated at 50 ℃ for 5 minutes. The molar extinction coefficient of pNP at pH 7 after the addition of Triton X-100 was $\varepsilon = 3.982 \times 10^3\ M^{-1}\ cm^{-1}$ whereas pNP without Triton X-100 was $7.11 \times 10^3\ M^{-1}\ cm^{-1}$. The mixture was subsequently used as the substrate for lipase. Next, a total of 1 mL solution containing the enzyme was added into a cuvette containing 2 mL of the substrate and incubated at 37 ℃ for 15 minutes. During the entire incubation process, the reaction was measured by a spectrophotometer at 410 nm absorbance against an enzyme-deactivated control. All experiments were run in triplicates. The rate of reaction was calculated by using Spectra Measurement software. One unit of enzyme was defined as 1 µmol of 4-nitrophenol enzymatically released from the substrate in one minute. Lipase activity was calculated as follows (Equation 3.2):

Absorbance = ε c L (Equation 3.2)

$$\frac{dc}{dt} = \frac{Absorbance}{t} \times \frac{1}{\varepsilon} \times \frac{1}{L} \times \frac{a}{b} = \frac{\mu mol}{ml.min} = \frac{U}{ml}$$

where

$\frac{dc}{dt}$ = rate of concentration change with time (μmol min^{-1})

$\frac{Absorbance}{t}$ = initial rate of enzyme activity (Abs min^{-1})

ε = Molar extinction coefficient (M^{-1} cm^{-1})

L = Light path length (1 cm)

a = total volume of enzymatic reaction (ml)

b = volume of sample (ml)

3.2.5 Screening of significant process variables

Full factorial design (FFD) was employed to screen for the effects of several process variables towards the efficiency of ATPS partitioning. The FFD was designed by using Minitab software (version 16), and a total of 195 runs was carried out. Table 3.3 gives the coded values of the factors in FFD. Extractive bioconversion was carried out by using the method mentioned in section 3.2.2 and the products formed were measured as described in the section 3.2.3 and section 3.2.4. Subsequently, the measurements were calculated to determine the partition coefficient and recovery yield of products formed. Analysis of variance (ANOVA) was used to determine the significance of each process variable in affecting the partitioning of the biomolecules. *P* values of less than 0.05 were considered significant in all analyses.

Table 3.3: Factors and value levels used in FFD

Variables (Factors)	Low value (-1)	Central Value (0)	High value (+1)
Molecular weights of PEG	3000	6000	8000
pH	6	7	8
Temperature (℃)	40	49	58
Tie-line length	28	32	38
Volume ratio (PEG:Salt)	30:70	50:50	70:30
Additive (NaCl) (%)	0	0.5	1

The six process variables were studied as they were believed to influence the partitioning of biomolecules in ATPS based on previous literatures (Mohammadi *et al*., 2008; Ooi *et al.*, 2009a; Ramyadevi *et al.*, 2012b; Su *et al.*, 2006). For PEG molecular weight, the central value was decided at PEG 6000 not only because PEG at this molecular weight has the highest selectivity against lipase in ATPS, but also because it acts as an intermediate molecular weight of PEG (Ooi *et al.*, 2009a & Su *et al.*, 2006). The levels of the low (-1) and high (+1) values were subsequently chosen according to Ooi *et al.* (2009a) and Ramyadevi *et al.* (2012b). For pH, in addition to the fact that the optimum pH of the lipase was pH7, high yield and high lipase activity were also observed by Ooi *et al*. (2009a) when pH range of 6.5 ~ 8.5 was used for lipase partitioning in ATPS. Moderate pH, neither too acidic nor too basic, was chosen, in order to prevent protein denaturation and at the same time, to promote environmental friendliness at the time of discharging the solution after the partitioning process (Ramyadevi *et al,* 2012b). pH 6, 7 and 8 were therefore chosen for analysis in this study. For temperature, 49℃ was chosen as the central value for investigation, as it corresponded to the optimum temperature of lipase as stated on the manufacturer's manual. The lipase is highly resistant to heat inactivation, with a half-life of more than

12 hours at 90-100^{o}C (Bisht *et al.*, 2013). Therefore, high temperatures were considered for analysis. However, temperatures of 60 ℃ and above were avoided to assure that the product obtained is purely biodegraded by biocatalyst instead of by thermal decomposition, since the melting point of PCL is at 60 ℃. Therefore, 58 ℃ was selected as the high (+1) value. Since the difference between high (+1) and central values is 9 ℃, it was decided that the difference between central and low (-1) values should be 9 ℃ too, and thus, 40 ℃ was selected as the low (-1) value. On the other hand, the levels of the three FFD values for tie-line length, volume ratio and concentration of neutral salt in the system were determined according to Mello *et al*. (2015) and Ramyadevi *et al.* (2012b).

3.2.6 Optimization

Based on the results obtained from the full factorial screening, tie-line length and volume ratio were selected for further optimization using the conventional method of one-factor-at-a-time, to determine the optimum operating conditions of extractive bioconversion of PCL. During the optimization process, the other variables were fixed at the following conditions: PEG molecular weight of 3000, pH 7 and 0 % additive. Temperature of 31, 33, 35, 37, 40, 49 and 58 ℃, tie-line lengths of 18, 23, 28, 32 and 38 (% w/w) as well as volume ratio of 30/70, 50/50, 70/30, 80/20, 90/10 were examined. Extractive bioconversion was carried out by using the method mentioned in section 3.2.2 and the products formed were measured as described in the section 3.2.3 and section 3.2.4. Samples with deactivated lipase and without lipase served as blanks. All experiments were run in triplicates. Eventually, measurements were calculated in order to determine the recovery yield of PCL and lipase in the upper and lower phases, respectively.

3.2.7 Calculations

The partition coefficient, *K*, was defined as the ratio of the lipase activity (or hydrolyzed PCL concentrations) in the two phases of ATPS (Equation 3.3) (Ooi *et al.*, 2009a).

$$K = \frac{A_T}{A_B} \qquad \text{Equation (3.3)}$$

where A_T and A_B are the lipase activities (in units/mL) or hydrolyzed PCL concentrations (in mg/mL) in the upper phase and lower phases, respectively.

Volume ratio, V_R, was defined as the ratio of volume in the upper phase (V_T) to that in the lower phase (V_B) (Equation 3.4) (Ooi *et al.*, 2009a):

$$V_R = \frac{V_T}{V_B} \qquad \text{Equation (3.4)}$$

The yield of hydrolyzed PCL in the upper phase was calculated using Equation 3.5 (Ooi *et al.*, 2009a).

$$\text{Yield of hydrolyzed PCL (\%)} = \frac{100}{1 + (\frac{1}{V_R} * \frac{1}{K})} \qquad \text{Equation (3.5)}$$

where V_R is the volume ratio of the upper phase to the lower phase and *K* is the partition coefficient.

The yield of lipase in the lower phase was calculated using Equation 3.6 (Ooi *et al.*, 2009a).

$$\text{Yield of lipase (\%)} = \frac{100}{1 + (V_R * K)} \qquad \text{Equation (3.6)}$$

where V_R is the volume ratio of the upper phase to the lower phase and *K* is the partition coefficient.

3.2.8 Qualitative analysis

3.2.8.1 Gas chromatography-tandem mass spectrometry (GC-MS/MS)

Gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis was carried out for identification of hydrolysed products formed after PCL hydrolysis in accordance to manufacturer's instructions, with slight modification (Gumel *et al.*, 2012). The hydrolyzed PCL was subjected to silylation to form stable sily derivatives which were more suitable for analysis using GC-MS/MS. The analysis was carried out in Agilent Triple Quadrapole 7000B GC-MS System (Agilent, USA) with a gas chromatograph fitted with a HP-5 methylphenysiloxane capillary column (30 m x 0.25 mm). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The oven temperature was programmed at 90 ℃ for 1 min, followed by 280 ℃ for 49 min. The injector temperature was set at 150 ℃ while the detector temperature was 250 ℃. A total of 2.0 µL aliquot was injected in splitless mode. The operating conditions of MS were: 70 eV ionization potential; ion source temperature of 290 ℃; quadruple of 100 ℃; solvent delay of 6.0 min; scan speed of 200 amu/s; scan range of 45-1,000 amu; and 3000 volts of EV voltage. Analysis of the data was done by using MassHunter Workstation software (Agilent, USA).

3.2.8.2 Thermogravimetric analysis (TGA)

Thermal stability of the control and hydrolyzed PCL was investigated by using thermogravimetric analysis (TGA) (Gumel *et al.*, 2012). For the TGA analysis, the sample was heated from 25 to 900 ℃ at the rate of 10 ℃ per min^{-1}. 10.0 mg sample was then loaded in the ceramic pan for acquisition of the thermal data.

3.2.8.3 Differential scanning calorimetry (DSC)

Differential scanning calorimetric (DSC) analysis was carried out following manufacturer's instructions with slight modification (Gumel *et al.*, 2012). Scanning was

performed by heating up the samples from -50 ℃ to 180 ℃, followed by cooling down to 30 ℃. The heating/cooling rate was fixed at 10 ℃ min^{-1} under nitrogen atmosphere. Prior to the sample placement in the cell, the sample was loaded on a tared aluminium pan and sealed with a pierced lid. A blank control comprising of an empty aluminium pan with pierced lid was also set up. The aluminium pans were then equilibrated at the starting temperature before subjected to the programmed heating-cooling process. The crystallization temperature (T_c) and melting temperature (T_m) of the polymer samples were then determined from the curve generated.

3.2.8.4 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used to determine the average molecular weight (M_n), weight average molecular weight (M_w), polydispersity index (PDI) and distribution of the control and hydrolyzed PCL (Gumel *et al.*, 2012). Styragel HR column (Waters Corporation, USA) was used for the high-resolution analysis of the low molecular weight compounds (Gumel *et al.*, 2012). The eluent used was tetrahydrofuran, which was pumped at a flow rate of 1 ml min^{-1} at 40 ℃. A 2.0 mg/ml sample was filtered through a 0.45 μm filter prior to injection into the machine. For each polymer sample, 100 μl of the filtered sample was injected. The instrument was calibrated using monodisperse polystyrene (PS) standards.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Phase diagram

The phase diagrams of PEG/salts ATPS were measured. Figure 4.1(*a*), (*b*) and (*c*) showed the phase diagrams of PEG 3000/salt system, PEG 6000/salt system and PEG 8000/salt system, respectively. A phase diagram is composed of a binodal curve and several tie-lines (see Figure 2.1). Mixture compositions under the binodal curve are homogeneous while those on top of the binodal lead to two aqueous phases which are immiscible. The lines linking the two points of the binodal are the tie-lines. As may be seen from Figure 4.1, the higher the PEG molecular weight, the lower the concentration required to achieve phase separation.

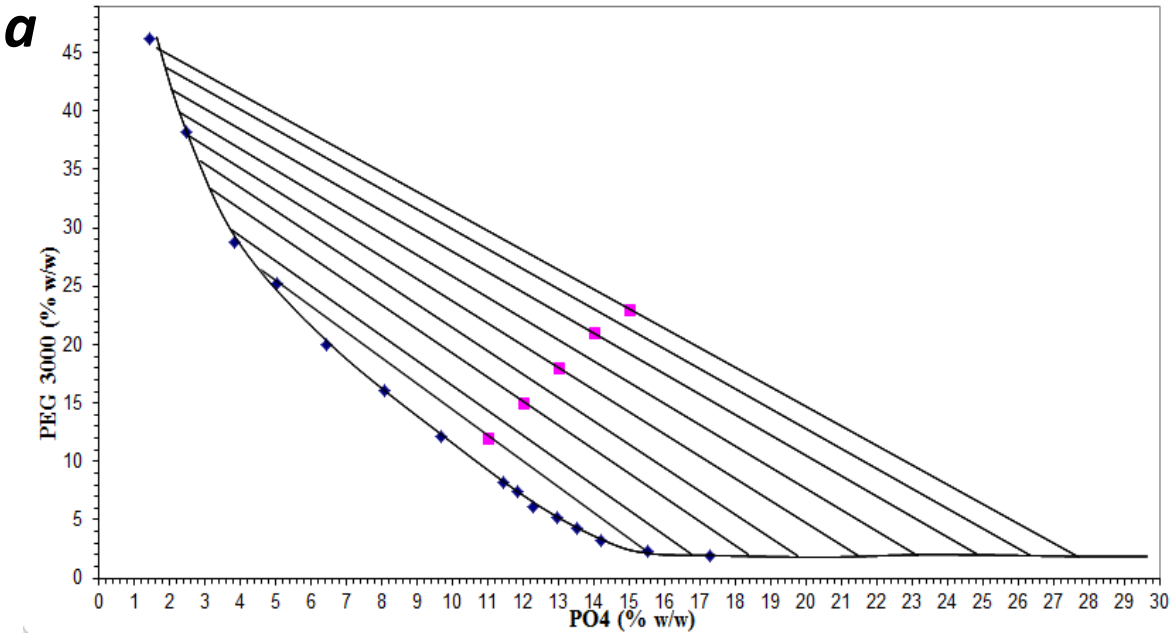
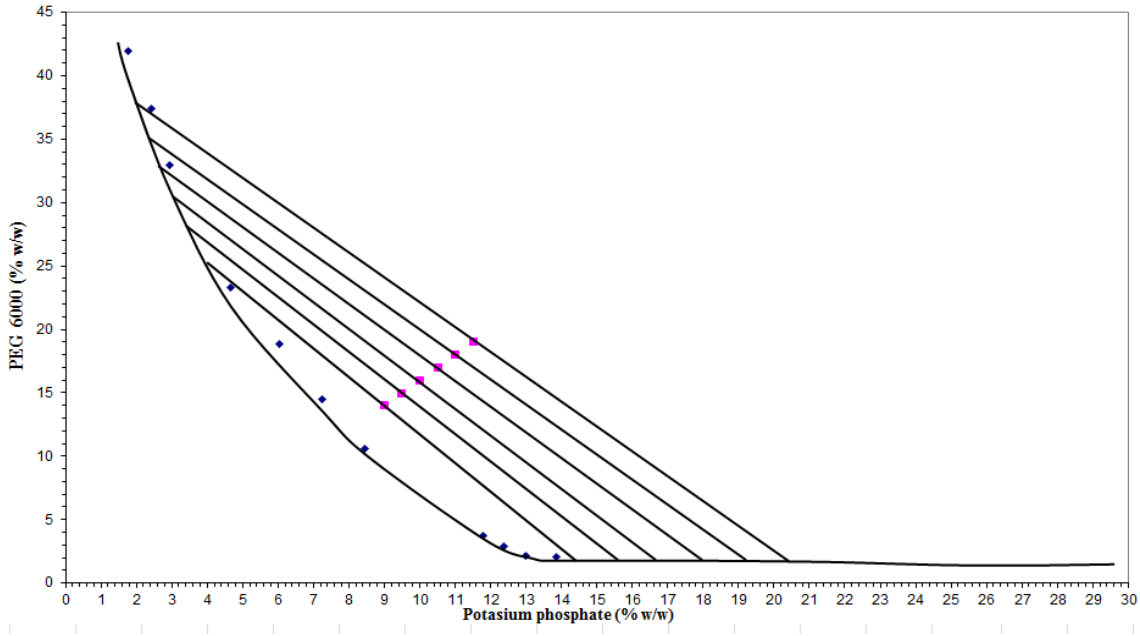


Figure 4.1: Phase diagrams of (*a*) PEG 3000, (*b*) 6000 and (*c*) 8000/potassium phosphate systems

b



c

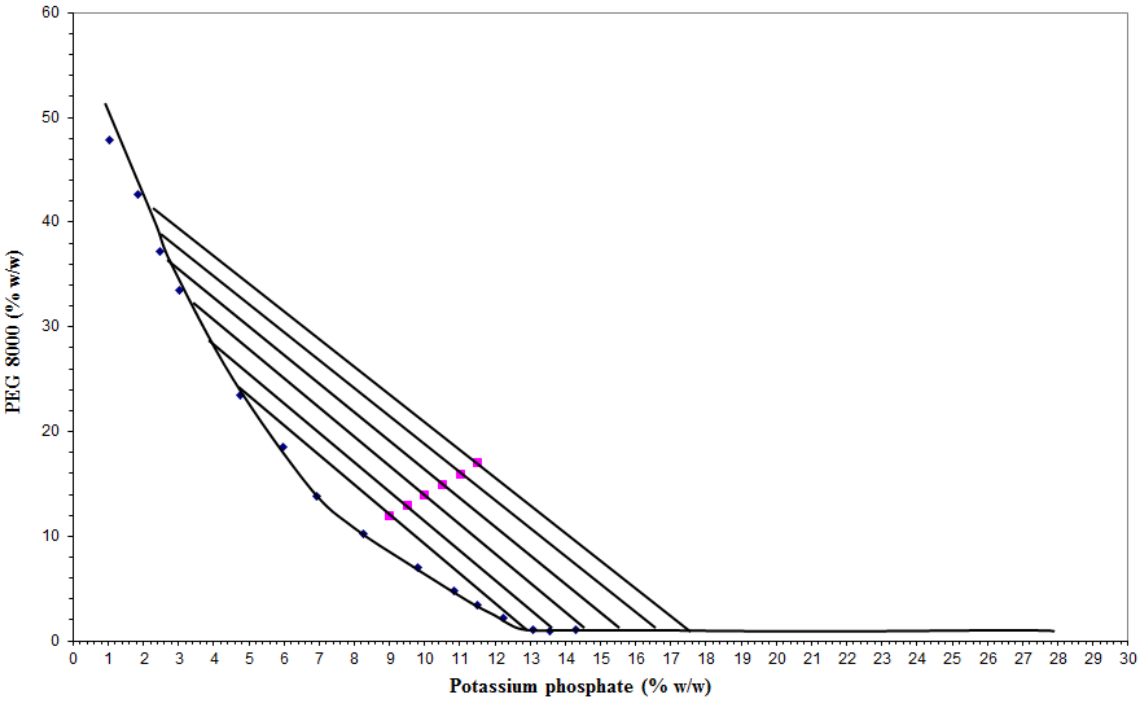


Figure 4.1, continued.

4.2 Residual analysis

Assessment of the fitted model was done by performing residual analysis with standardized residuals. The values of the residuals were calculated by subtracting model fitted values from the experimental values (Equation 4.1). The residual plots of the full factorial design models in the upper and lower phases were shown in Figure 4.2 and Figure 4.3 respectively.

Residuals = Experimental values – Model fitted values (Equation 4.1)

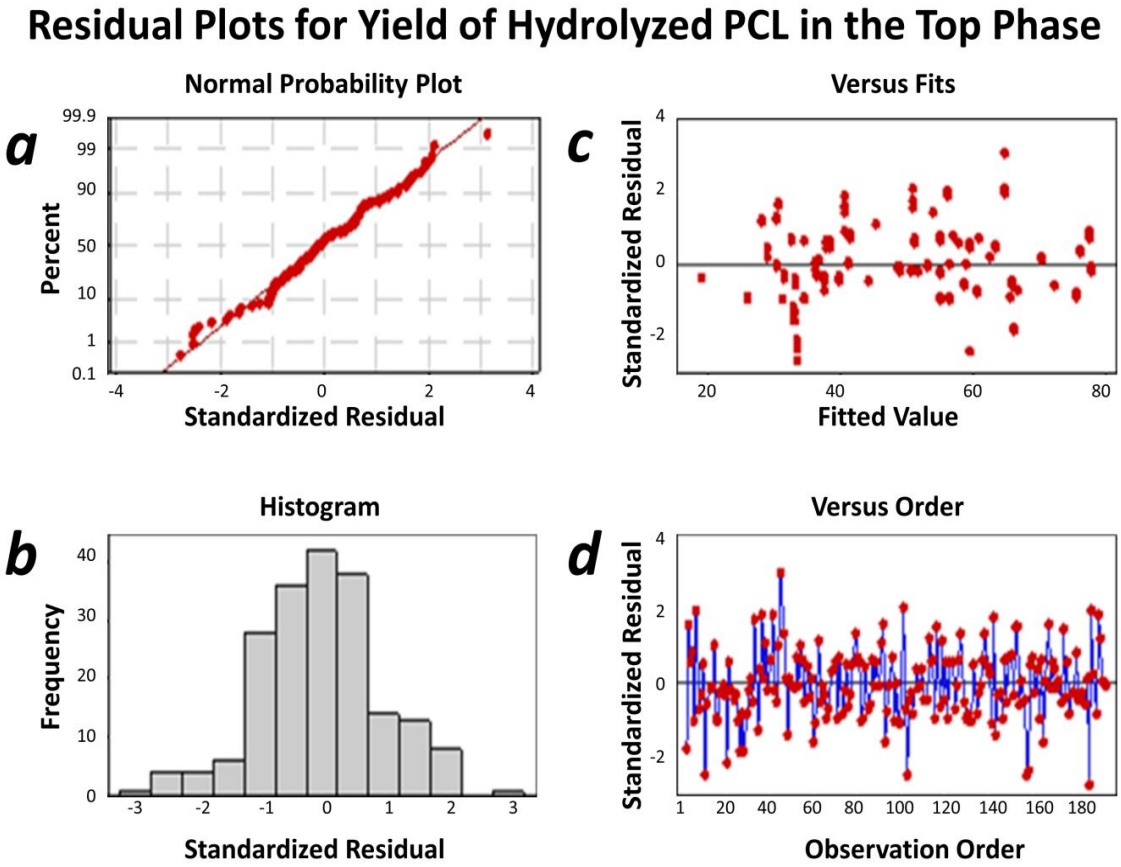


Figure 4.2: Residual plots of the full factorial design model for the effect of parameters studied on the yield of hydrolyzed PCL in the upper phase

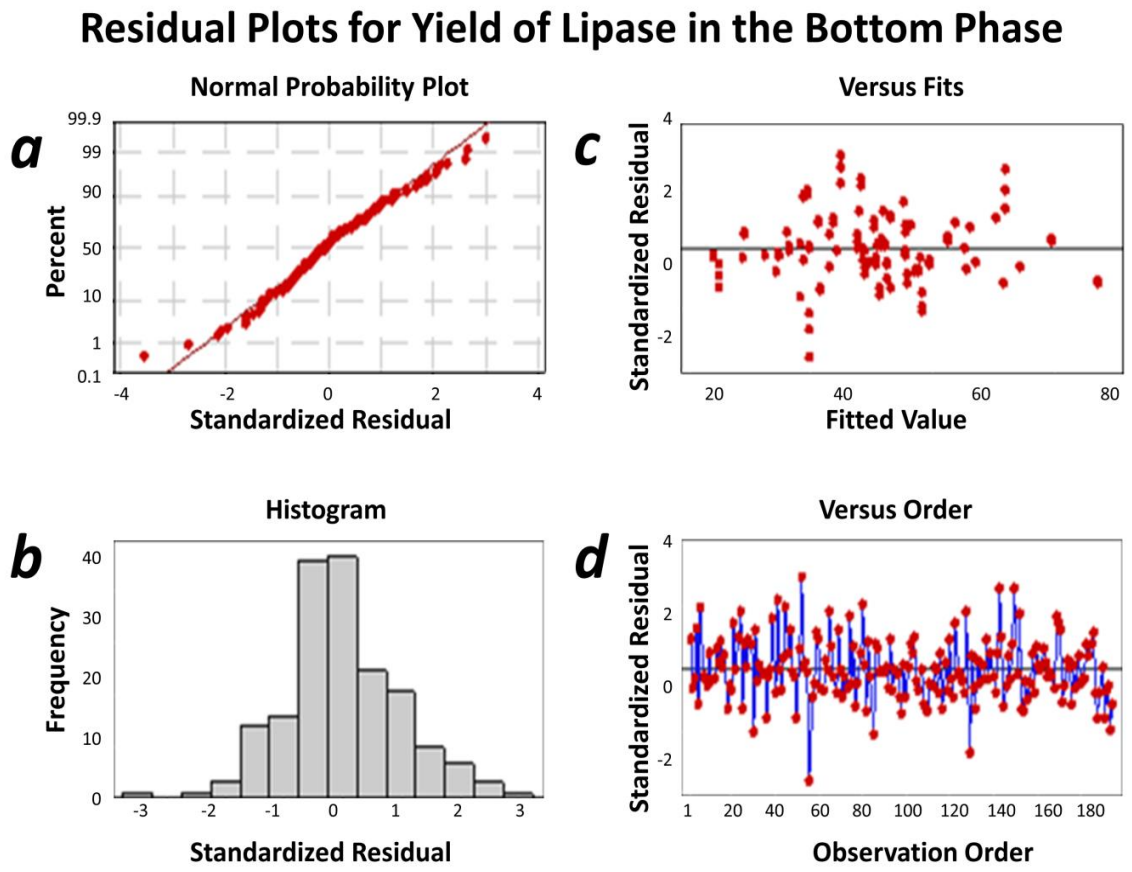


Figure 4.3: Residual plots of the full factorial design model for the effect of parameters studied on the yield of lipase in the lower phase

Assumption of normality was justified based on the observation that residuals were scattered narrowly around the standardized line for both upper and lower phases as shown in Figure 4.2 (*a*) and Figure 4.3 (*a*) respectively. This observation was further supported by the appearance of histograms in Figure 4.2 (*b*) and Figure 4.3 (*b*), which revealed bell-shape distributions. The normal distribution of the residuals signifies that the experimental data errors were due to random events, rather than non-random factors (Connors, 2002). Additionally, no strong evidence of systematic error in the experimental data for both the upper and lower phases, as illustrated by the random scattering of the residual points in almost equal numbers below and above the standardized zero line in the plots of standardized residuals versus fitted values (Figure 4.2 (*c*) and Figure 4.3 (*c*) respectively). Finally, based on the plots of standardized

residual versus observation order in Figure 4.2 (*d*) and Figure 4.3 (*d*), the data obtained was expected to be independent of the order of its collection for both phases of the ATPS, as indicated by the absence of strong pattern of data scattering.

4.3 Significance of process variables studied

Screening was performed on the six main factors included in this study (i.e. molecular weight of PEG, tie-line length (TLL), pH, bioconversion temperature, volume ratio (V_R), and the presence of NaCl additive) in order to evaluate the significance of each factor, as well as of combinations of factors, on the partitioning of hydrolyzed PCL into the upper phase and lipase into the lower phase. A total of 195 runs were included, and the yields of hydrolyzed PCL and lipase were shown in Table 4.1. The effect of each factor and combinations of the factors was illustrated in the normal probability plots in Figure 4.4 (for upper phase) and Figure 4.5 (for lower phase), where deviation from the normality indicates the presence of significance influence. The corresponding ANOVA *P* values of the effects were shown in Table 4.2 (upper phase, single factors analysis), Table 4.3 (upper phase, two-way interaction analysis), Table 4.4 (upper phase, three-way interaction analysis), Table 4.5 (lower phase, single factors analysis), Table 4.6 (lower phase, two-way interaction analysis), and Table 4.7 (lower phase, three-way interaction analysis).

For the upper phase, temperature, V_R and additive appeared to be the significant single factors which could affect the mean percentage of recovery of hydrolyzed PCL, each with a *P* value of less than 0.001. Analysis of two-way interactions between the factors revealed that PEG molecular weight-pH, PEG molecular weight-temperature, PEG molecular weight-V_R, pH-temperature, pH-additive, temperature-TLL, temperature-additive, and TLL-V_R interactions could significantly influence the

partitioning, with *P* values of 0.006, < 0.001, 0.009, < 0.001, 0.035, 0.008, < 0.001 and < 0.001 respectively. In addition, the following three-way interactions were shown to be significant for influencing the partitioning of hydrolyzed PCL in the upper phase: PEG molecular weight-pH-temperature ($P < 0.001$), PEG molecular weight-pH-additive ($P < 0.001$), PEG molecular weight-temperature-additive ($P < 0.001$), PEG molecular weight-TLL-additive ($P = 0.005$), PEG molecular weight-V_R-additive ($P = 0.021$), PEG molecular weight-temperature-TLL ($P < 0.001$), pH-temperature-additive ($P = 0.005$), pH-TLL-V_R ($P < 0.001$), temperature-V_R-additive ($P < 0.001$) and TLL-V_R-additive ($P < 0.001$).

On the other hand, for the lower phase, single factors which could significantly influence the partitioning of lipase were temperature ($P < 0.001$), TLL ($P < 0.001$) and V_R ($P < 0.001$). Besides, the effects of two-way interactions between PEG molecular weight and pH ($P < 0.001$), PEG molecular weight and temperature ($P < 0.001$), PEG molecular weight and V_R ($P = 0.031$), PEG molecular weight and additive ($P < 0.001$), pH and temperature ($P < 0.001$), pH and TLL ($P < 0.001$), pH and V_R ($P = 0.007$), temperature and V_R ($P = 0.013$), as well as V_R and additive ($P = 0.032$) were also significant. For three-way interaction, PEG molecular weight-pH-TLL, PEG molecular weight-pH-V_R, PEG molecular weight-pH-additive, pH-TLL-V_R and temperature-TLL-V_R interactions were shown to affect the partitioning of lipase significantly, with all having a *P* value of less than 0.001 except the PEG molecular weight-pH-V_R interaction ($P = 0.008$).

Table 4.1: The 195 runs performed for the screening of significant process variables and the associated yields

StdOrder	RunOrder	CenterPt	Blocks	PEG molecular weight	pH	Temp	TLL	V_R	Additive	Yield of PCL in upper phase	Yield of lipase in lower phase
58	1	1	1	8000	6	40	38	2.3333	1	50.0009	52.5472
107	2	1	1	3000	8	40	38	0.4328	1	45.5032	68.7812
138	3	1	1	8000	6	40	38	0.4328	0	38.5203	54.8080
161	4	1	1	3000	6	40	28	0.4328	1	48.5741	84.0231
38	5	1	1	8000	6	58	28	0.4328	1	16.5909	45.5021
154	6	1	1	8000	6	40	38	2.3333	0	83.3402	67.0004
85	7	1	1	3000	6	58	28	2.3333	0	66.5594	32.5460
96	8	1	1	8000	8	58	38	2.3333	0	75.6633	25.9902
64	9	1	1	8000	8	58	38	2.3333	1	64.8970	19.7342
63	10	1	1	3000	8	58	38	2.3333	1	37.0231	33.8982
16	11	1	1	8000	8	58	38	0.4328	0	39.3642	35.6744
32	12	1	1	8000	8	58	38	2.3333	0	77.0468	25.8814
180	13	1	1	8000	8	40	28	2.3333	1	51.9855	56.5150
179	14	1	1	3000	8	40	28	2.3333	1	55.0366	64.3749
88	15	1	1	8000	8	58	28	2.3333	0	67.0734	49.3497
78	16	1	1	8000	6	58	38	0.4328	0	21.9266	52.6723
5	17	1	1	3000	6	58	28	0.4328	0	50.0342	47.2247
114	18	1	1	8000	6	40	28	2.3333	1	58.8135	39.6750
168	19	1	1	8000	8	58	28	0.4328	1	28.7045	45.6374
137	20	1	1	3000	6	40	38	0.4328	0	14.0002	51.1776
108	21	1	1	8000	8	40	38	0.4328	1	40.1820	69.0866
182	22	1	1	8000	6	58	28	2.3333	1	47.5475	47.6482

Table 4.1, continued

110	23	1	1	8000	6	58	38	0.4328	1	14.9923	62.8904
53	24	1	1	3000	6	58	28	2.3333	1	52.7607	56.5692
49	25	1	1	3000	6	40	28	2.3333	1	47.4634	41.5585
186	26	1	1	8000	6	40	38	2.3333	1	49.6442	51.5935
150	27	1	1	8000	6	58	28	2.3333	0	59.7231	52.7371
122	28	1	1	8000	6	40	38	2.3333	1	49.3654	52.5498
152	29	1	1	8000	8	58	28	2.3333	0	68.4020	48.7996
170	30	1	1	8000	6	40	38	0.4328	1	34.7180	39.4500
45	31	1	1	3000	6	58	38	0.4328	1	30.4913	63.5474
80	32	1	1	8000	8	58	38	0.4328	0	39.9136	35.7431
136	33	1	1	8000	8	58	28	0.4328	0	67.0639	52.5322
111	34	1	1	3000	8	58	38	0.4328	1	21.6598	48.2232
60	35	1	1	8000	8	40	38	2.3333	1	67.2078	32.9940
189	36	1	1	3000	6	58	38	2.3333	1	73.6699	23.6202
93	37	1	1	3000	6	58	38	2.3333	0	71.8090	30.7941
48	38	1	1	8000	8	58	38	0.4328	1	38.5788	40.1050
15	39	1	1	3000	8	58	38	0.4328	0	47.0312	54.1430
82	40	1	1	8000	6	40	28	2.3333	0	67.0085	50.3395
90	41	1	1	8000	6	40	38	2.3333	0	82.2954	69.0006
118	42	1	1	8000	6	58	28	2.3333	1	47.7480	47.6431
39	43	1	1	3000	8	58	28	0.4328	1	33.0252	51.3830
51	44	1	1	3000	8	40	28	2.3333	1	55.0366	65.7968
26	45	1	1	8000	6	40	38	2.3333	0	92.8322	67.0003
120	46	1	1	8000	8	58	28	2.3333	1	66.8650	40.9498
121	47	1	1	3000	6	40	38	2.3333	1	64.3967	59.2717
175	48	1	1	3000	8	58	38	0.4328	1	20.6829	48.3231

Table 4.1, continued

157	49	1	1	3000	6	58	38	2.3333	0	71.8606	30.6325
50	50	1	1	8000	6	40	28	2.3333	1	58.7115	37.1285
116	51	1	1	8000	8	40	28	2.3333	1	52.0717	56.3811
27	52	1	1	3000	8	40	38	2.3333	0	63.8839	72.6397
195	53	0	1	6000	7	49	32	1	0.5	41.1938	36.9198
115	54	1	1	3000	8	40	28	2.3333	1	55.1510	64.7286
190	55	1	1	8000	6	58	38	2.3333	1	57.3411	43.6695
28	56	1	1	8000	8	40	38	2.3333	0	61.7769	7.1043
128	57	1	1	8000	8	58	38	2.3333	1	64.0334	16.7331
105	58	1	1	3000	6	40	38	0.4328	1	34.6481	63.0402
57	59	1	1	3000	6	40	38	2.3333	1	64.2651	59.0488
100	60	1	1	8000	8	40	28	0.4328	1	18.6231	79.6108
123	61	1	1	3000	8	40	38	2.3333	1	45.5902	43.5647
75	62	1	1	3000	8	40	38	0.4328	0	41.3892	59.0811
131	63	1	1	3000	8	40	28	0.4328	0	29.1224	82.7435
194	64	0	1	6000	7	49	32	1	0.5	41.2646	36.4760
181	65	1	1	3000	6	58	28	2.3333	1	52.2753	56.5631
76	66	1	1	8000	8	40	38	0.4328	0	25.9518	62.1162
149	67	1	1	3000	6	58	28	2.3333	0	66.4889	32.8036
87	68	1	1	3000	8	58	28	2.3333	0	79.8169	27.1263
173	69	1	1	3000	6	58	38	0.4328	1	32.6663	63.4982
162	70	1	1	8000	6	40	28	0.4328	1	43.8574	65.6129
187	71	1	1	3000	8	40	38	2.3333	1	46.6167	41.0260
95	72	1	1	3000	8	58	38	2.3333	0	84.3476	21.8070
89	73	1	1	3000	6	40	38	2.3333	0	53.7759	37.8246
79	74	1	1	3000	8	58	38	0.4328	0	46.9995	54.2932

Table 4.1, continued

145	75	1	1	3000	6	40	28	2.3333	0	62.8211	45.6108
20	76	1	1	8000	8	40	28	2.3333	0	53.2075	63.3889
68	77	1	1	8000	8	40	28	0.4328	0	33.7533	61.1849
17	78	1	1	3000	6	40	28	2.3333	0	62.9733	45.6173
184	79	1	1	8000	8	58	28	2.3333	1	66.9580	40.9540
91	80	1	1	3000	8	40	38	2.3333	0	63.9710	64.3490
55	81	1	1	3000	8	58	28	2.3333	1	60.4975	39.6519
177	82	1	1	3000	6	40	28	2.3333	1	47.5052	41.0461
24	83	1	1	8000	8	58	28	2.3333	0	67.2183	49.3609
188	84	1	1	8000	8	40	38	2.3333	1	68.0969	32.6854
156	85	1	1	8000	8	40	38	2.3333	0	60.0743	20.1757
148	86	1	1	8000	8	40	28	2.3333	0	53.7810	63.8335
130	87	1	1	8000	6	40	28	0.4328	0	43.8589	72.5547
7	88	1	1	3000	8	58	28	0.4328	0	56.3274	48.2849
62	89	1	1	8000	6	58	38	2.3333	1	56.5135	43.6916
193	90	0	1	6000	7	49	32	1	0.5	41.2008	36.0413
176	91	1	1	8000	8	58	38	0.4328	1	38.3525	40.5563
171	92	1	1	3000	8	40	38	0.4328	1	45.9166	68.6582
164	93	1	1	8000	8	40	28	0.4328	1	18.4228	79.3275
167	94	1	1	3000	8	58	28	0.4328	1	30.7661	51.0986
135	95	1	1	3000	8	58	28	0.4328	0	56.4438	48.3855
6	96	1	1	8000	6	58	28	0.4328	0	47.9909	54.5623
102	97	1	1	8000	6	58	28	0.4328	1	16.8134	48.0101
106	98	1	1	8000	6	40	38	0.4328	1	36.5294	44.9927
14	99	1	1	8000	6	58	38	0.4328	0	21.8537	53.3342
59	100	1	1	3000	8	40	38	2.3333	1	45.5837	41.0224

Table 4.1, continued

8	101	1	1	8000	8	58	28	0.4328	0	70.0684	51.5258
84	102	1	1	8000	8	40	28	2.3333	0	52.9957	63.9019
191	103	1	1	3000	8	58	38	2.3333	1	37.0281	33.4505
174	104	1	1	8000	6	58	38	0.4328	1	15.0211	62.8195
83	105	1	1	3000	8	40	28	2.3333	0	48.9577	55.9277
103	106	1	1	3000	8	58	28	0.4328	1	30.4632	51.0981
98	107	1	1	8000	6	40	28	0.4328	1	42.4753	65.0330
163	108	1	1	3000	8	40	28	0.4328	1	29.3161	77.0345
178	109	1	1	8000	6	40	28	2.3333	1	58.8487	39.5654
192	110	1	1	8000	8	58	38	2.3333	1	63.6233	12.7233
41	111	1	1	3000	6	40	38	0.4328	1	33.9879	63.0373
104	112	1	1	8000	8	58	28	0.4328	1	27.9313	45.5851
112	113	1	1	8000	8	58	38	0.4328	1	39.3151	40.5278
34	114	1	1	8000	6	40	28	0.4328	1	43.8574	65.6375
146	115	1	1	8000	6	40	28	2.3333	0	67.2037	50.3768
140	116	1	1	8000	8	40	38	0.4328	0	25.9598	60.0676
77	117	1	1	3000	6	58	38	0.4328	0	54.8665	29.4457
3	118	1	1	3000	8	40	28	0.4328	0	27.5651	82.0601
139	119	1	1	3000	8	40	38	0.4328	0	41.3728	59.0811
46	120	1	1	8000	6	58	38	0.4328	1	15.0231	62.3002
74	121	1	1	8000	6	40	38	0.4328	0	38.2422	55.3974
166	122	1	1	8000	6	58	28	0.4328	1	17.3916	47.9456
172	123	1	1	8000	8	40	38	0.4328	1	40.1323	69.0933
126	124	1	1	8000	6	58	38	2.3333	1	56.4613	43.6652
21	125	1	1	3000	6	58	28	2.3333	0	66.5229	32.5111

Table 4.1, continued

71	126	1	1	3000	8	58	28	0.4328	0	56.0718	45.4102
18	127	1	1	8000	6	40	28	2.3333	0	67.0085	50.3198
33	128	1	1	3000	6	40	28	0.4328	1	53.3758	89.3422
92	129	1	1	8000	8	40	38	2.3333	0	61.7693	15.1151
158	130	1	1	8000	6	58	38	2.3333	0	57.0637	51.5860
153	131	1	1	3000	6	40	38	2.3333	0	53.2597	37.8225
113	132	1	1	3000	6	40	28	2.3333	1	47.5686	41.4910
142	133	1	1	8000	6	58	38	0.4328	0	22.0016	54.5231
81	134	1	1	3000	6	40	28	2.3333	0	62.8116	45.5023
86	135	1	1	8000	6	58	28	2.3333	0	59.6322	53.4342
160	136	1	1	8000	8	58	38	2.3333	0	75.8519	25.8647
52	137	1	1	8000	8	40	28	2.3333	1	52.9209	58.9589
159	138	1	1	3000	8	58	38	2.3333	0	84.0245	21.3224
56	139	1	1	8000	8	58	28	2.3333	1	66.8650	40.9643
124	140	1	1	8000	8	40	38	2.3333	1	68.0965	32.5707
23	141	1	1	3000	8	58	28	2.3333	0	79.3242	27.1221
12	142	1	1	8000	8	40	38	0.4328	0	25.0392	60.5158
97	143	1	1	3000	6	40	28	0.4328	1	57.4727	95.9342
36	144	1	1	8000	8	40	28	0.4328	1	20.5841	79.7564
132	145	1	1	8000	8	40	28	0.4328	0	33.8209	61.0364
19	146	1	1	3000	8	40	28	2.3333	0	49.0508	54.9333
94	147	1	1	8000	6	58	38	2.3333	0	57.0611	51.5860
10	148	1	1	8000	6	40	38	0.4328	0	39.0817	54.7844
129	149	1	1	3000	6	40	28	0.4328	0	35.6881	71.1658
155	150	1	1	3000	8	40	38	2.3333	0	64.0048	69.2011

Table 4.1, continued

134	151	1	1	8000	6	58	28	0.4328	0	48.6980	54.2342
117	152	1	1	3000	6	58	28	2.3333	1	52.3025	55.5814
141	153	1	1	3000	6	58	38	0.4328	0	55.3861	29.5964
13	154	1	1	3000	6	58	38	0.4328	0	55.1300	29.0209
144	155	1	1	8000	8	58	38	0.4328	0	39.3642	35.6052
101	156	1	1	3000	6	58	28	0.4328	1	37.5318	43.4007
169	157	1	1	3000	6	40	38	0.4328	1	33.8219	63.0882
127	158	1	1	3000	8	58	38	2.3333	1	37.2810	33.8737
9	159	1	1	3000	6	40	38	0.4328	0	11.8615	50.9939
66	160	1	1	8000	6	40	28	0.4328	0	42.8211	72.7378
183	161	1	1	3000	8	58	28	2.3333	1	61.6570	39.6147
54	162	1	1	8000	6	58	28	2.3333	1	47.1129	47.6472
2	163	1	1	8000	6	40	28	0.4328	0	42.0231	72.5547
30	164	1	1	8000	6	58	38	2.3333	0	57.0234	49.5860
70	165	1	1	8000	6	58	28	0.4328	0	47.6706	53.4342
47	166	1	1	3000	8	58	38	0.4328	1	18.4228	48.3293
29	167	1	1	3000	6	58	38	2.3333	0	71.2860	30.6692
43	168	1	1	3000	8	40	38	0.4328	1	45.7893	68.8328
143	169	1	1	3000	8	58	38	0.4328	0	47.6204	54.6023
44	170	1	1	8000	8	40	38	0.4328	1	40.1323	69.0608
109	171	1	1	3000	6	58	38	0.4328	1	32.6882	63.4244
99	172	1	1	3000	8	40	28	0.4328	1	29.3590	77.8870
37	173	1	1	3000	6	58	28	0.4328	1	37.4998	47.8348
67	174	1	1	3000	8	40	28	0.4328	0	29.0186	82.1234
72	175	1	1	8000	8	58	28	0.4328	0	65.0323	51.6524

Table 4.1, continued

4	176	1	1	8000	8	40	28	0.4328	0	33.2913	61.6068
119	177	1	1	3000	8	58	28	2.3333	1	60.6428	39.6054
25	178	1	1	3000	6	40	38	2.3333	0	53.6173	37.3827
69	179	1	1	3000	6	58	28	0.4328	0	49.6873	47.2278
147	180	1	1	3000	8	40	28	2.3333	0	48.9577	55.5296
31	181	1	1	3000	8	58	38	2.3333	0	85.8787	21.0767
40	182	1	1	8000	8	58	28	0.4328	1	27.9313	47.6396
65	183	1	1	3000	6	40	28	0.4328	0	36.5212	71.1513
133	184	1	1	3000	6	58	28	0.4328	0	49.8494	47.2204
1	185	1	1	3000	6	40	28	0.4328	0	34.9965	71.1715
185	186	1	1	3000	6	40	38	2.3333	1	63.9822	59.0375
73	187	1	1	3000	6	40	38	0.4328	0	8.8615	50.9926
61	188	1	1	3000	6	58	38	2.3333	1	74.6887	23.6474
151	189	1	1	3000	8	58	28	2.3333	0	79.2064	27.1314
22	190	1	1	8000	6	58	28	2.3333	0	59.7238	52.7934
125	191	1	1	3000	6	58	38	2.3333	1	73.4327	23.6241
11	192	1	1	3000	8	40	38	0.4328	0	42.1892	59.5750
165	193	1	1	3000	6	58	28	0.4328	1	37.8897	47.9789
42	194	1	1	8000	6	40	38	0.4328	1	36.8231	40.5249
35	195	1	1	3000	8	40	28	0.4328	1	29.9687	77.0397

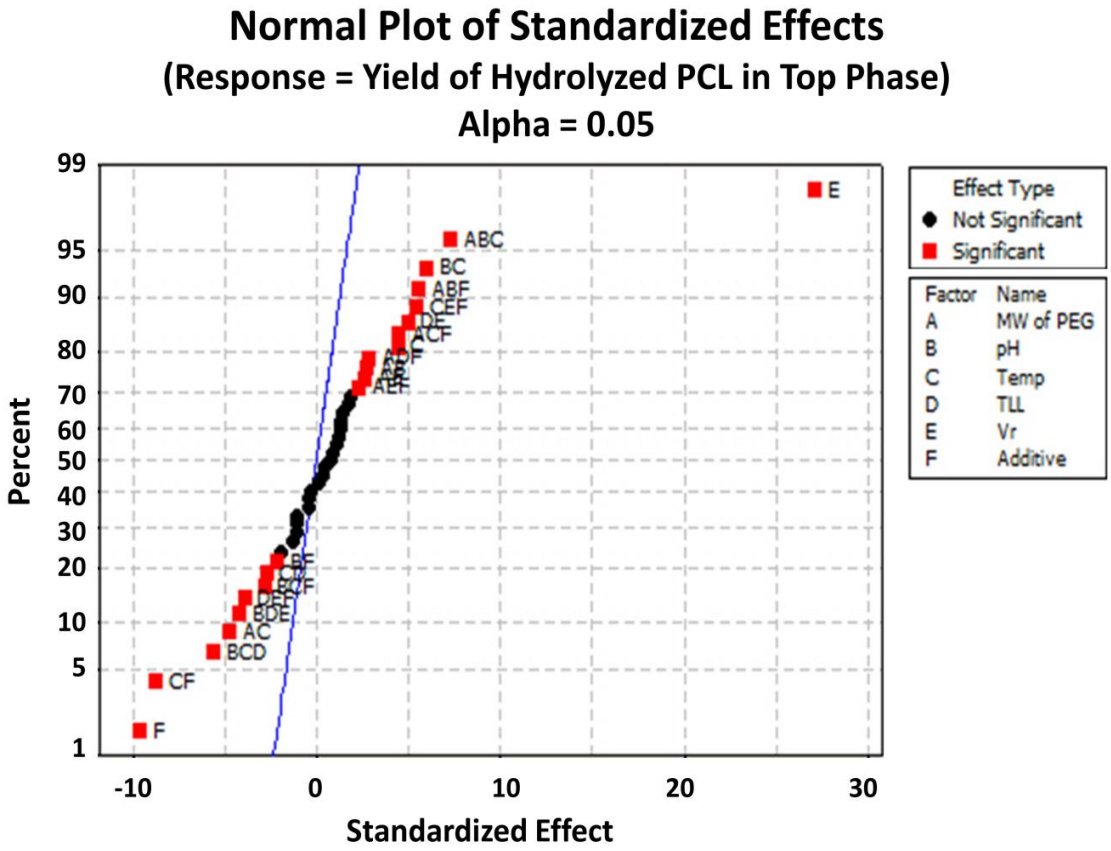


Figure 4.4: Normal plot for the effect of the process variables on partitioning of hydrolyzed PCL in the upper phase

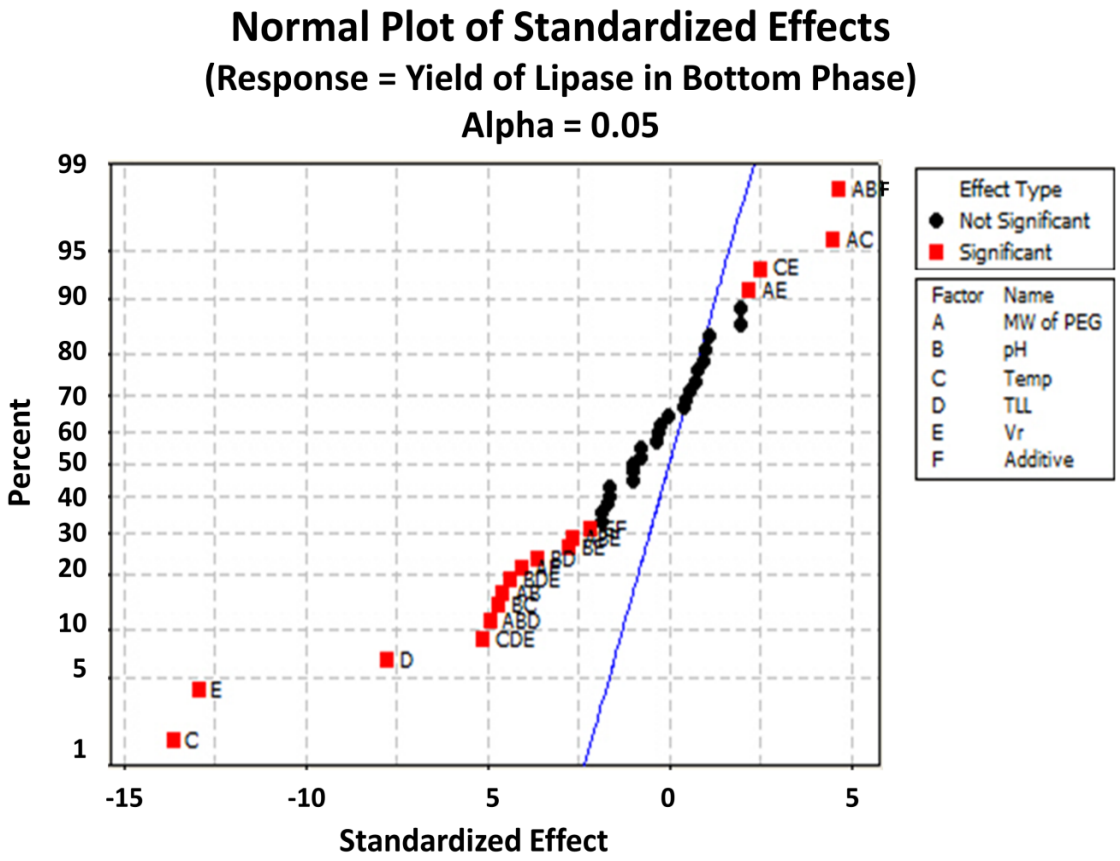


Figure 4.5: Normal plot for the effect of the process variables on partitioning of lipase in the lower phase

Table 4.2: ANOVA of the effect of single factors on the partitioning of hydrolyzed PCL in upper phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG	1	45.5	45.5	1.13	0.290
pH	1	46.2	46.2	1.15	0.286
Temperature	1	793.4	793.4	19.71	<0.001
TLL	1	6.1	6.1	0.15	0.697
V_R	1	29683.9	29683.9	737.51	<0.001
Additive	1	3706.9	3706.9	92.10	<0.001

S = 6.34420; R-Sq = 89.43 %; R-Sq(adj) = 86.50 %

*Significant at 95 % confidence level

Table 4.3: ANOVA of the effect of two-way interactions between the factors on the partitioning of hydrolyzed PCL in upper phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG*pH	1	310.6	310.6	7.72	0.006
MW of PEG*Temp	1	900.2	900.2	22.37	<0.001
MW of PEG*TLL	1	10.6	10.6	0.26	0.609
MW of PEG*V_R	1	283.0	283.0	7.03	0.009
MW of PEG*Additive	1	2.8	2.8	0.07	0.793
pH*Temp	1	1462.8	1462.8	36.34	<0.001
pH*TLL	1	136.8	136.8	3.40	0.067
pH*V_R	1	5.4	5.4	0.13	0.714
pH*Additive	1	182.9	182.9	4.55	0.035
Temp*TLL	1	295.0	295.0	7.33	0.008
Temp*V_R	1	68.5	68.5	1.70	0.194
Temp*Additive	1	3064.4	3064.4	76.14	<0.001
TLL*V_R	1	996.4	996.4	24.76	<0.001
TLL*Additive	1	131.0	131.0	3.25	0.073
V_R*Additive	1	63.0	63.0	1.56	0.213

*Significant at 95 % confidence level

Table 4.4: ANOVA of the effect of three-way interactions between the factors on the partitioning of hydrolyzed PCL in upper phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG*pH*Temp	1	2136.2	2136.2	53.08	<0.001
MW of PEG*pH*TLL	1	83.4	83.4	2.07	2.07
MW of PEG*pH*V_R	1	1.3	1.3	0.03	0.858
MW of PEG*pH*Additive	1	1251.9	1251.9	31.11	<0.001
MW of PEG*Temp*TLL	1	144.7	144.7	3.60	0.060
MW of PEG*Temp*V_R	1	47.7	47.7	1.18	0.278
MW of PEG*Temp*Additive	1	810.7	810.7	20.14	<0.001
MW of PEG*TLL*V_R	1	54.7	54.7	1.36	0.246
MW of PEG*TLL*Additive	1	331.8	331.8	8.24	0.005
MW of PEG*V_R*Additive	1	217.9	217.9	5.41	0.021
pH*Temp*TLL	1	1256.6	1256.6	31.22	<0.001
pH*Temp*V_R	1	3.7	3.7	0.09	0.762
pH*Temp*Additive	1	324.7	324.7	8.07	0.005
pH*TLL*V_R	1	699.1	699.1	17.37	<0.001
pH*TLL*Additive	1	42.7	42.7	1.06	0.305
pH*V_R*Additive	1	73.7	73.7	1.83	0.178
Temp*TLL*V_R	1	35.7	35.7	0.89	0.348
Temp*TLL*Additive	1	22.8	22.8	0.57	0.453
Temp*V_R*Additive	1	1215.6	1215.6	30.20	<0.001
TLL*V_R*Additive	1	615.3	615.3	15.29	<0.001

*Significant at 95 % confidence level

Table 4.5: ANOVA of the effect of single factors on the partitioning of lipase in lower phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG	1	37.9	37.9	0.63	0.428
pH	1	155.9	155.9	2.60	0.109
Temperature	1	11153.1	11153.1	186.36	<0.001
TLL	1	3619.6	3619.6	60.48	<0.001
V_R	1	10053.0	10053.0	167.97	<0.001
Additive	1	227.4	227.4	3.8	0.053

S = 7.73619; R-Sq = 81.55 %; R-Sq(adj) = 76.46 %

*Significant at 95 % confidence level

Table 4.6: ANOVA of the effect of two-way interactions between the factors on the partitioning of lipase in lower phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG*pH	1	1265.1	1265.1	21.14	<0.001
MW of PEG*Temp	1	1224.2	1224.2	20.46	<0.001
MW of PEG*TLL	1	58.6	58.6	0.98	0.324
MW of PEG*V_R	1	284.2	284.2	4.75	0.031
MW of PEG*Additive	1	989.8	989.8	16.54	<0.001
pH*Temp	1	1313.1	1313.1	21.94	<0.001
pH*TLL	1	790.9	790.9	13.21	<0.001
pH*V_R	1	452.4	452.4	7.56	0.007
pH*Additive	1	4.7	4.7	0.08	0.780
Temp*TLL	1	230.7	230.7	3.86	0.051
Temp*V_R	1	379.0	379.0	6.33	0.013
Temp*Additive	1	14.1	14.1	0.24	0.628
TLL*V_R	1	3.6	3.6	0.06	0.807
TLL*Additive	1	17.9	17.9	0.30	0.585
V_R*Additive	1	280.5	280.5	4.69	0.032

*Significant at 95 % confidence level

Table 4.7: ANOVA of the effect of three-way interactions between the factors on the partitioning of lipase in lower phase.

Source	**Degrees of freedom**	**Sum of Squares (SS)**	**Mean Square**	**F value**	***P* value**
MW of PEG*pH*Temp	1	55.6	55.6	0.93	0.337
MW of PEG*pH*TLL	1	1460.1	1460.1	24.40	<0.001
MW of PEG*pH*V_R	1	428.6	428.6	7.16	0.008
MW of PEG*pH*Additive	1	1289.1	1289.1	21.54	<0.001
MW of PEG*Temp*TLL	1	0.0	0.0	0.0	0.977
MW of PEG*Temp*V_R	1	72.3	72.3	1.21	0.273
MW of PEG*Temp*Additive	1	155.5	155.5	2.60	0.109
MW of PEG*TLL*V_R	1	209.3	209.3	3.50	0.063
MW of PEG*TLL*Additive	1	63.2	63.2	1.06	0.306
MW of PEG*V_R*Additive	1	35.3	35.3	0.59	0.444
pH*Temp*TLL	1	36.2	36.2	0.60	0.438
pH*Temp*V_R	1	8.0	8.0	0.1	0.715
pH*Temp*Additive	1	174.7	174.7	2.92	0.090
pH*TLL*V_R	1	1154.5	1154.5	19.29	<0.001
pH*TLL*Additive	1	58.8	58.8	0.98	0.323
pH*V_R*Additive	1	30.5	30.5	0.51	0.477
Temp*TLL*V_R	1	1578.3	1578.3	26.37	<0.001
Temp*TLL*Additive	1	11.1	11.1	0.18	0.668
Temp*V_R*Additive	1	56.2	56.2	0.94	0.334
TLL*V_R*Additive	1	208.7	208.7	3.49	0.064

*Significant at 95 % confidence level

4.4 Main effect analysis

The trends by which the individual variables affected the partitioning of hydrolyzed PCL and lipase in the upper and lower phases were shown in the main effect plots in Figure 4.6 and 4.7, respectively. For the upper phase, a positive relationship was observed for pH, temperature and V_R, in such a way that the higher the values of the process variables, the higher the yield of hydrolyzed PCL obtained. On the contrary, PEG molecular weight, TLL and additive showed a negative relationship with the recovery of the hydrolyzed PCL. On the other hand, for the lower phase, a negative relationship was observed for all the factors studied, except additive.

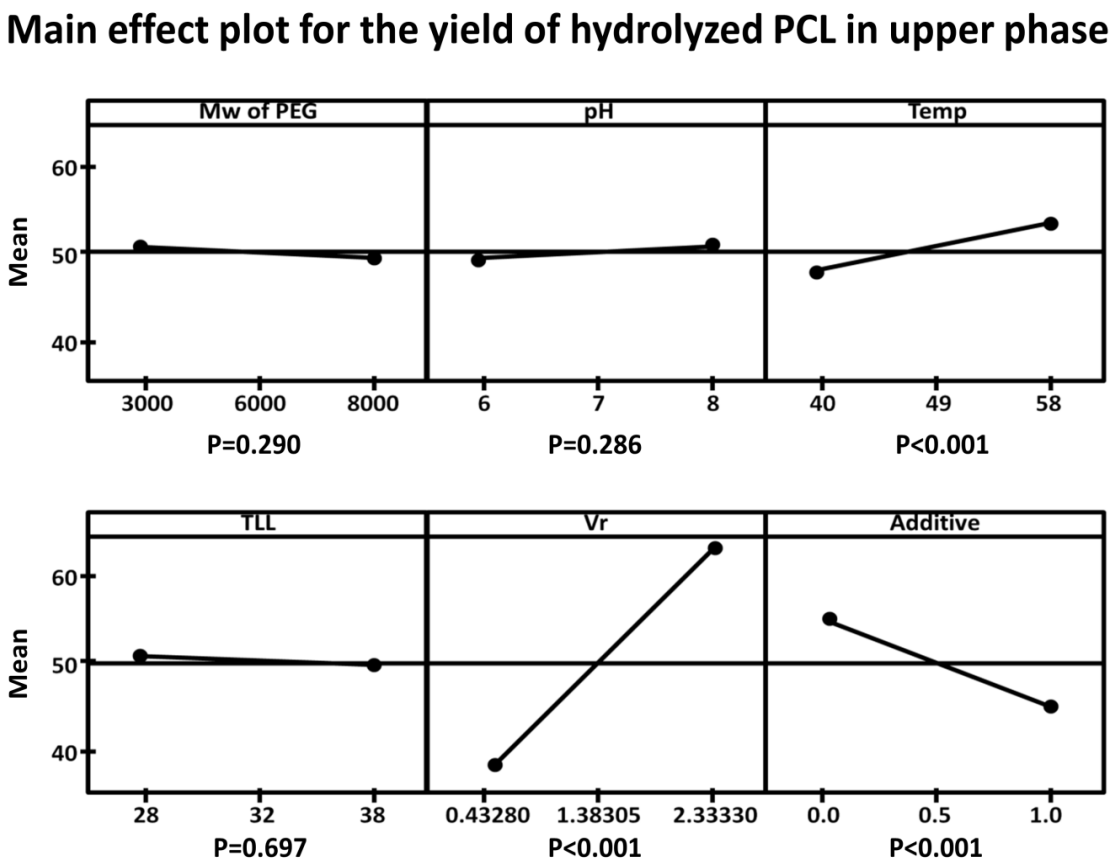


Figure 4.6: Main effect plot for the partitioning of hydrolyzed PCL in the upper phase. (a) Molecular weight of PEG, (b) pH, (c) Temperature, (d) TLL, (e) V_R, (f) Additive

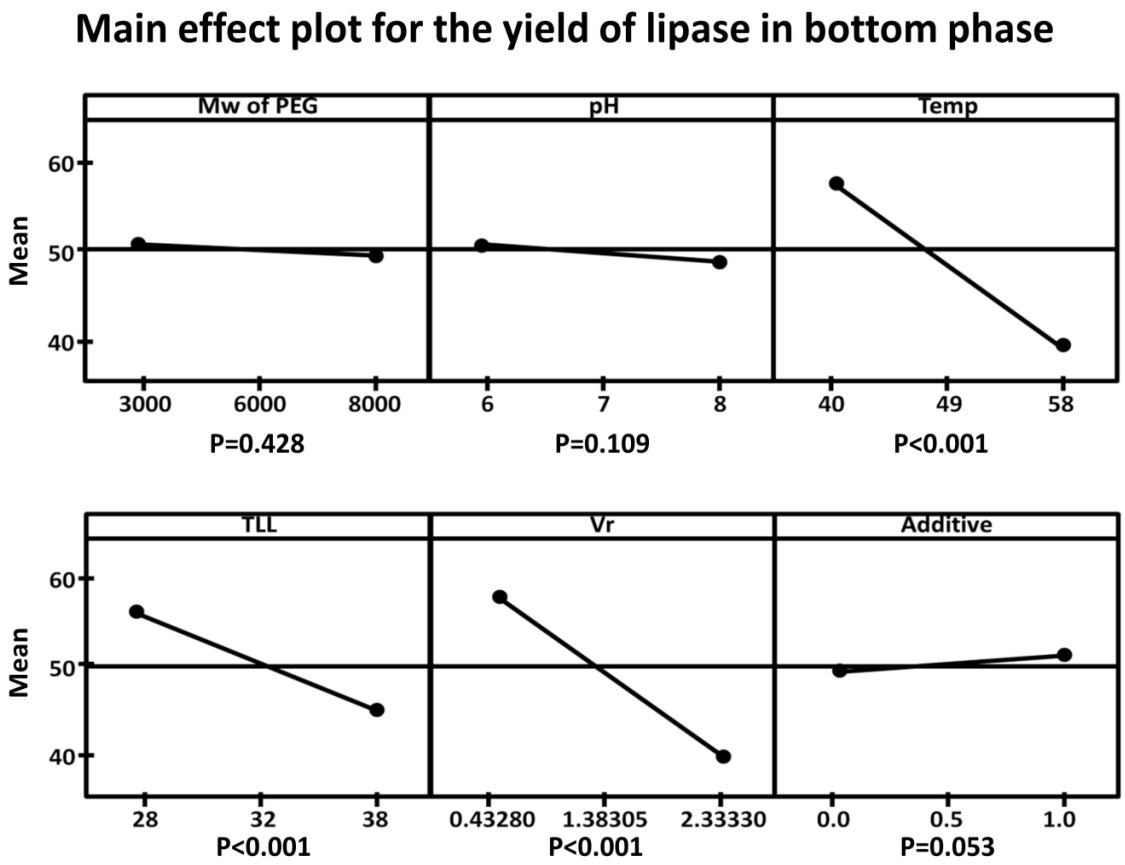


Figure 4.7: Main effect plot for the partitioning of lipase in the lower phase.

(a) Molecular weight of PEG, (b) pH, (c) Temperature, (d) TLL, (e) V_R, (f) Additive

4.4.1 Effect of molecular weight of polymer

Figure 4.6 (*a*) showed the effect of PEG molecular weight, in the range of 3000 to 8000, on the mean percentage of hydrolyzed PCL yield in the upper phase. The mean percentage of the product yield showed an insignificant decreasing trend with an increase in PEG molecular weight. Therefore, the highest yield of hydrolyzed PCL was obtained in a system containing PEG of molecular weight of 3000, and vice versa. Within the range of PEG molecular weight used in this study, the negative effect of PEG molecular weight on the yield of hydrolyzed PCL was, however, not statistically significant, as illustrated by the result of ANOVA test (P = 0.290) (Table 4.2).

Generally, if a phase-forming polymer is substituted with a lower molecular weight fraction of the same polymer, the interaction between the polymer and the biomolecules tends to increase, leading to an enhanced affinity of the biomolecule to the said phase (Raja *et al*., 2011). Therefore, an increase in the molecular weight of the phase-forming polymer tends to promote the partitioning of the desired product into the phase opposite to that in which the polymer is enriched. Our observation in the present study, in which a lower yield of hydrolyzed PCL in the upper phase was associated with a higher molecular weight of PEG in the same phase, was in concordance with this general rule. We postulate that the increase in PEG molecular weight in the upper phase encouraged the transfer of the hydrolyzed PCL into the lower phase, hence resulting in its low yield.

The phenomenon observed may be attributed to the volume exclusion effect and the hydrophobic nature of the phase-forming polymer (Almeida *et al*., 1998; Su *et al*., 2006). The increase in the molecular weight of the polymer corresponds to the increase in its chain length, which, in turn, leads to the formation of a more compact polymeric conformation with more extensive intramolecular hydrophobic interactions. Collectively, these intramolecular hydrophobic bonds create repulsive forces (Forciniti *et al*., 1991; Knowles *et al*., 2011; Zhu *et al*., 2001), which cause the reduction of the free volume available in the upper phase to accommodate the biomolecules (Bhat & Timasheff, 1992; Ibarra-Herrera *et al*., 2011; Mehrnoush *et al*., 2012; Nalinanon *et al*., 2009; Raja *et al*., 2011; Yucekan & Onal, 2011). The biomolecules are therefore forced to leave the upper phase and enter the lower phase.

In addition, the transfer of a solute molecule into an aqueous medium is also governed by the total interaction energy between the biomolecule and the phase-

forming polymer (Zaslavsky, 1995). The weak interaction between high molecular weight PEG and the biomolecules adopts a positive free energy value ($DG_{transfer} > 0$, with entropic changes overcome enthalpy changes), leading to entropically unfavorable conditions, which further drives the partition of the biomolecules into the lower phase (Nalinanon *et al*., 2009). On the contrary, the strong interaction between PEG of low molecular weights and the biomolecules could result in an enthalpic force, which facilitates the transfer of the biomolecules into the upper phase (Tubio *et al*, 2007).

Furthermore, interfacial tension, which is affected indirectly by the molecular weight of the phase-forming polymer, has been reported to play an important role in influencing the partitioning behavior of biomolecules in ATPS (Bamberger *et al*., 1984; Ratanapongleka, 2010; Saravanan *et al*., 2007). The increase in the molecular weight of the polymer may result in an increase in the hydrophobicity of the PEG-rich phase as a result of its long polymeric chain. This intensification of the hydrophobicity could increase the interfacial tension between the phases, which in turn, interferes with the partitioning of the biomolecules, in such a way that the particles are to be excluded from the upper phase (Srinivas *et al*., 2002; Wu *et al*., 1996). In addition, the increasing molecular weight of the polymer may produce a system with a higher viscosity which may not be suitable for the partitioning and recovery of biomolecules (Zhao *et al*., 2013).

Similar to our observation, many studies have shown results in line with the general rule described above. For example, in a study involving the extraction of alcohol dehydrogenase (ADH) from yeast in PEG-salt system, Madhusudhan *et al*. (2008) showed a significant reduction of free volume in the PEG phase as the molecular weight of the PEG was increased from 600 to 20000, which caused the selective

partitioning of the product into the lower phase. Besides, in another study which involved protein partitioning in a PEG-maltodextrin ATPS, the product yield in the upper phase was found to decrease with an increase in PEG molecular weight from 4000 to 10000, an observation which the researchers claimed to be due to the hydrophobic interaction between the polymer and the product (Ramyadevi *et al*., 2012a). Apart from that, Srinivas *et al*. (2002) found that the low interfacial tension attributed by low molecular weight polymer played a role in promoting the preferential partition of the desired product to the PEG-rich phase. In addition, Zhu *et al*. (1996) reported that in a polymer-salt system, the yield of urokinase tended to decrease in the PEG-rich phase as polymer molecular weight was increased, which was also in agreement with the abovementioned general rule. The general rule was also observed by Prodanović and Antov (2008), who showed that the partitioning of *Penicillium cyclopium* pectinases in the upper phase of an ATPS was negatively affected by the molecular weight of the phase-forming polymer.

The effect of PEG molecular weight on the mean percentage of lipase yield in the lower phase was also investigated in the present study, and the results are shown in Figure 4.7 (*a*). A negative relationship between the molecular weight of PEG and yield of lipase in the lower phase was observed. Therefore, the higher the molecular weight of PEG used, the lower the yield of lipase obtained, and vice versa. However, the effect of PEG molecular weight on the yield of lipase was not statistically significant, as demonstrated by the result of ANOVA test (P = 0.428) (Table 4.5). The lack of statistical significance could be attributed to the small molecular size of lipase (approximately 34 kDa), as the effect of PEG molecular weight on the behavior of partitioning is usually apparent only for proteins of larger molecular sizes (>50 kDa) (Almeida *et al*., 1998).

Interestingly, the trend by which PEG molecular weight influenced lipase partitioning did not follow the general rule stated above. However, violation of the abovementioned general rule was not uncommon, and several researchers have reported a similar finding as ours. Almeida *et al*. (1998), for example, showed an increased affinity of the cutinase for the upper phase (and therefore reduced the yield in the lower phase) as the PEG molecular weight was increased. We postulate that the violation of the general rule in our study was due to the fact that the volume exclusion effect imparted by PEG molecular weight was dominant to the electrostatic effect caused by the phase-forming salt. Therefore, as the molecular weight of PEG increases, the hydrolyzed PCL was partitioned into, and occupied, the lower phase, which led to the reduction of the volume available for the lipase in the lower phase. Furthermore, we hypothesized that the hydrolyzed PCL could form intermolecular interactions such as ion-dipole force and/or ion-induced dipole force with the ions of salts. The ability to form these intermolecular interactions could favor the retention of the hydrolyzed PCL over lipase in the lower phase, forcing lipase to move to the upper phase and/or interphase of the ATPS, due to the influence of volume exclusion effect as well as salting out effect (Grossman and Gainer, 1988). Together, these proposed mechanisms caused the reduction of lipase yield in the lower phase with an increasing molecular weight of PEG.

In addition, the transfer of lipase to the upper phase instead of its retention in the lower phase could be facilitated by the presence of interaction between lipase and PEG, which was assumed to be dominant over the volume exclusion effect formed by the PEG of in the upper phase (Spelzini *et al*., 2005). Interaction between protein and PEG has been shown to affect the partitioning of the former in PEG-phosphate and PEG-sodium sulphate systems (Haghtalab *et al*., 2003). Such an interaction could, however,

result in both positive and negative yields as the molecular weight of PEG was increased (Haghtalab *et al*., 2003). The contrary results could be explained by the solution behavior of PEG (Spelzini *et al*., 2005). PEG is able to form either a fully-extended structure or a compact structure, which are stabilized by varying degrees of intramolecular hydrophobic bonds. These bonds discourage the polymer to interact with the solvent, but at the same time increase its affinity to interact with the hydrophobic portions of proteins (Spelzini *et al*., 2005). As the difference in hydrophobicity between the two phases increases, the partition of the proteins tends to be facilitated into the upper phase (Ramyadevi *et al*., 2012a). Therefore, the ability to form flexible structures allows PEG to exert both positive and negative effects on the yield of the protein of interest.

Furthermore, we propose that the presence of electrostatic interaction between the lipase and PEG could be another underlying factor which contributed to the displacement of lipase from lower phase in our study. This postulation was first suggested by Xia *et al.* (1993), who hypothesized that an electrostatic interaction could be formed between the protonated carboxyl groups of pepsinogen and the oxygen ether of PEG. Increasing PEG molecular weight increased this interaction, which resulted in a better hydration and higher solubility of the pepsinogen in the PEG phase, causing the protein to transfer towards the PEG-rich phase. In addition, the repulsion force created by the negatively-charged salt ions constituting the lower phase of a PEG-salt ATPS could generate repulsion forces for proteins of the same charge, which further drove the transfer of the latter to the upper phase. The same mechanisms could be responsible for the preferential migration of lipase to the upper phase in the present study, thereby reducing its yield in the lower phase.

4.4.2 Effect of pH of the ATPS system

Figures 4.6 (*b*) and 4.7 (*b*) showed the effects of the pH of the ATPS system, in the range of pH 6.0 – 8.0, on the mean percentage of hydrolyzed PCL and lipase in the upper and lower phases, respectively. The system pH was initially selected for analysis in the present study as it has been previously suggested that manipulations of the pH could influence the partitioning of biomolecules in a readily predictable manner (Zaslavsky, 1995; Forciniti *et al*., 1991). Generally, we observed a positive relationship between the pH of the system and the yield of the hydrolyzed PCL in the upper phase. On the other hand, a negative relationship was found between the system pH and lipase yield in the lower phase. Therefore, at pH 8.0, the mean percentage of the hydrolyzed PCL in the upper phase was the highest whereas that of the lipase in the lower phase was the lowest, and vice versa.

Similar to the present study, the observation that the system pH was positively associated with the yield of products in the upper phase has been found in several other previous reports. For example, Mohammadi & Omidinia (2008) showed that the recovery of phenylalanine dehydrogenase in the upper phase was the highest when the system pH was increased from 5.8 to 8.0. In our study, the positive relationship between the system pH and the yield of hydrolyzed PCL could be explained by the coincidence between the higher end of pH range studied and the optimum pH for lipase stability and activity. It has been shown previously that maximum stability and enzymatic activity of *B. cepacia* lipase was achieved at pH 8.0 – 9.0 (Padilha *et al*., 2012). Therefore, at this pH, PCL hydrolysis occurred at a more rapid rate compared to the other pH examined in this study, thereby generating a higher yield of the hydrolyzed PCL. The higher rate of hydrolysis could indirectly contribute to the eventual greater recovery of the products,

which were preferentially partitioned into the upper phase due either to the hydrophobic interaction with PEG, or the salting-out effect in the lower phase.

The effect of pH on the partitioning of the biomolecules could also be explained by the fact that pH could alter the charge of the biomolecules in the system (Mohammadi *et al*., 2007; Raja *et al*., 2011). The value of pH, relative to the isoelectric points (pI) of the biomolecule, determines the net charge of the biomolecule in the solution (Barbosa *et al*., 2011). When pH is greater than, lesser than or equal to its pI, the biomolecule charges negative, positive and neutral respectively (Raja *et al*., 2011). Several previous studies have demonstrated that at a higher pH, the negatively charged biomolecule preferentially partitioned into the upper phase (Schmidt *et al*., 1994; Franco *et al*., 1996), due to the enhanced hydrophobic and electrostatic interactions between the biomolecule and PEG, in which PEG tends to act as positively charged and attracts the negatively charged biomolecules to the upper phase (Albertsson, 1986; Franco *et al*., 1996; Huddleston *et al*., 1991a; Ramyadevi, 2012a; Yücekan & Önal, 2011; Tanuja *et al*., 1997). *Burkholderia* lipase has a pI value of 6.3 and is therefore negatively charged at a pH higher than this (Ishimoto *et al*., 2001). Therefore, at a high pH, lipase had the tendency to enter the upper phase and move out from the lower phase, leading to its reduced yield in the lower phase in the present study. Our results was in agreement with that observed by Ooi *et al*. (2009a), who demonstrated the increased migration of *Burkholderia* lipase to the upper phase when the pH was raised from 6.5 to 8.5, presumably due to the influence of the protein charge. However, the result of ANOVA in our study illustrated that the effect of the system pH on the partitioning of the biomolecules was not statistically significant (upper phase, $P = 0.286$, Table 4.2; lower phase, $P = 0.109$, Table 4.5). One possible explanation for this observation is that the hydrophobic and electrostatic interactions between the lipase and phase-forming

PEG was limited by hydrolyzed PCL, whose transfer into the upper phase was also enhanced at high pH. Therefore, the tendency of the negatively-charged lipase to partition into the upper phase at high pH was restricted.

Besides, pH could influence the partitioning of the biomolecules by modifying the ratio of the charged and uncharged species in the system (Ramyadevi, 2012a). This ratio reflects the overall interactions of the charged and uncharged particles in the system, which play an equally important role as the net charge of the biomolecule in influencing the partition behavior, particularly for large biomolecules (Zaslavsky, 1995; Ramyadevi, 2012a). The unequal distribution of cations and anions in the system may also cause the formation of an electrical double layer near the interface, which influences the partitioning behavior of the charged biomolecules (Ramyadevi, 2012a). The Henderson-Hasselbalch equation could be used to predict the said ratio, such that the higher the pH of the system, the higher the ratio of A^- molar concentration to HA molar concentration, where HA refers to an undissociated weak acid while A^- is the conjugate base of the acid (Yong *et al*., 2010). As the concentration of A^- increases, repulsion or salting-out effects in the lower phase would be enhanced, which facilitate the transfer of lipase to the upper phase, hence reducing its yield in the lower phase (Macros *et al*., 1999; Ishimoto *et al*., 2001). Additionally, as pH also influences the partition of various salts and ions, phase composition could be altered with a change in pH (Mohammadi *et al*., 2007; Raja *et al*., 2011; Zhu *et al*., 1996). This could result in the modifications to the electrostatic [- difference between the two phases, which in turn, influences the partitioning behavior as well as the partition coefficient (Mohammadi *et al*., 2007; Zhu *et al*., 1996).

Apart from that, pH may also affect the partitioning behavior of the biomolecules by inducing aggregation to the biomolecules (Zaslavsky, 1995; Ratanapongleka, 2010). Protein aggregation may occur if the pH of the system is close to the isoelectric point of the protein. Saravanan *et al*. (2006) showed that when the pH of the system was increased from 6 to 8 (which was shifted nearer to the isoelectric point of the protein examined in the study), the transfer of the protein into the PEG-containing upper phase was facilitated. We postulate that in the present study, a similar increase in the rate of lipase aggregation occurred with the increasing pH, as the pH of the system became closer to the isoelectric point of *Burkholderia* lipase. Aggregation of the lipase increased the partitioning of the enzyme into the upper phase, thereby reducing its yield in the lower phase. A shift in pH can also affect the intramolecular bonds of the biomolecule, resulting in conformational changes, which could in turn, alter the partitioning behavior of the biomolecule (Zhu *et al*., 1996). In addition, although protein denaturation can also affect yield of lipase in upper and lower phase, this event was unlikely in the present study, as the pH used was close to the neutral pH and the optimum pH of the enzyme. All the above postulations could provide an explanation for the influence of pH on the increased and decreased yield of hydrolyzed PCL and lipase in the upper and lower phases respectively, as observed in the present study. Nevertheless, further quantitative and qualitative works are needed in order to confirm the postulations.

4.4.3 Effect of temperature

In this study, two different temperatures were involved throughout the process of extractive bioconversion, namely the temperature of bioconversion using lipase, and the temperature of product recovery using ATPS. Changes in the latter could result in the modification of tie-line length (TLL) (de Oliveira *et al*., 2008; Regupathi *et al*., 2011).

Since TLL is one of the main factors investigated in this study, the abovementioned changes could complicate the experimental design and analysis, which could eventually lead to misleading interpretations of data. Therefore, the present study focused only on the effect of temperature of bioconversion on the final yield of biomolecules, with the procedure of ATPS partitioning performed at room temperature. Nonetheless, despite not being investigated in the present study, the effect of partitioning temperature on the final yield of product has been more or less well-established, with a higher temperature typically associated with increased yields of biomolecules (Noorashikin *et al*., 2012; Xavier *et al*., 2013; Xia *et al*., 2011), although there are notable exceptions (Saravanan *et al*., 2006).

The effect of bioconversion temperature on the yield of hydrolyzed PCL in the upper phase was shown in Figure 4.6 (*c*), while that on the yield of lipase in the lower phase was shown in Figure 4.7 (*c*). Generally, an increase in the temperature was associated with a higher yield of PCL monomers and oligomers. For lipase, on the other hand, a negative relationship was observed, such that the higher the temperature, the lower was its yield. Remarkably, the effect of the bioconversion temperature was statistically significant, with $P < 0.001$ in both of the phases (Tables 4.2 and 4.5).

The most important influence of bioconversion temperature on the final yield of the biomolecules was probably associated with the activity and stability of lipase. As a general rule of thumb, a rise in temperature increases the activity of an enzyme, until a certain point where the enzyme undergoes denaturation and conformational changes, thereby losing its active site specificity (Biazus *et al*., 2009; Curvelo Santana *et al*., 2010). For many enzymes, the reaction rate approximately doubles with every 10 ℃ increase in the temperature (Wolfenden *et al*., 1999). In our study, three bioconversion

temperatures were selected and examined for their effects on the yield of biomolecules. The temperatures were 40, 49 and 58 ℃, each of which differed substantially from one another and could therefore influence the enzymatic activity, although verification of this hypothesis was beyond the scope of the present study. The possible increase in enzymatic activity could result in a greater amount of PCL being hydrolyzed in our study, which contributed to the increased recovery of PCL monomers and oligomers in the upper phase at high temperatures.

The link between the increased rate of bioconversion (and hence the increased initial amount of hydrolyzed PCL) and the ultimate product recovery was, however, not straightforward. Nevertheless, it is possible that for ATPS systems with high and low initial loads of PCL monomers and oligomers, the overall balance of intermolecular interactions between the hydrolyzed PCL and phase-forming components was different. The different loads could also contribute to different degrees of phase ratio changes, which in turn affected the partitioning efficiency of not only the hydrolyzed PCL, but also the lipase (Amid *et al*., 2014). This eventually resulted in different yields of the biomolecules in systems with different temperatures of bioconversion.

In addition to the increase rate of reaction, a higher temperature is also typically associated with a reduced stability of enzymes at prolonged durations. The stability of an enzyme can be measured based on its half-life (Doran, 1995; Vieille & Zeikus, 2001). Generally, the half-life of enzymes in a high temperature system is shorter than that in a system of low temperature, provided that the solvent used in both systems was identical (Doran, 1995; Marangoni, 2002). At any given temperature and system composition, the half-lives of lipases from different organisms are different, and there is currently a paucity of information on the half-lives of *Burkholderia* lipase in PEG/phosphate

systems at the three temperatures examined in the present study. However, the half-lives of lipases are normally much shorter than the 72-hour incubation period during the process of bioconversion in our study (Nawani *et al*., 2006; Rahman *et al*., 2005; Salameh & Wiegel, 2010; Zhang *et al*., 2010). Thus, it is possible that by the end of the incubation period, a greater fraction of lipase had been degraded in a system of higher temperatures, which contributed to its reduced recovery and yield in the lower phase of the said system.

4.4.4 Effect of tie-line length

The effect of tie-line length (TLL) on the yields of hydrolyzed PCL in the upper phase and lipase in the lower phase was also investigated in this study, and the results were shown in Figures 4.6 (*d*) and 4.7 (*d*) respectively. For both the upper and lower phases, a negative relationship was observed between the TLL and the yield of the respective biomolecules. Although TLL influenced the partitioning of hydrolyzed PCL in the upper phase and lipase in the lower phase in the same direction, the magnitude of the influence was different. The results of the ANOVA test indicated that the effect of TLL on hydrolyzed PCL yield in the upper phase did not reach statistical significance (P = 0.697), while that on lipase yield in the lower phase was highly significant ($P < 0.001$) (Tables 4.2 and 4.5).

Partitioning of biomolecules in an ATPS relies considerably on, among other factors, the concentration of the phase-forming components (Khayati, 2013; Navapara *et al*., 2011). An increase in salt concentration, for example, promotes the salting out effect, which increases the tendency of biomolecules to move out from the salt-rich phase (Mehrnoush *et al*., 2011; Wang *et al*., 2010). Considering the fact that TLL corresponds positively to the concentrations of phase-forming components in both

phases of ATPS (see Equation 4.2), it is justifiable to implicate the role of TLL in the modification of partition behavior of biomolecules.

$(TLL)^2 = (A_p)^2 + (A_s)^2$ (Equation 4.2)

where A_p is the difference in polymer concentration (in % w/v) between upper and lower phases and A_s is the difference in salt concentration (in % w/v) between the said phases

Based on the equation above, a higher TLL therefore signifies an increased concentration of PEG in the upper phase and phosphate salt in the lower phase in the present study. Our observation that a high TLL was associated with a low yield of hydrolyzed PCL and lipase in the respective phases can thus be explained as follows. At high TLL, the increased PEG concentration denotes that a greater number of polymer units were available to form various types of interaction with appropriate biomolecules (Raja *et al*., 2011). For example, when lipase acquired a net negative charge at high pH, it could be attracted to the upper phase where PEG acted as a positively charged compound (Huddleston *et al*., 1991a; Tanuja *et al*., 1997). Once in the upper phase, the hydrophobic part of the lipase could also form hydrophobic interactions with the polymer, thereby occupying the free volume available for other biomolecules, such as PCL monomers and oligomers, whose interactions with the PEG were weak. The hydrolyzed PCL was then forced to leave the upper phase, thereby causing its reduced yield. The accumulation of lipase on the upper phase also explained its low recovery in the lower phase.

The entry of lipase into the upper phase in ATPS with high TLL was not due entirely to the increase in PEG concentration. A higher TLL also signifies the increase

in the concentration of salt in the lower phase. The high concentration of salt ions increased the demand of water molecules, thus decreasing the number of water molecules available for interaction with the charged part of lipase (Prasad, 2010). This ultimately promoted hydrophobic interactions among lipases, which caused the coagulation of the enzyme and its eventual evacuation from the salt-rich lower phase in a process termed "salting out" (Mehrnoush *et al*., 2011; Prasad, 2010; Wang *et al*., 2010). Additionally, the retention of lipase in the lower phase was further discouraged by the fact that an increase in TLL was also associated with a drop in the free volume available for biomolecules in the lower phase (Mehrnoush *et al*., 2012). Compared to the reduction of free volume in the upper phase as described above, the free volume drop in the phosphate-rich lower phase was relatively more dramatic. The effect of TLL was therefore more apparent in the lower phase, which explains why the lower phase was significantly affected by TLL while the upper phase was not significantly affected.

4.4.5 Effect of volume ratio

Figures 4.6 (*e*) and 4.7 (*e*) respectively showed the effect of volume ratio (V_R) on the mean percentage of hydrolyzed PCL in the upper phase and lipase in the lower phase. A positive relationship was observed in the upper phase, with a higher V_R corresponded to a greater yield of hydrolyzed PCL. A contrasting observation was seen in the lower phase, in which the higher the V_R, the lower the lipase yield. Notably, despite exerting its influence in opposing directions in the upper and lower phases, the effect of V_R on the yield of the respective biomolecules was statistically significant, with ANOVA *P* values of less than 0.001 in both phases (Tables 4.2 and 4.5).

The mechanism underlying the above observation is rather straightforward. As mentioned in Chapter 3.2.7, V_R refers to the ratio of volume in the upper phase to that in

the lower phase. Therefore, as V_R increases, the free volume available for accommodating the solute molecules in the upper phase increases, while that in the lower phase decreases (Ooi *et al*., 2009b). The increase in the free volume in the upper phase encouraged a higher amount of water molecules to enter the said phase, thus extending its solubility limit. Hence, in the present study, the entry of both hydrolyzed PCL and lipase into the upper phase, and the corresponding evacuation of both the biomolecules from the lower phase, was facilitated with an increase in V_R. In addition, ε-caprolactone monomers and oligomers, being polar molecules, can form hydrogen bonds with the water molecules which entered the upper phase. This further strengthened the retention of the hydrolyzed PCL in the upper phase as the V_R of the system was increased. Together, these mechanisms resulted in the enhancement of the yield of the hydrolyzed PCL in the upper phase, and the reduction of the recovery of lipase in the lower phase.

Apart from that, the specific activity of certain enzymes, such as alcohol dehydrogenases, has been shown to be positively influenced by the V_R of the system (Madhusudhan *et al*., 2008). Although much further works are warranted, it remains a possibility that the increase in V_R was also associated with a higher activity of lipase in the present study, which rendered the hydrolysis of PCL more effective during the bioconversion process. This produced a greater amount of PCL monomers and oligomers, which consequently led to an increase in the yield of hydrolyzed PCL in the upper phase in our study.

4.4.6 Effect of NaCl additive

The effect of NaCl additive on the partitioning of hydrolyzed PCL in the upper phase and lipase in the lower phase was shown in Figures 4.6 (*f*) and 4.7 (*f*), respectively.

Generally, the higher the concentration of the NaCl additive in the upper phase, the lower the yield of the hydrolyzed PCL. On the contrary, a positive relationship was observed in the lower phase, such that a higher yield of lipase was obtained in a system containing a higher concentration of the neutral salt additive. The effect of the NaCl additive on the yield of hydrolyzed PCL in the upper phase was statistically highly significant ($P < 0.001$) (Table 4.2). However, its effect on lipase yield in the lower phase was not significant, although the P value was close to the borderline of statistical significance ($P = 0.053$) (Table 4.5). Nevertheless, the lack of statistical significance for the effect of NaCl additive on lipase partitioning was not unexpected. It has been previously shown that when the pH of an ATPS is near to the isoelectric point of a protein, the effect of the salt additive on its partitioning would not be substantial (Gu, 2000). In this study, the systems of pH 6.0 to 8.0 were used, all of which were reasonably near to the isoelectric point of *Burkholderia* lipase, which is around 6.3.

The role of additive in influencing ATPS partitioning was initially suspected, and eventually investigated, in the present study because additives can present long range effects to the characteristics of aqueous solutions (Zaslavsky, 1995). Since the structure of water relies heavily on relatively weak forces such as hydrogen bonds, its spatial arrangement and distribution of the intermolecular interaction energies could be easily affected by the addition of various types of solutes (Zaslavsky, 1995). Thus, the presence of different types and concentrations of additive could result in variations to the ionic composition, charge distribution, solvent polarity and other properties of aqueous solutions, which could in turn, affect biomolecule partitioning in ATPSs. In fact, various types of salt have been used as additives in several ATPSs, and have been shown to influence the partitioning of various biomolecules (Ferreira & Teixeira, 2011; Luechau *et al*., 2009; Srinivas, 2000). Addition of salts has also been shown to reduce

the concentration of polymer required for phase separation in ATPSs, which further assures its importance in biomolecule partitioning.

The trend by which NaCl affected the partitioning of hydrolyzed PCL and lipase in the present study could be explained by the modification of electrostatic potential difference by the additive (Zaslavsky, 1995). Addition of low concentrations of univalent salts such as NaCl has been shown to alter the ionic composition of the system without causing significant impact to the position of the binodal (Diamond & Hsu, 1992). Since different ions have different affinities to the two phases, changes in ionic composition can disturb the distribution of cations and anions present in the system. Alterations to the ionic distribution can create electrostatic potential difference across the interface, which upsets the electrical interaction and repulsion between the molecules present, thus affecting the distribution of polyelectrolytes or charged molecules (Madeira *et al*., 2005; Pfennig & Schwerin, 1995). The higher the concentration of NaCl additive added, the greater the electrostatic potential difference, which leads to the enhanced interaction between the biomolecules and the salt-rich lower phase (Raja & Murty, 2013). This mechanism caused a greater amount of the hydrolyzed PCL to move out from the upper phase in the present study, thereby reducing its yield, as the concentration of the NaCl additive was increased from 0.1 M to 1.0 M. Similarly, the increase in the concentration of the NaCl additive corresponded to a greater electrostatic potential difference, which exerted a strong effect on the partitioning of lipase since the enzyme contains a large number of charged amino acid residues. The greater electrostatic potential difference encouraged the partitioning of lipase into the lower phase, thus facilitating its recovery.

Besides, the effect of NaCl additive on the partitioning of the biomolecules can also be explained with the concept of free volume. As NaCl dissociates in the aqueous solution, the Cl^- anion produced tends to be attracted to the PEG-containing upper phase, as the polymer inclines to act as a positively charged entity (Huddleston *et al*., 1991a; Tanuja *et al*., 1997). With the entry of Cl^- anions into the upper phase, the free volume available for the biomolecules in the said phase decreases. Additionally, as mentioned above, the addition of neutral salt could disrupt the distribution of ions in the system. Some of the phase-forming salt molecules could be brought to the interface or the upper phase, and dominate the free volume available. Other than that, in the present study, we propose that some of the lipases entered the upper phase and form hydrophobic interactions with PEG, thereby further taking up the free volume available. The transfer of the hydrolyzed PCL into the upper phase was thus restricted by the lack of free volume available, leading to its low yield in the upper phase as NaCl was added into the system. The migration of the hydrolyzed PCL into the lower phase could also be promoted by its intermolecular interactions (such as ion-dipole force and/or ion-induced dipole force) with the ions of salts in the lower phase, as well as by the volume exclusion effect aroused from the lipase-PEG interaction in the upper phase. The presence of the proposed lipase-PEG interaction also signifies that a reduced amount of lipase could be partitioned into the lower phase, which probably explains the lack of significance of NaCl addition with the yield of lipase in the lower phase.

Apart from that, the properties of a biomolecule could be altered by the additive added, thereby influencing the partitioning behavior of the biomolecule. A protein, for example, could form various interactions with the additive added, or undergo conformational modifications in response to the altered properties of the aqueous solutions (Zaslavsky, 1995). The salt ions generated during dissociation of the additive

in aqueous solutions could also decrease the solubility of biomolecules (Raja & Murty, 2013). For example, addition of salt has been shown to reduce the solubility of various proteins, such as serum albumin and ceruloplasmin, in PEG. We postulate that the solubility of lipase in PEG was also negatively affected by the addition of NaCl. Hence, when high concentrations of NaCl were added into the ATPS, the transfer of lipase to the PEG-rich upper phase was discouraged, which explains its high yield in the lower phase.

4.5 One-factor-at-a-time optimization for recovery of hydrolyzed PCL and lipase

The results from the full factorial screening indicated that bioconversion temperature, V_R and additive were the factors which could significantly affect the partitioning and recovery of hydrolyzed PCL in the upper phase. On the other hand, for the partitioning of lipase in lower phase, the significant factors were bioconversion temperature, V_R and TLL. Further optimization of the significant factors is important to identify the most ideal conditions at which extractive bioconversion of PCL in ATPS occur.

For the purpose of optimization, apart from the factor being optimized, all other factors were fixed at a level where the balance between high yields of hydrolyzed PCL in the upper phase and lipase in the lower phase could be attained. The factors were optimized sequentially, starting with temperature and subsequently TLL and V_R. Notably, the addition of salt additive in the system was excluded from the optimization process. This is because the presence of additives caused strong decline in the yield of the hydrolyzed PCL in the upper phase, and increased the yield of lipase in the lower phase only slightly. Therefore, a system without the NaCl additive was preferred, since a high yield of hydrolyzed PCL in the upper phase could be obtained while not causing

serious trade-off to the yield of lipase in the lower phase. Hence, in all optimization work, systems without the presence of NaCl were used.

4.5.1 Optimization of temperature

Optimization of temperature was performed in a system composed of 19 % (w/w) PEG 3000 and 8.1 % (w/w) potassium phosphate at pH 7.0, TLL of 28 % (w/w) and V_R of 70/30. A total of seven temperatures were selected for the optimization *viz.* 31, 33, 35, 37, 40, 49 and 58 ℃. The trends by which the temperatures affected the recovery of the hydrolyzed PCL in the upper phase and lipase in the lower phase were shown in Figure 4.8. For the upper phase, a positive trend was observed between temperature and the hydrolyzed PCL recovery, where the temperatures of 31, 33, 35, 37, 40, 49 and 58 ℃ corresponded to 36.1, 38.3, 50.2, 60.1, 62.1, 64.8 and 66.0 % yields respectively. The rate of enzymatic hydrolysis increased with an increase in temperature (Wolfenden *et al*., 1999), hence higher the amount of products generated. This contributed to an overall higher initial load for partitioning in ATPS, which translated into an increased product recovery (Lin *et al*., 2012).

On the other hand, the yield of lipase was relatively constant at the lower temperatures, followed by a positive trend at slightly higher temperatures, until a certain point where an opposite trend was observed. The yield of lipase in the lower phase was 31.8, 31.7, 32.1, 42.3, 45.5, 41.3 and 32.5 % at 31, 33, 35, 37, 40, 49 and 58 ℃ respectively. At lower temperature ranges, it is suggested that most of the lipases were attracted to the upper phase due to the availability of free volume in the upper phase, resulting in the low yields in the lower phase. The free volume in the upper phase could only accommodate a limited amount of lipase, thus leading to the relatively constant yield at these temperatures. At higher temperatures, the enzymatic rate increased,

leading to the increase in the amount of hydrolyzed PCL in the upper phase. The entry of hydrolyzed PCL into the upper phase reduced the free volume available for lipase, thereby forcing the latter to move into the lower phase, which increased its yields. However, the stability of enzyme at temperatures above 40 ℃ was low (Nawani *et al.*, 2006; Rahman *et al.*, 2005; Salameh & Wiegel, 2010; Zhang *et al.*, 2010). Consequently, after the long incubation period of 72 hours, much of the lipase lost their activities, which led to its lower initial load for partitioning and hence, a reduced yield at temperatures above 40 ℃.

Considering the tradeoff between yields of hydrolyzed PCL in the upper phase and lipase in the lower phase, 40 ℃ was deemed as the optimum temperature for the extractive bioconversion.

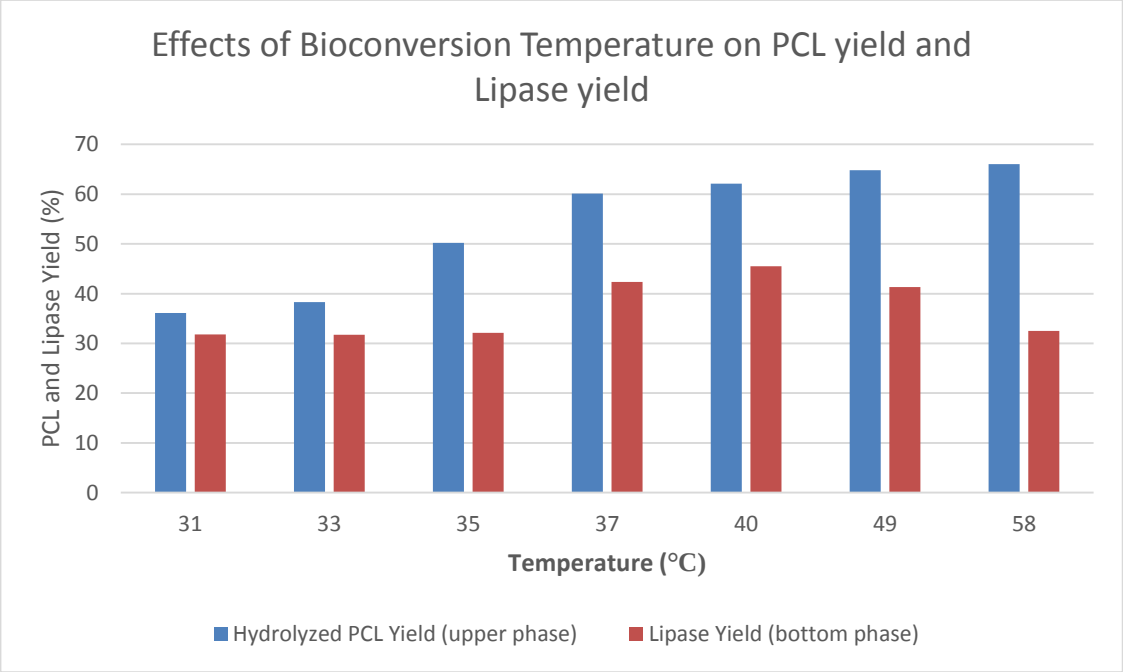


Figure 4.8: Effects of bioconversion temperature on the yields of hydrolyzed PCL in the upper phase and lipase in the lower phase

4.5.2 Optimization of TLL

TLL was optimized in a system composed of 19 % (w/w) PEG 3000 and 8.1 % (w/w) potassium phosphate at pH 7.0, temperature of 40 ℃ and V_R of 70/30. Five TLLs were investigated, namely 18 %, 23 %, 28 %, 32 % and 38 % (w/w). The effect of TLL on hydrolyzed PCL and lipase yields in the upper and lower phases respectively was shown in Figure 4.9. Generally, the yield of both hydrolyzed PCL and lipase initially increased with an increase in TLL, until a certain point where an opposite trend was observed. At the five TLLs, the yields of hydrolyzed PCL in the upper phase were 58.0, 65.8, 63.5, 58.9, and 52.3 % respectively, while the yields of lipase in the lower phase were 39.0, 40.2, 49.4, 41.3, and 36.0 %. Considering the balance between the yield of hydrolyzed PCL and lipase in their respective phases, a TLL of 28 % (w/w) was considered as optimum.

Based on the observation that an increase in TLL corresponded to the increase in PEG and salt concentrations, the above trend can be explained as follows: an increase in PEG concentrations at high TLL signifies the availability of more polymer units for various kinds of interactions with biomolecules (Raja *et al*., 2011). Therefore, initially, the PCL monomers and oligomers could interact more frequently with the PEG through, for example, hydrogen bonding, thereby favoring their partitioning into the upper phase. The preference of the upper phase to accommodate hydrolyzed PCL over lipase at this stage caused the transfer of the latter into the lower phase, resulting in its increased yield. Moreover, at this initial TLL, the concentration of salt was still low in the lower phase. Therefore, salting out effect was not apparent, and most of the lipase molecules continue to be retained in the lower phase. Competition between hydrolyzed PCL and lipase in the upper phase was weak, which facilitated the initial increment of PCL yield in the upper phase with increasing TLL.

However, as TLL keep increasing, the salting out effect and the corresponding free volume reduction of the lower phase became more pronounced (Mehrnoush *et al*., 2012). Hence, lipase molecules moved out from the lower phase and entered the upper phase, where they competed with PCL monomers and oligomers for interaction with PEG. The interaction between lipase and PEG was stronger than that between hydrolyzed PCL and PEG. This was due to the fact that *Burkholderia* lipase was slightly negatively charged at pH 7 since it has a pI value of 6.3 (Ishimoto *et al*., 2001). Hence, it could attract to the PEG which acts as a positively charged polymer. Moreover, the high salt concentration in the lower phase led to the hydration of lipases, which exposed their hydrophobic parts and therefore, encouraged their hydrophobic interactions with the PEG (Bonomo *et al*., 2006). Lipases therefore occupied the free volume in the upper phase, which resulted in removal of hydrolyzed PCL from the upper phase through volume exclusion. Thus, as TLL increased, the yield of lipase in the lower phase as well as the yield of hydrolyzed PCL in the upper phase decreased.

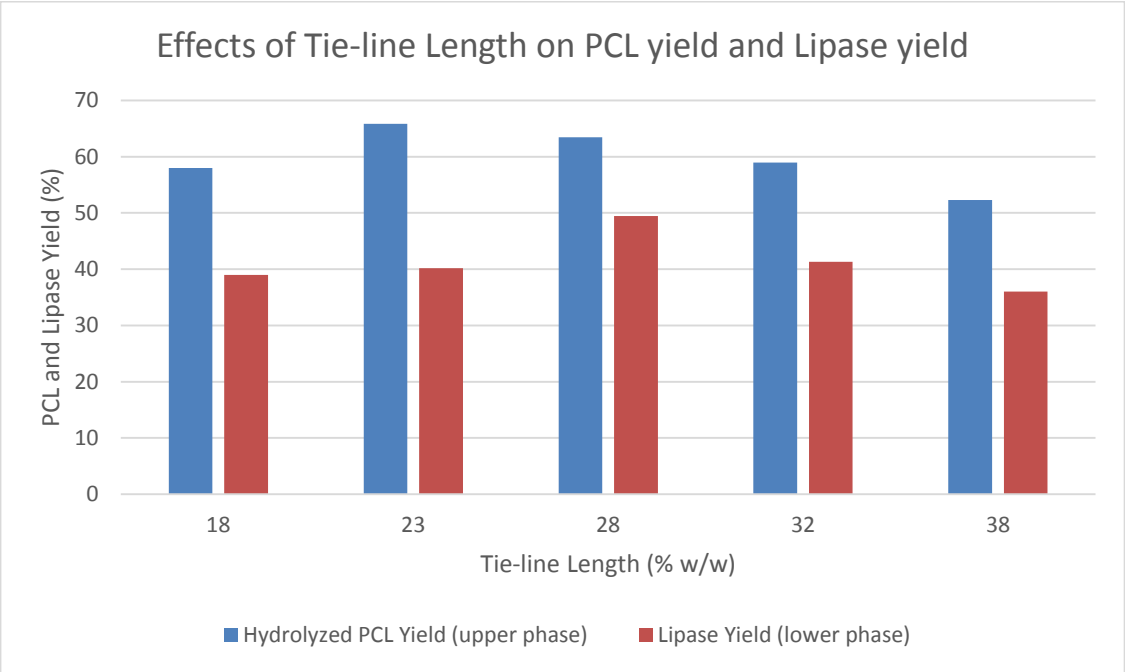


Figure 4.9: Effects of tie-line length (TLL) on the yields of hydrolyzed PCL in the upper phase and lipase in the lower phase

4.5.3 Optimization of volume ratio

Volume ratio (V_R) is another parameter which was chosen for the one-factor-at-a-time optimization for the recovery of hydrolyzed PCL and lipase. Optimization of the process was performed with five different V_Rs, namely 30/70, 50/50, 70/30, 80/20, and 90/10, in a system composed of 19 % (w/w) PEG 3000 and 8.1 % (w/w) potassium phosphate at pH 7.0, temperature of 40 ℃ and TLL of 28 % (w/w). The results of the optimization were shown in Figure 4.10. The yield of hydrolyzed PCL in the upper phase generally increased with increased V_R, until a certain point where a reduced yield was observed. The mean percentages of hydrolyzed PCL recovery in the upper phase at the five V_Rs were 30.3, 42.5, 63.7, 79.8, and 69.3 %, respectively. On the other hand, the yield of lipase in the lower phase initially decreased with increased V_R. The yield eventually became constant as the V_R continue to increase. The yields of lipase in the lower phase were 78.5, 46.2, 41.2, 42.0, and 42.0 % at the five V_Rs above. Therefore, the optimum condition for the extractive bioconversion was achieved with a V_R of 80/20, where the highest yield of hydrolyzed PCL could be obtained from the upper phase, without significant loss to the yield of lipase in the lower phase.

The initial increase in the yield of hydrolyzed PCL as V_R was increased could be due to direct increase in the free volume of the upper phase. An increased free volume permitted the retention of a greater number of the hydrolyzed PCL, thereby increasing its recovery percentage. The initial increase in V_R also caused the free volume reduction in the lower phase, thereby forcing the evacuation of lipase molecules from the lower phase resulting in reduced recovery. However, as V_R continue to increase, the yield of lipase in the lower phase became relatively constant. Although free volume reduction continue to occur, the overall dynamic interactions between other system parameters (e.g. pH, temperature, PEG molecular weight, etc) exerted a strong influence on the

partitioning behavior, which preferentially retained the lipase in the lower phase or the interface. Nevertheless, at the V_R of 90/10, the scarcity of free volume in the lower phase or interface resulted in the entry of some lipase molecules into upper phase, subsequently competing with hydrolyzed PCL for interaction with the phase-forming polymer. The increased entry of lipase could saturate the upper phase, leading to the random removal of biomolecules from the phase, resulting in the reduced recovery yield of hydrolyzed PCL.

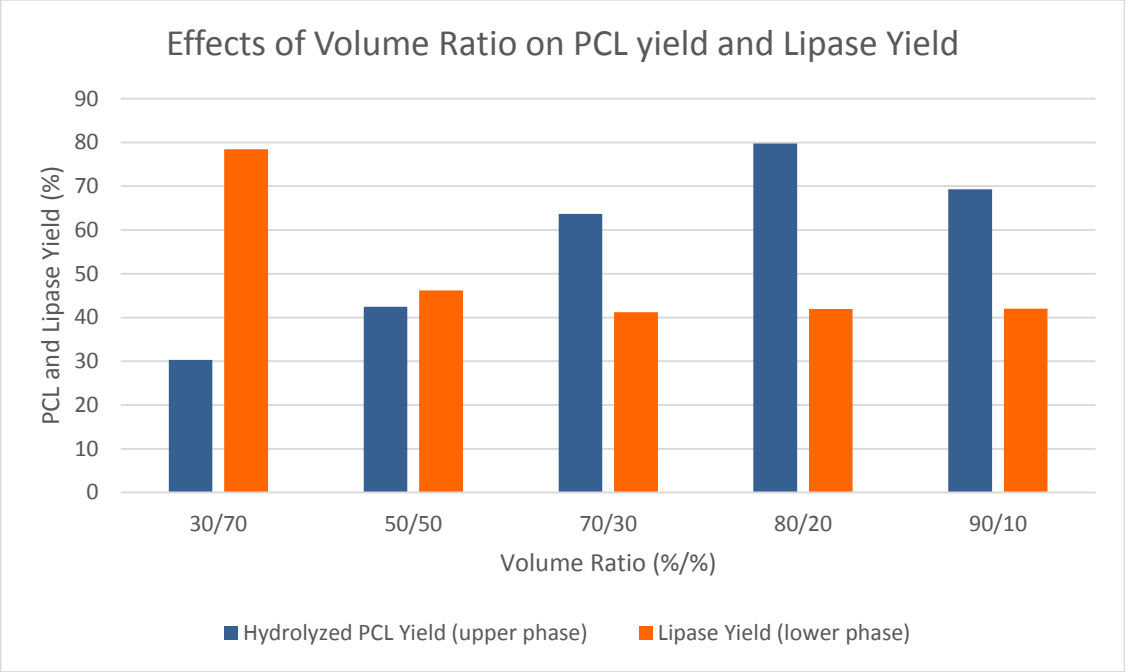


Figure 4.10: Effects of volume ratio (V_R) on the yields of hydrolyzed PCL in the upper phase and lipase in the lower phase

4.5.4 Concluding optimum conditions for recovery of hydrolyzed PCL and lipase

In this study, the applicability of ATPS in extractive bioconversion of PCL to its monomers and oligomers using *B. cepacia* lipase was demonstrated. The optimum conditions to achieve high yields of hydrolyzed PCL in the upper phase and lipase in the lower phase were: 19 % (w/w) PEG 3000, 8.1 % (w/w) potassium phosphate, no NaCl additive, TLL 28 % (w/w), V_R 80:20, pH 7.0 and 40 ℃ (Table 4.8). Under these

conditions, 79.8 % hydrolyzed PCL and 42.0 % lipase were recovered from the upper and lower phases respectively.

Table 4.8: Optimum conditions for recovery of hydrolyzed PCL and lipase

Parameter	Molecular weight of PEG	pH	Temperature	V_R	TLL (w/w) (%)	NaCl additive (%)
Optimum condition	PEG 3000	7.0	40 ℃	80:20	28	0

4.6 Properties of hydrolyzed PCL

4.6.1 Gas chromatography–tandem mass spectrometry (GC-MS/MS)

GC-MS/MS was carried out to identify the products formed after hydrolysis of PCL. GC-MS/MS is a highly specific, sensitive and selective technique for routine quantitative analysis of complex mixtures, and is able to remove matrix interference for providing reliable confirmation of mixture components (Thakur *et al*., 2010). Figure 4.11 showed the GC-MS/MS chromatograms of the samples obtained in upper phase (*a*) and lower phase (*b*) of the ATPS. Comparison of the two chromatograms revealed a common peak (*labeled "1"*) in both phases. However, a small peak (*labeled "2"*) was found specifically at the upper phase. Analysis with MassHunter Workstation software (Agilent, USA) indicated that the peak labeled "*1*" corresponded to ε-caprolactone monomers, whereas the peak labeled "*2*" corresponded to ε-caprolactone dimers (Figure 4.12). Control without lipase was also analyzed with GC-MS, and no similar peaks were observed in the chromatogram (data not shown). This provides an indication that the formation of the ε-caprolactone monomers and oligomers was due to action of lipase, and that ATPS could serve as an efficient method for recovery of the hydrolyzed products in extractive bioconversion of PCL.

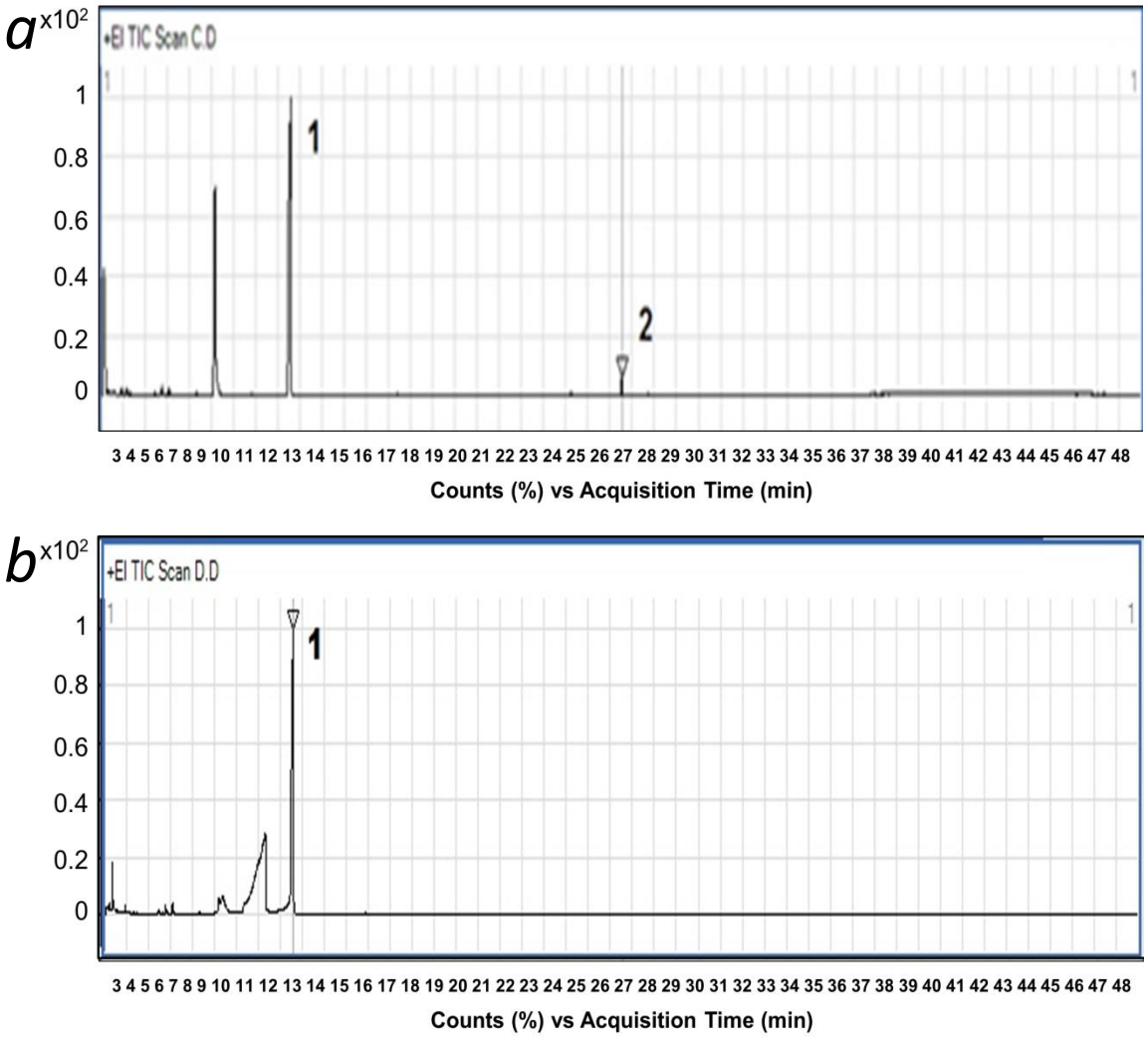


Figure 4.11: GC-MS/MS chromatograms of the samples obtained in upper phase (*a*) and lower phase (*b*) of the ATPS

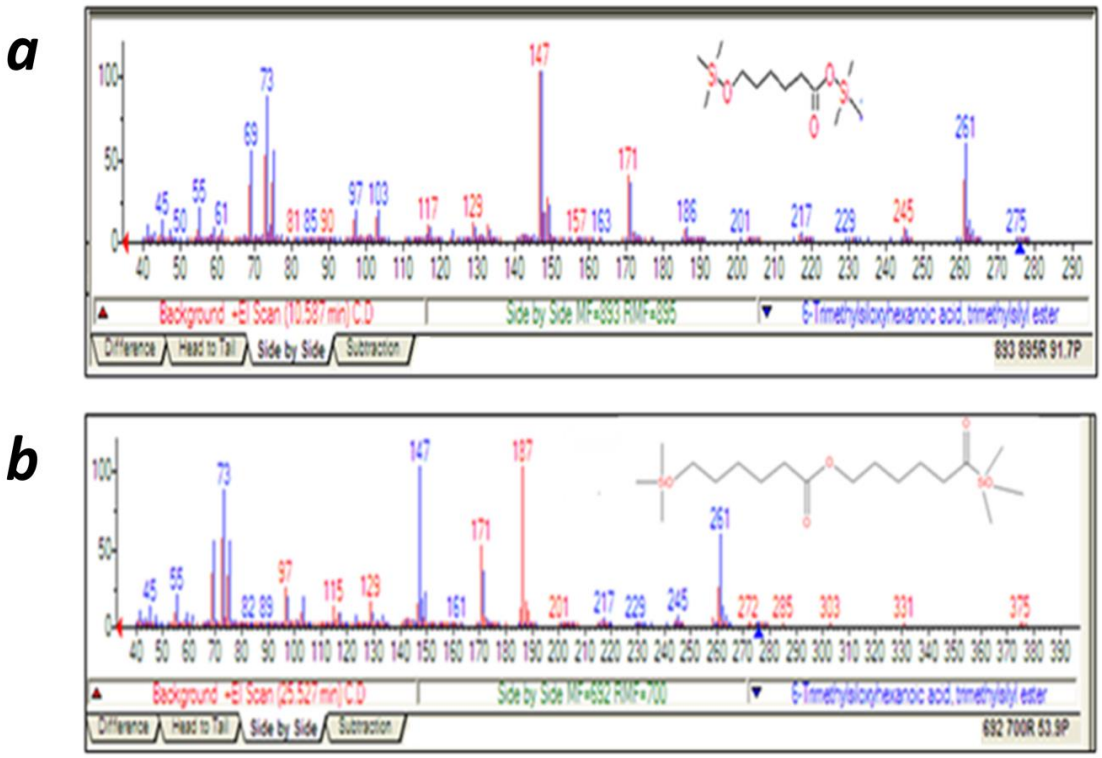


Figure 4.12: Analysis with MassHunter Workstation software revealed that the peaks labeled "*1*" and "*2*" corresponded to ε-caprolactone monomer (*a*) and dimer (*b*), respectively. The molecular weight of the silylated monomer was 261, while that of the silylated dimer was 375

4.6.2 Thermogravimetric analysis analysis (TGA)

The thermal stability of PCL was investigated by using thermogravimetric analysis (TGA), which was performed on the residual reactant after the completion of hydrolysis at 40, 49 and 58 ℃. The intact PCL (i.e. samples without undergoing hydrolysis) was also analyzed as a control. The losses of sample weight as a function of temperature were shown in Figure 4.13. It was observed that samples from different hydrolysis temperatures underwent weight loss starting from different heating temperatures. For the control sample, the weight loss was observed to start from approximately 380 ℃.

On the other hand, the recorded onsets of weight loss for excess reactants from 40, 49 and 58 ℃ hydrolysis reactions were approximately 372, 360, and 295 ℃ respectively. Therefore, the higher the temperature of hydrolysis, the lower the temperature at which the onset of weight loss was observed. A possible explanation for this observation is that the rate of hydrolysis increases with temperature (Al-Zuhair *et al*., 2003). Therefore, at high temperatures, most of the PCL had been hydrolyzed, thus leaving only a small amount of polymers intact. The small amount of intact polymer required less energy for breaking the necessary bonds to trigger decomposition, which corresponded to a lower onset temperature. Additionally, it was also observed that the weight of all the samples were relatively constant prior to the weight loss temperatures mentioned above. This observation indicates that the samples tested contained no volatiles such as solvent or moisture, and the weight loss was therefore due entirely to the decomposition of PCL (Sin *et al*., 2011).

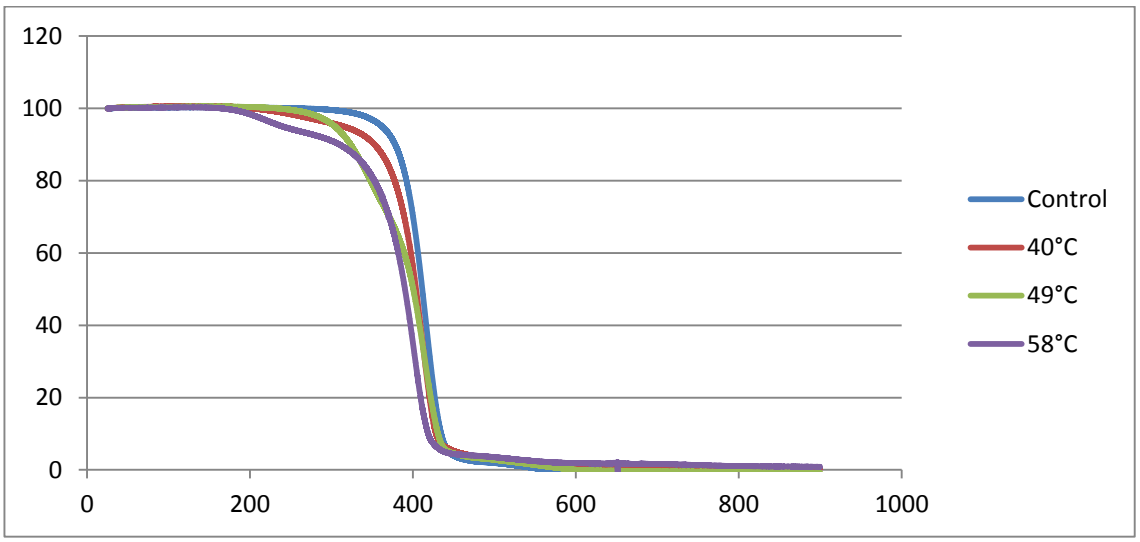


Figure 4.13: TGA thermogram showing the weight losses of PCL hydrolyzed at 40, 49 and 58 ℃, as well as unhydrolyzed control

4.6.3 Differential scanning calorimetry (DSC)

DSC was performed on excess reactants from 40, 49 and 58 ℃ hydrolysis reactions, as well as the unhydrolyzed control sample, to determine their melting and crystallization temperatures. The chromatograms were shown in Figure 4.14. Both melting and crystallization temperatures decreased with the increase in the hydrolysis temperature. The melting temperature of the excess reactants from 40, 49 and 58 ℃ hydrolysis reactions were 72.1, 70.7 and 70.6 ℃ respectively, while the crystallization temperature recorded for the three samples were 33.8, 33.2 and 32.7 ℃. Interestingly, while the melting temperature of the unhydrolyzed control sample was higher than those of the hydrolysis reactants (72.6 ℃), its crystallization temperature was lower than the other samples (30.1 ℃).

At high temperature ranges, the rate of hydrolysis was increased, which generated a higher amount of ε-caprolactone monomers and oligomers in the resulting reactants. The decreasing trend of the melting temperature with an increase in hydrolysis temperature could be explained by the fact that the presence of larger amounts of hydrolyzed products could disrupt the attractive forces that stabilize the polymer (Brittain & Bruce, 2006). As such, less energy was required to break the forces, which eventually contributed to the lower melting point (Brittain & Bruce, 2006). Additionally, the presence of these hydrolyzed products resulted in a lower molecular weight of the sample, which is typically associated with a lower melting and crystallization temperatures (Sperling, 2005). This is because the initiation of nucleation and nuclear density increment, which represent key steps in crystallization, is positively influenced by the molecular weight of a polymer (Li *et al.*, 2014). Therefore, samples hydrolyzed at high temperatures in this study, which had a lower molecular weight,

initiated the above steps later during the cooling down process, which translated into a lower temperature of crystallization.

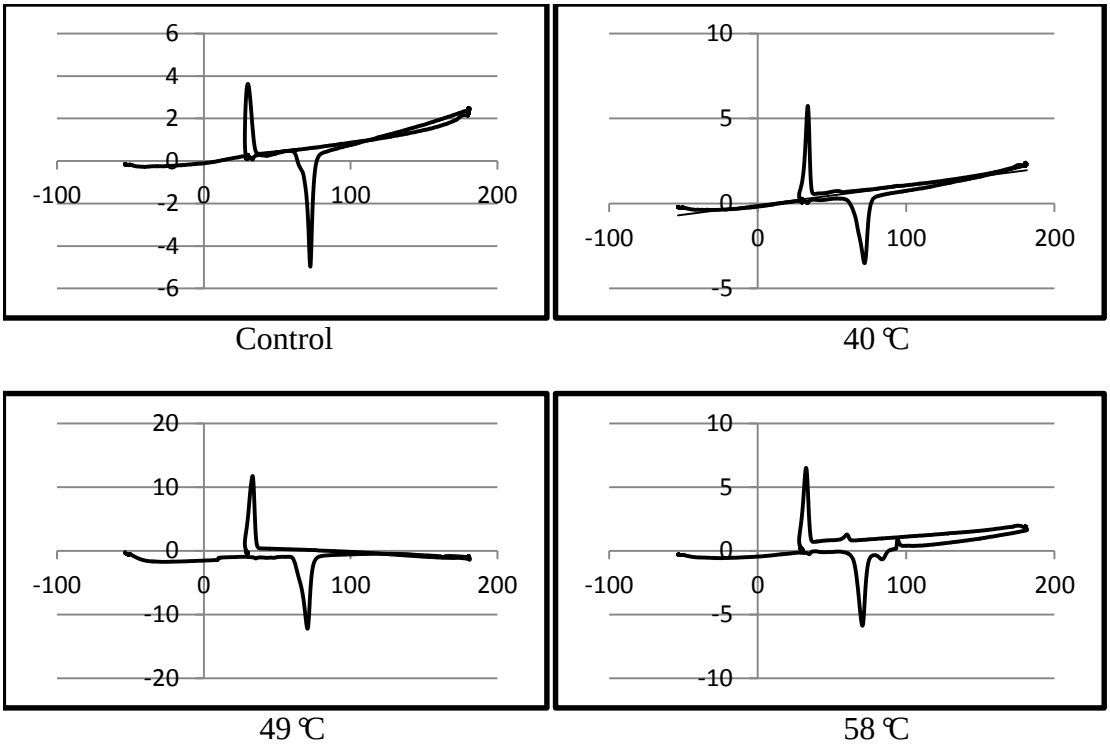


Figure 4.14: DSC curves for products hydrolyzed at 40, 49 and 58 ℃ and unhydrolyzed control

4.6.4 Gel permeation chromatography (GPC)

GPC was used to characterize the number averaged molecular weight (*Mn*), the weight averaged molecular weight (*Mw*) and the polydispersity index (PDI) of the residual reactants from hydrolysis of PCL at 40, 49 and 58 ℃, as well as the control sample. The average molecular weights were determined against a polystyrene (PS) calibration curve. A decreased *Mn* values was observed with the increase in hydrolysis temperature (Table 4.9). The *Mn* of the control sample was 43,293 g mol^{-1}, while those of the excess reactants from 40, 49 and 58 ℃ hydrolysis of PCL were 42,904, 32,460 and 2,734 g mol^{-1} respectively. The reduction in Mn values was primarily caused by the chain

scission of the ester linkages of PCL at moderately high hydrolysis temperature (Sin *et al*., 2010; Sin *et al*., 2011). Besides, the *Mw* for the four samples above were 78,262, 65,120, 51,725, and 4,438 g mol^{-1} respectively (Table 4.9). An increase in the PDI of the degraded PCL samples was also observed as hydrolysis temperature was increased from 40 to 58 ℃ (Table 4.9). This indicated that the hydrolyzed samples showed a broader molecular weight distribution, which could be explained by the generation of low molecular weight hydroxyalkanoic acids of varying sizes as the rate of hydrolysis was increased (Sin *et al*., 2011). It was postulated that the lipase employed a random chain scission mechanism to catalyze the enzymatic hydrolysis of the polymeric backbone, since broadening of the molecular weight distribution and rapid decrease of molecular weight were observed (Kunioka & Doi, 1989; Montano-Herrera *et al*., 2014).

Table 4.9: GPC analysis of PCL samples hydrolyzed at different temperatures

Sample	**GPC analysis**		
	M_n[a] **(Da)**	M_w[b] **(Da)**	***PDI***[c]
Control	43,293	78,262	1.81
PCL remaining at 40 ℃	42,904	65,120	1.52
PCL remaining at 49 ℃	32,460	51,725	1.59
PCL remaining at 58 ℃	2,734	4,438	1.62

[a] Number averaged molecular weight

[b] Weight averaged molecular weight

[c] Polydispersity index, defined by M_w/M_n

CHAPTER 5

CONCLUSION

In this study, we have successfully demonstrated the applicability of ATPS in extractive bioconversion of PCL to its monomers and oligomers using *Burkholderia cepacia* lipase. Results from the FFD screening demonstrated that among the six factors investigated, temperature of bioconversion, V_R and concentration of NaCl additive could significantly influence the partitioning of the hydrolyzed PCL into the upper phase. On the other hand, the partitioning of the lipase into the lower phase was significantly affected by temperature, TLL and V_R.

Inclusion of NaCl additive in the system resulted in drastic reduction in the yield of hydrolyzed PCL in the upper phase, but did not cause much alteration to the yield of lipase. Therefore, it was reasonably clear that a system without the presence of NaCl was desirable. Hence, concentration of NaCl additive was not subjected to the further optimization work, while bioconversion temperature, TLL and V_R were optimized using one-factor-at-a-time strategy. Optimization was done in a system composed of 19 % (w/w) PEG 3000 and 8.1 % (w/w) potassium phosphate at pH 7.0 without the presence of NaCl additive. It was found that the most optimum conditions, which allowed acceptable tradeoff between yields of hydrolyzed PCL in the upper phase and lipase in the lower phase, were at a temperature of 40 ℃, TLL of 28 % (w/w), and V_R of 80:20. Under these conditions, 79.8 % hydrolyzed PCL and 42.0 % lipase was recovered from the upper and lower phases respectively (Table 4.8).

Qualitative analysis, which included GC-MS/MS, TGA, DSC and GPC, were used to characterize the products formed after the extractive bioconversion. GC-MS/MS

analysis showed that the products obtained were ε-caprolactone monomers and oligomers. On the other hand, TGA analysis demonstrated that the onset of PCL decomposition was lower when the temperature of hydrolysis was higher. Analysis by DSC revealed that the melting and crystallization temperatures generally decreased with increase in the temperature at which PCL hydrolysis was performed. Besides, GPC analysis showed that as the temperature of hydrolysis was increased, the molecular weight of the PCL samples was decreased while the polydispersity index was increased. The summarized physical and chemical properties of the products were shown in Table 5.1.

Table 5.1: Physical and chemical properties of the products

Physical and chemical properties	**Main findings**
Molecular weight of :	
Silylated monomer	261
Silylated dimer	375
Onset of weight loss:	
40 ℃ hydrolysis reaction	372 ℃
49 ℃ hydrolysis reaction	360 ℃
58 ℃ hydrolysis reaction	295 ℃
Melting temperature:	
40 ℃ hydrolysis reaction	72.1 ℃
49 ℃ hydrolysis reaction	70.7 ℃
58 ℃ hydrolysis reaction	70.6 ℃

Table 5.1, continued

Crystallization temperature:	
40 ℃ hydrolysis reaction	33.8 ℃
49 ℃ hydrolysis reaction	33.2 ℃
58 ℃ hydrolysis reaction	32.7 ℃
M_n[a] **(Da)**	
PCL remaining at 40 ℃	42,904
PCL remaining at 49 ℃	32,460
PCL remaining at 58 ℃	2,734
M_w[b] **(Da)**	
PCL remaining at 40 ℃	65,120
PCL remaining at 49 ℃	51,725
PCL remaining at 58 ℃	4,438
PDI[c]	
PCL remaining at 40 ℃	1.52
PCL remaining at 49 ℃	1.59
PCL remaining at 58 ℃	1.62

[a] Number averaged molecular weight

[b] Weight averaged molecular weight

[c] Polydispersity index, defined by M_w/M_n

In conclusion, we have successfully established the optimum conditions for extractive bioconversion of PCL using *B. cepacia* lipase in an ATPS and characterized the products recovered.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE WORK

Although an ATPS for extractive bioconversion of PCL using *B. cepacia* lipase has been established in the present study, the yields of both hydrolyzed PCL and lipase was not exceptionally high. In order to improve the development of the system and apply it in the industrial scale, much more work could be done, but are not included in the present study due mainly to time and cost restrictions. The following experiments are recommended for future work.

Firstly, factors other than PEG molecular weight, TLL, pH, bioconversion temperature, V_R, and NaCl additive can be investigated for their significance in influencing the partitioning of the hydrolyzed PCL and lipase. Examples of factors which can be studied include temperature of ATPS partitioning, and the use of other additives, which have been previously found to exert significant effects to the partitioning of other biomolecules. In addition, since lipases from different organisms have different specificities and activities, the use of lipases from other organisms for the extractive bioconversion can be considered, given the fact that efficiency of enzyme could potentially influence biomolecule partitioning.

Besides, the partitioning of the hydrolyzed PCL and lipase can be studied in ATPSs composed of other phase-forming components. This is because interaction of hydrolyzed PCL and lipase with the phase-forming components represents a major determinant for the partitioning behavior of the biomolecules, and it is expected that the interaction would be dissimilar in systems containing different types of phase-forming components. It is possible that in ATPSs made of other phase-forming components, the

partitioning of hydrolyzed PCL into the upper phase and lipase into the lower phase would be enhanced. Therefore, it is recommended that the study be conducted in other types of ATPS. Several other polymer/salt-based systems, such as EOPO/ammonium sulfate and Ucon 50-HB5100/sodium citrate systems, have been shown to be effective for recovery of biomolecules and can be considered for extractive bioconversion of PCL. Apart from polymer/salt-based system, the extractive bioconversion can also be investigated in other types of ATPSs, such as polymer/polymer-based ATPSs or alcohol/salt-based ATPS.

In addition, although the significant factors which could affect the partitioning of the hydrolyzed PCL and lipase have been successfully established in the present study, the mechanisms by which these factors contribute to the differential partitioning behavior is less well understood. Most of the mechanisms described in Chapter 4 are purely postulations from published reports in the literature. Therefore, it is recommended that the specific mechanisms by which each significant factor affects the partitioning be studied. It may be helpful to study the partitioning kinetics as well. Understanding the specific mechanisms and kinetics of partitioning allows us to design a better strategy for recovery of the biomolecules in the future.

Finally, PCL and its monomers and oligomers are mainly used in large-scale manufacturing sectors. As such, the various advantages of ATPS, such as low cost and energy requirements, can only be maximized and fully appreciated when the extractive bioconversion is utilized in the industrial scale. To be applicable in the industrial scale, scaling up is necessary. It is thus recommended that experiments involving scaling up of the system be done in the future.

References

Abbott, N. L., Blankschtein, D., & Hatton, T. A. (1990). On protein partitioning in two-phase aqueous polymer systems. *Bioseparation*, *1*, 191-225.

Abdel-Motaal, F. F., El-Sayed, M. A., El-Zayat, S. A., & Ito, S. (2013). Biodegradation of poly (ε-caprolactone) (PCL) film and foam plastic by *Pseudozyma japonica* sp. nov., a novel cutinolytic ustilaginomycetous yeast species. *3 Biotech*. doi: 10.1007/s13205-013-0182-9.

Albertsson, P. Å. (1986). *Partition of cell particles and macromolecules.* New York: Wiley.

Albertsson, P. Å., Johansson, G., & Tjerneld, F. (1990). Aqueous two-phase separations. In: Asenjo, J. A. (Ed), *Separation Processes in Biotechnology* (pp. 287-327). New York: Marcel Dekker.

Alhir, S., Markakis, P., Chandan, R. C. (1990). Lipase of *Penicillium caseicolum. Journal of Agricultural and Food Chemistry, 38*(3), 598-601.

Ali, F. B., Kang, D. J., Kim, M. P., Cho, C. H., & Kim, B. J. (2014). Synthesis of biodegradable and flexible, polylactic acid based, thermoplastic polyurethane with high gas barrier properties. *Polymer International*, 63(9), 1620-1626.

Almeida, M. C., Ven âncio, A, Teixeira, J. A, & Aires-Barros, M. R. (1998). Cutinase purification on poly(ethylene glycol)-hydroxypropyl starch aqueous two-phase systems. *Journal of Chromatography B: Biomedical Sciences and Applications*, 711(1-2), 151–159.

Al-Zuhair, S., Hasan, M., & Ramachandran, K. B. (2003). Kinetics of the enzymatic hydrolysis of palm oil by lipase. *Process Biochemistry*, 38, 1155-1163.

Amid, M., Manap, Y., & Zohdi, N. K. (2014). A novel aqueous two phase system composed of a thermo-separating polymer and an organic solvent for purification of thermo-acidic amylase enzyme from red pitaya (*Hylocereus polyrhizus*) peel. *Molecules*, 19, 6635-6650

Amir, R. M., Shabbir M. A., Khan, M. R., Hussainm S. (2012). Interesterification of fats and oils-a review. *Pakistan Journal of Food Science, 22*(3), 143-153.

Ananthapadmanabhan, K. P., & Goddard, E. D. (1987). Aqueous biphase formation in polyethylene oxide-inorganic salt systems. *Langmuir, 3*, 25-31.

Andersson, E., & Hahn-Hägerdal, B. (1990). Bioconversions in aqueous two-phase systems. *Enzyme and Microbial Technology, 12*, 242-254.

Antony, J. (2014*). Design of experiments for engineers and scientists (2nd ed).* Elsevier Ltd, London.

Antov, M. G. (2005). Bioseparations in aqueous two-phase systems. *Acta Periodica Technologica* (*APTEFF*), 36(266), 145–154.

Aravindan, R., Anbumathi, P., & Viruthagiri, T. (2007). Lipase application in food industry. *Indian Journal of Biotechnology, 6*, 141-158.

Arnau, C., Ramon, R., Casas, C., & Valero, F. (2010). Optimization of the heterologous production of a Rhizopus oryzae lipase in Pichia pastoris systemusingmixed substrates on controlled fed-batch bioprocess. *Enzyme and Microbial Technology, 46*, 494–500.

Asenjo, J. A., & Andrews, B. A. (2012). Aqueous two-phase systems for protein separation: phase separation and application. *Journal of Chromatography A, 1238, 1*- 10.

Bailey, W. J., Ni, Z., & Wu, S. R. (1982). Synthesis of poly-ϵ-caprolactone via a free radical mechanism. Free radical ring-opening polymerization of 2-methylene-1,3-dioxepane. Journal of Polymer Science: *Polymer Chemistry Edition*, 20(11), 3021-3030.

Balasubramaniam, D. (2003). *Lysozyme separation from tobacco extract by aqueous two -phase extraction* (unpublished doctoral dissertation). Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Bamberger, S., Seaman, G. V. F., Sharp, K. A., & Brooks, D. E. (1984). The effects of salts on the interfacial tension of aqueous dextran poly(ethylene glycol) phase systems. *Journal of Colloid and Interface Science, 99*(1), 194–200.

Banik, R. M., & Santhiagu, A. (2002). Extractive fermentation for enhanced gellan-hydrolysing enzyme production by Bacillus thuringiensis H14. *Journal of Microbiology and Biotechnology, 1*8(8), 715-720.

Banik, R. M., Santhiagu, A., Kanari, B., Sabarinath, C., & Upadhyay, S. N. (2003). Technological aspects of extractive fermentation using aqueous two-phase systems. *World Journal of Microbiology and Biotechnology 19*, 337-348.

Barbosa, J. M., Souza, R. L., Fricks, A. T., Zanin,G. M., Soares, C. M. F. S., & Lima, A. S. (2011). Purification of lipase produced by a new source of *Bacillus* in submerged fermentation using an aqueous two-phase system. *Journal of Chromatography B, 879*(32), 3853-3858.

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

Becker, J. S., Thomas, O. R. T., Franzreb, M. (2009). Protein separation with magnetic adsorbents in micellar aqueous two-phase systems. *Separation and Purification Technology,* 65, 46-53.

Beijerinck, M. W. (1896). Uber eine Eigentumlichkeit der loslichen Starke. *Zentralblatt for Bakteriologie, Parasiten und Infektionskrankheiten, 2*, 679-699.

Bhat, R., & Timasheff, S. N. (1992). Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Science*, 1(9), 1133–1143.

Bisht, D., Yadav, S. K., & Darmwal, N. S. (2013). An oxidant and organic solvent tolerant alkaline lipase by *P. aeruginosa* mutant: downstream processing and biochemical characterization. *Brazilian Journal of Microbiology, 44*(4), 1305-1314.

Boisclair, M. D., Egan, D. A., Huberman, K., & Infantino, R. (2004). High-throughput screening in industry. In: Teicher, B. A & Andrews, P. A (Eds), *Anticancer Drug Development Guide* (pp. 23-39). New York, United States. Humana Press.

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.

Bradoo, S., Rathi, P., Saxena, R. K., & Gupta, R. (2002). Microwave-assisted rapid characterization of lipase selectivities. *Journal of Biochemical and Biophysical Methods, 51*, 115-120.

Brittain, H. G., & Bruce, R. D. (2006). Thermal analysis. In S. Ahuja & N. Jespersen (Eds.), *Comprehensive analytical chemistry: Modern instrumental analysis* (pp. 63-109). Amsterdam: Elsevier.

Cabezas, H. (1996). Theory of phase formation in aqueous two-phase systems. *Journal of Chromatography B: Biomedical Science and Application, 680*(1-2), 3-30.

Cabral, J. M. S. (2007). Cell partitioning in aqueous two-phase polymer systems. In: Kumar, A., Galaev, I., & Mattiasson, B. (Eds), *Cell Separation* (pp. 151-171). Berlin, Heidelberg: Springer-Verlag.

Carlsson, A. J., Bauer, P., Ma, H., & Widersten, M. (2012). Obtaining optical purity for product diols in enzyme-catalyzed epoxide hydrolysis: contributions from changes in both enantio- and regioselectivity. *Biochemistry, 51*, 7627-7637.

Castillo, B., Bansal, V., Ganesan, A., Halling, P., Secundo, F., Ferrer, A., Griebenow, K., & Barletta, G. (2006). On the activity loss of hydrolyses in organic solvents: II. A mechanistic study of subtilisin Carlsberg. *BMC Biotechnology, 6*(51).

Castro, B. D. & Aznar, M. (2005). Liquid-liquid equilibrium of water + PEG 8000 + magnesium sulfate or sodium sulfate aqueous two-phase systems at 35 ℃: experimental determination and thermodynamic modeling. *Brazilian Journal of Chemical Engineering, 22*(2), 463-470.

Celik, A., Kemikli, N., Ozturk, R., Muftuoglu, A. E., & Yilmaz, F. (2009). Synthesis, characterization and thermal properties of novel star polymer consisting of poly (ε-caprolactone) arms emanating from an octa-functional porphyzine core. *Reactive & Functional Polymers, 69*, 705-713.

Chen, J. P., & Lee, M. S. (1995). Enhanced production of *Serratia marcescens* chitinase in PEG/dextran aqueous two-phase systems. *Enzyme and Microbial Technology. 17*, 1021-1027.

Connors, K. A. (2002). *Thermodynamics of pharmaceutial systems: An introduction for students of pharmacy.* New Jersey: John Wiley & Sons.

Cramer, S. M., & Holstein, M. A. (2011). Downstream bioprocessing: recent advances and future promise. *Current Opinion in Chemical Engineering, 1*, 27-37.

Cunha, M. T., & Aires-Barros, M. R. (2000). Large-scale extraction of proteins. In: Hatti-Kaul, R. (Ed), *Aqueous Two-Phase Systems: Methods and Protocols* (pp. 391-409). Totowa, New Jersey: Humana Press.

Daugulis, A. J. (1994). Integrated fermentation and recovery processes. *Current Opinion. In Biotechnology, 5*, 192-195.

de Oliveira, R. M., dos Reis Coimbra, J. S., Francisco, K. R., Minim, L. A., da Silva, L. H. M., & Pereira, J. A. M. (2008). Liquid-liquid equilibrium of aqueous two-phase systems containing poly(ethylene) glycol 4000 and zinc sulfate at different temperatures. *Journal of Chemical & Engineering Data*, 53(4), 919-922.

Diamond, A. D., & Hsu, J. T. (1992). Aqueous two-phase systems for biomolecule separation. *Advances in Biochemical Engineering/Biotechnology*, 47, 89-135.

Doran, P. M. (1995). *Bioprocess Engineering Principles*. San Diego: Academic Press.

Dupont, J., de Souza, R. F., & Suarez, P. A. Z. (2002). Ionic liquid (molten salt) phase organometallic catalysis. *Chemical Reviews, 102*, 3667-3692.

Ebnesajjad, S. (2013). *Handbook of biopolymers and biodegradable plastics: properties, processing and applications*. Oxford, United Kingdom: Elsevier.

Euromonitor International. (2011). *Enzymes: Sustainable alternatives with remarkable scope.* London.

Farruggia, B., Nerli, B., Di Nuci, H., Rigatusso, R., & Picó, G. (1999). Thermal features of the bovine serum albumin unfolding by polyethylene glycols. *International Journal of Biological Macromolecules*, 26(1), 23–33.

Ferreira, L. A., & Teixeira, J. A. (2011). Salt effect on the aqueous two-phase system PEG 8000-sodium sulfate. *Journal of Chemical & Engineering Data*, 56, 133-137.

Ferrer, M., Soliveri, J., Plou, F. J., Lopez-Cortes, N., Reyes-Duarte, D., Christensen, M., Copa-Patino, J. L., & Ballesteros, A. (2005). Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida Antarctica* B, and their antimicrobial properties. *Enzyme and Microbial Technology, 36*(4), 391-398.

Forciniti, D., Hall, C. K., & Kula, M. R. (1991). Protein partitioning at the isoelectric point: influence of polymer molecular weight and concentration and protein size. *Biotechnology and Bioengineering*, 38(9), 986–94.

Franco, T. T., Andrews, A. T., & Asenjo, J. A. (1996). Conservative chemical modification of proteins to study the effects of a single protein property on partitioning in aqueous two-phase sytems. *Biotechnology and Bioengineering*, *49*(3), 290-299.

Freeman, A., Woodley, J. M., & Lilly, M. D. (1993). *In situ* product removal as a tool for bioprocessing. *Nature Biotechnology, 11*, 1007-1012.

Frost, G. M., & Moss, D. A. (1987). Production of enzymes by fermentation. In: Rehm, H.-J., Reed, G. (Eds), *Biotechnology* (pp. 65-211). Weinheim: VCH.

Garca-Perez, A. I., Sancho, P., & Pinilla, M. (1998). Surface and metabolic properties of microcytic and macrocytic human anaemic red blood cells detected in polymer aqueous two-phase systems. *Journal of Chromatography B: Biomedical Sciences and Applications, 711*, 301-307.

Garg, N., Galaev, I. Y., & Mattiasson, B. (1996). Dye-affinity techniques for bioprocessing: recent developments. *Journal of Molecular Recognition, 9*, 259-274.

Ghosh, P. K., Saxena, R. K., Gupta, R., Yadav, R. P., & Davidson, S. (1996). Microbial lipases: production and applications. *Science Progress, 79*(2), 119-157.

Giunchedi, P., Conti, B., Maggi, L., & Conte, U. (1994). Cellulose acetate butyrate and polycaprolactone for ketoprofen spray-dried microsphere preparation. *Journal of Microencapsulation*, 11(4), 381-393.

Gnanasekaran, D., Madhavan, K., & Reddy, B. S. R. (2009). Developments of polyhedral oligomeric silsesquioxanes (POSS), POSS nanocomposites and their applications: a review. *Journal of Scientific & Industrial Research, 68*, 437-464.

Goja, A. M., & Hong Yang, A. M. G. (2013). Aqueous two-phase extraction advances for bioseparation. *Journal of Bioprocessing & Biotechniques*, 04(01), 1–8.

Greve, A., & Kula, M. R. (1991). Phase diagrams of new aqueous phase systems composed of aliphatic alcohols, salts and water. *Fluid Phase Equilibria, 62*, 53-63.

Grossman, P. D., & Gainer, J. L. (1988). Correlation of aqueous two-phase partitioning of proteins with changes in free volume. *Biotechnology Progress, 4*(1), 6–11.

Gu, T. (2000). Liquid-liquid partitioning methods for bioseparations. In S. Ahuja (Ed.), *Handbook of bioseparations* (pp. 239-364). New York: Academic Press.

Gumel, A. M., Annuar, M. S. M., & Heidelberg,T. (2012). Effect of carbon substrates on biodegradable polymer composition and stability produced by *Delftia tsuruhatensis* Bet002 isolated from palm oil mill effluent. *Polymer Degradation and Stability, 97*, 1224-1231.

Gumel, A. M., Annuar, M. S. M., Heidelberg, T., & Chisti, Y. (2011). Lipase mediated synthesis of sugar fatty acid esters. *Process Biochemistry, 46*, 2079-2090.

Gündüz, U. (2000). Partitioning of bovine serum albumin in an aqueous two-phase system: optimization of partition coefficient. *Journal of Chromatography B: Biomedical Sciences and Applications, 43*(1-2), 259-262.

Guo, P., Xiao, X., El-Gohary, Y., Paredes, J., Prasadan, K., Shiota, C., Wiersch, J., Welsh, C., & Gittes, G. K. (2013). A simplified purification method for AAV variant by polyethylene glycol aqueous two-phase partitioning. *Bioengineering, 4*(2), 103-106.

Gupta, M. N. (2000). *Methods in non-aqueous enzymology*. New York, United States: Springer Science & Business Media.

Gupta, R., Bradoo, S., & Saxena, R. K. (1999). Aqueous two-phase systems: an attractive technology for downstream processing of biomolecules. *Current Science, 77,* 520-523.

Gupta, R., Gupta, N., & Rathi, P. (2004). Bacterial lipases: An overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology, 64*, 763-781.

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*, 6632-6633.

Haghtalab, A., Mokhtarani, B., & Maurer, G. (2003). Experimental results and thermodynamic modeling of the partitioning of lysozyme , bovine serum albumin , and α-amylase in aqueous two-phase systems of peg and (K2HPO4 or Na2SO4). *Journal of Chemical & Engineering Data*, 46, 1170–1177.

Harris, D. P., Andrews, A. T., Wright, G., Pyle, D. L., & Asenjo, J. A. (1997). The application of aqueous two-phase systems to the purification of pharmaceutical proteins from transgenic sheep milk. *Bioseparation, 7*, 31-37.

Harris, J. M. (1992). *Poly (ethylene glycol) chemistry: biotechnical and biomedical applications.* New York, United States of America: Springer Science & Business.

Harris, J. M., & Chess, R. B. (2003). Effect of pegylation on pharmaceuticals. *Nature Review Drug Discovery, 2*, 214-221.

Hart, R. A., Ogez, J. R., & Builder, S. E. (1995). Use of multifactorial analysis to develop aqueous two-phase systems for isolation of non-native IGF-I. *Bioseparation, 5*, 113-121.

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology, 39*, 235-251.

Hatti-Kaul, R. (1999). *Methods in Biotechnology: Aqueous Two-Phase systems: Methods and Protocols.*

Hatti-Kaul, R. (2000a). Aqueous two-phase systems: a general overview. In: Hatti-Kaul, R. (Ed), *Aqueous Two-Phase Systems: Methods and Protocols* (pp. 1-10). Totowa, New Jersey: Humana Press.

Hatti-Kaul, R. (2000b). Extractive bioconversion in aqueous two-phase systems. In: Hatti-Kaul, R. (Ed), *Aqueous Two-Phase Systems: Methods and Protocols* (pp. 413-417). Totowa, New Jersey: Humana Press.

Hatti-Kaul, R. (2001). *Aqueous two phase systems. Molecular Biotechnology, 19*(3), 269-277.

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*, 143-149.

Houde, A., Kademi, A., & Leblanc, D. (2004). Lipases and their industrial applications: An overview. *Applied Biochemistry and Biotechnology, 118* (1-3), 155-170.

Hua, D. L., & Xu, P. (2011). Recent advances in biotechnological production of 2-phenylethanol. *Biotechnology Advances, 29*(6), 654-660.

Huddleston, J. G., Ottomar, K. W., Ngonyani, D. M., & Lyddiatt, A. (1991a). Influence of system and molecular parameters upon fractionation of intracellular proteins from *Saccharomyces* by aqueous two-phase partition. *Enzyme and Microbial Technology*, 13, 24-32.

Huddleston, J., Veide, A., Kohler, K., Flanagan, J., Enfors, S., & Lyddiatt, A. (1991b).The molecular basis of partitioning in aqueous two-phase systems. *Trends in Biotechnology, 9,* 381.

Hustedt, H., Kroner, K. H., & Kula, M. R. (1985). Applications of phase partitioning in biotechnology. In: Walter, H., Brooks, D.E., & Fisher, D. (Eds), *Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses and Application in Biotechnology* (pp. 529-587). Orlando, Florida: Academic Press.

Hutmacher, D. W., Schantz, T., Zein I., Ng, K. W., Teoh, S. H., & Tan, K. C. (2001). Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. *Journal of Biomedical Materials Research*, 55(2), 203-216.

Ibarra-Herrera, C. C., Aguilar, O., & Rito-Palomares, M. (2011). Application of an aqueous two-phase systems strategy for the potential recovery of a recombinant protein from alfalfa (Medicago sativa). *Separation and Purification Technology, 77*(1), 94–98.

Idris, A. & Bukhari, A. (2011). Immobilized *Candida Antarctica* lipase B: hydration, stripping off and application in ring opening polyester synthesis. *Biotechnology Advances, 30*, 550-563.

Igarashi, L., Kieckbusch, T. G., & Franco, T. T. (2004). Xylanase mass transfer studies in an aqueous two-phase systems using spray and sieve plate columns. *Bioprocess and Biosystems Engineering, 26*, 151-159.

Ishimoto, R. Manabu, S., & Kawai, F. (2001). Screening and characterization of trehalose-oleate hydrolyzing lipase. *FEMS Microbiology Letters, 195*, 231-235.

Jaeger, K. E., & Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology, 16*, 396.

Jaeger, K. E., Dijkstra, B. W., & Reetz, M.T. (1999). Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology, 53*, 315-351.

Jaeger, K. E., Ransac, S., Dijkstra, B.W., Colson, C., Van Heuvel, M., & Misset, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, *15*, 29-63.

James, J. J., Lakshmi, B. S., Raviprasad, V., Ananth, M. J., Kangueane, P., & Gautam, P. (2013). Insights from molecular dynamics simulations into pH-dependent enantioselective hydrolysis of ibuprofen esters by *Candida rugosa* lipase. *Protein Engineering, Design and Selection*, *16*(12), 1017-1024.

Johansson, G., & Reczey, K. (1998). Concentration and purification of *β-glucosidase* from *Aspergillus niger* by using aqueous two-phase partitioning. *Journal of Chromatography B: Biomedical Sciences and Applications,* 711, 161-172.

Kalaivani, S., & Regupathi, I. (2013). Partitioning studies of α-lactalbumin in environmental friendly poly (ethylene glycol)-citrate salt aqueous two phase systems. *Bioprocess and Biosystem Engineering*, 36(10), 1475-1483.

Kamihira, M., Kaul, R., & Mattiasson, B. (1992). Purification of recombinant protein A by aqueous two-phase extraction integrated with affinity precipitation. *Biotechnology and Bioengineering, 40*, 1381-1387.

Kammoun, R., Chouayekh, H., Abid, H., Naili, B., & Bejar, S. (2009). Purification of CBS 819.72 α-amylase by aqueous two-phase systems: modelling using Response Surface Methodology. *Biochemical Engineering Journal, 46*, 306-312.

Kariduraganavar, M. Y., Kittur, A. A., & Kamble, R. R. (2014). Polymer synthesis and processing. In S. G. Kumbar, C. T. Laurencin & M. Deng (Eds.), *Natural and Synthetic Biomedical Polymers* (pp. 1-31). Burlington, USA: Elsevier.

Kepka, C., Lemmens, R., Vasi, J., Nyhammar, T., & Gustavsson, P. E. (2004). Integrated process for purification of plasmid DNA using aqueous two-phase systems combined with membrane filtration and lid bead chromatography. *Journal of Chromatography A, 1057*(1-2), 115-124.

Kfoury, G., Raquez, J. M., Hassouna, F., Odent, J., Toniazzo, V., Ruch, D., & Dubois, P. (2013). Recent advances in high performance poly(lactide): from "green" plasticization to super-tough materilas via (reactive compounding). *Frontiers in Chemistry, 1*(32). Advance online publication. doi: 10.3389/fchem.2013.00032.

Khayati, G. (2013). Optimization of propionic acid extraction by aqueous two-phase system using response surface methodology. *Chemical Engineering Communications*, 200(5), 667-677.

Kitajyo, Y., Imai, T., Sakai, Y., Tamaki, M., Tani, H., Takahasji, K., Narumi, A., Kaga, H., Kaneko, N., Satoh, T., & Kakuchi, T. (2007). Encapsulation-release property of amphiphilic hyperbranched d-glucan as a unimolecular reverse micelle. *Polymer, 48*, 1237-1244.

Knowles, D. B., Lacroix, A. S., Deines, N. F., Shkel, I., & Record, M. T. (2011). Separation of preferential interaction and excluded volume effects on DNA duplex and hairpin stability. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(31), 12699-12704.

Kopperschlager, G. (1994). Affinity extraction with dye ligands. *Methods in Enzymology, 228*, 121-136.

Kopperschlager, G., & Birkenmeier. (1990). Affinity partitioning and extraction of proteins. *Bioseparation, 1*, 235-25.

Koshimune M, Takamatsu K, Nakatsuka H, Inui K, Yamano Y, & Ikada Y. (2003). Creating bioabsorbable Schwann cell coated conduits through tissue engineering. *Bio-Medical Materials and Engineering*, 13(3), 223-229.

Kumar, K., & Sawant K. (2013). Encapsulation of exemestane in polycaprolactone nanoparticles: optimization, characterization, and release kinetics. *Cancer Nanotechnology, 4*(4-5), 57-71.

Kunioka, M., Tamaki, A., & Doi, Y. (1989). Crystalline and thermal properties of bacterial copolyesters: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Macromolecules. 22*, 694-697.

Labet, M. & Thielemans, W. (2009). Synthesis of polycaprolactone: a review. *Chemical Society Reviews, 38*(12), 3484-3504).

Lacerda, V. G., Mageste, A. B., Santos, I. J. B., da Silva, L. H. M., & da Silva, M. D. C. H. (2009). Separation of Cd and Ni from Ni-Cd batteries by an environmentally safe methodology employing aqueous two-phase systems. *Journal of Power Sources, 193,* 908-913.

Larsson, M., Arasaratnam, V., & Mattiasson, B. (1989). Integration of bioconversion and downstream processing: starch hydrolysis in an aqueous two-phase system. *Biotechnology and Bioengineering, 33*, 758-766.

Li, L., Liu, J., Ren, Z., & Yan, S. (2014). Crystallization behavior of disproportionately high and low molecular weight PEO blends. *Chinese Journal of Polymer Science*, 32(9), 1199-1209.

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,* 58-62.

Li, X., Lian, Z., Dong, B., Xu, Y., Yong, Q., & Yu, S. (2011). Extractive bioconversion of xylan for production of xylobiose and xylotriose using a PEG6000/sodium citrate aqueous two-phase system. *Korean Journal of Chemical Engineering, 28*(9), 1897-1901.

Li, Z. G., Teng, H., & Xiu, Z. L. (2010). Aqueous two-phase extraction of 2,3-butanediol from fermentation broths using an ethanol/ammonium sulphate system. *Process Biochemistry, 45* (5), 731-737.

Li, Z., Liu, X., Pei, Y., Wang, J., & He, M. (2012). Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins. *Green Chemistry, 14*, 2941-2950.

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 and Technology, 47*(7), 1023–1030.

Ling, T. C., & Ng, H. S. (2013). *Extractive bioconversion of cyclodextrins by Bacillus cereus cyclodextrin glycosyltransferase using aqueous two-phase system* [Abstract]. Proceedings of the World Biotechnology Congress 2013, Boston, USA. Retrieved May 18, 2014, from http://www.worldbiotechcongress.com/abstracts/SL/SL-03-30 2013_074_TauChuanLING.htm.

Liu, P., Song, J., He, L., Liang X., & Ding, H. (2009). Properties of alkoxysilane functionalized polycaprolactone/polydimethylsiloxane-modified epoxy resin composites: Effect of curing temperature and compositions. *Journal of Applied Polymer Science, 114*(2), 811–817.

Lu, M., & Tjerneld, F. (1997). Interaction between tryptophan residues and hydrophobically modified dextran: effect on partitioning of peptides and proteins in aqueous two-phase systems. *Journal of Chromatography A, 766*, 99-108.

Luechau, F. Ling, T. C., & Lyddiatt, A. (2009). Selective partition of plasmid DNA and RNA in aqueous two-phase systems by the addition of neutral salt. *Separation and Purification Technology*, *68*(1), 114-118.

Lye, G. J., Asenjo, J. A., & Pyle, D. L. (1995). Extraction of lysozyme and ribonuclease-a using reverse micelles: Limits to protein solubilization. *Biotechnology and Bioengineering, 47*, 509-519.

Maafi, E. M., Malek, F., & Tighzert, L. (2009). Synthesis and characterization of new polyurethane based on polycaprolactone. *Journal of Applied Polymer Science, 115*(6), 3651–3658.

Madeira, P. P., Xu, X., Teixeira, J. A., & Macedo, E. A. (2005). Prediction of protein partition in polymer/salt aqueous two-phase systems using the modified Wilson model. *Biochemical Engineering Journal*, *24*, 147-155.

Madhusudhan, M. C., Raghavarao, K. S. M. S., & Nene, S. (2008). Integrated process for extraction and purification of alcohol dehydrogenase from Baker's yeast involving precipitation and aqueous two phase extraction. *Biochemical Engineering Journal, 38*, 414-420.

Marangoni, A. G. (2002) Lipases: Structure, function, and properties. In T. M. Kuo & H. W. Gardner (Eds.), *Lipid Biotechnology* (pp. 357-386). New York: Marcel Dekker.

Marco, R. P., Alejandro, N., Enrique, G., & Leobardo, S. C. (2000). Aroma compounds recovery from mycelial cultures in aqueous two-phase processes. *Journal of Chromatography B, 743*, 403–408.

Mattiasson, B. (1983). Applications of aqueous two-phase systems in biotechnology. *Trends in Biotechnology, 1*, 16-20.

McClean, S. & M. Callaghan (2009). *Burkholderia cepacia* complex: Epithelial cell-pathogen confrontations and potential for therapeutic intervention. Journal of *Medical Microbiology, 58,* 1-12.

Mehrnoush, A., Mustafa, S., & Yazid, A. M. M . (2011). 'Heat-treatment aqueous two phase system' for purification of serine protease from kesinai (*Streblus asper*) leaves. *Molecules, 16,* 10202-10213.

Mehrnoush, A., Mustafa, S., Sarker, M. Z. I., & Yazid, A. M. M. (2012). Optimization of serine protease purification from mango (*Mangifera indica cv. Chokanan*) peel in polyethylene glycol/dextran aqueous two phase system. *International Journal of Molecular Sciences, 13*(3), 3636-3649.

Mello, B. P. R., de Oliveira, T. F., Castiglioni, G. L., Soares, J. M. S., & Ulhoa, C. J. (2015). Application of lipase from burkholderia cepacia in the degradation of agro-industrial effluent. *Water Science and Technology, 1*(7), 957-964.

Middleton, J. C., & Tipton, A. J. (2000). Synthetic biodegradable polymers as orthopedic devices. *Biomaterials, 21*(23), 2335-2346.

Minuth, T., Thömmes, J., & Kula, M. R. (1996). A closed concept for purification of the membrane-bound cholesterol oxidase from *Nocardia rhodochrous* by surfactant-based cloud-point extraction, organic-solvent extraction and anion-exchange chromatography. *Biotechnology and Applied Biochemistry, 23,* 107-116.

Mohammadi, H. S. & Omidinia, E. (2007). Purification of recombinant phenylalanine dehydrogenase by partition in aqueous two-phase systems. *Journal of Chromatography B, 854*, 273–278.

Mohammadi, H. S., Omidinia, E., & Taherkhani, H. (2008). Rapid one-step separation and purification of recombinant phenylalanine dehydrogenase in aqueous two-phase systems. *Iranian Biomedical Journal, 12*(2), 115-122.

Montano-Herrera, L., Pratt, S., Arcos-Hernandez, M. V., Halley, P. J., Lant, P. A., Werker, A., & Laycock, B. (2014). In-line monitoring of thermal degradation of pha during melting processing by near-infrared spectroscopy. *New Biotechnology, 31*(4), 357-363.

Morenweiser, R. (2005). Downstream processing of viral vectors and vaccines. *Gene Therapy, 12*, S103-S110.

Nalinanon, S., Benjakul, S., Visessanguan, W., & Kishimura, H. (2009). Partitioning of protease from stomach of albacore tuna (Thunnus alalunga) by aqueous two-phase systems. *Process Biochemistry, 44*(4), 471–476.

Navapara, R. D., Avhad, D. N., & Rathod, V. K. (2011). Application of response surface methodology for optimization of bromelain extraction in aqueous two-phase system. *Separation Science and Technology, 46*(11), 1838-1847.

Nawani, N., Singh, R., & Kaur, J. (2006). Immobilization and stability studies of a lipase from thermophilic *Bacillus sp*: The effect of process parameters on immobilization of enzyme. *Electronic Journal of Biotechnology*, *9*(5). doi: 10.2225/vol9-issue5-fulltext-9

Ng, H. S., Ooi, C. W., Mokhtar, M. N., Show, P. L., Ariff, A., Tan, J. S., & Ling, T. C. (2013). Extractive bioconversion of cyclodextrins by Bacillus cereus cyclodextrin glycosyltransferase in aqueous two-phase system. *Bioresource Technology*, *142*, 723–6.

Ng, K. W., Achuth, H. N., Moochhala, S., Lim, T. C., & Hutmacher, D. W. (2007). *In vivo* evaluation of an ultra-thin polycaprolactone film as a wound dressing. *Journal of Biomaterials Science-Polymer Edition, 18*, 925-938.

Ng, K. W., Hutmacher, D. W., Schantz, J. T., Ng, C. S., Too, H. P., Lim, T. C., Phan, T. T., & Teoh, S. H. (2001). Evaluation of ultra-thin poly(epsilon-caprolactone) films for tissue-engineered skin. *Tissue Engineering*, *7*(4), 441-455.

Noorashikin, M. S., Mohamad, S., & Abas, M. R. (2012). *Ionic liquid with β-cyclodextrin based aqueous two phase extraction of parabens in water samples*. Proceedings from the 3rd International Conference on Chemistry and Chemical Engineering. Singapore: IACSIT Press.

Ohlsson, R., Hentschel, C. C., & Williams, J. G. (1978).A rapid method for the isolation of circular DNA using an aqueous two-phase partition system. *Nucleic Acids Research, 5,* 583–590.

Ooi, C. W. (2010). *Direct Recovery of Lipase derived from Burkholderia sp. ST8* (unpublished doctoral dissertation). Universiti Putra Malaysia, Malaysia.

Ooi, C. W., Hii, S. L., Mustapa Kamal, S. M., Ariff, A., & Ling, T. C. (2011a) Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from Burkholderia pseudomallei, *Process Biochemistry, 46*, 68-73.

Ooi, C. W., Tan, C. P., Hii, S. L., Ariff, A., Ibrahim, S., & Ling, T. C.(2011b), Primary recovery of lipase derived from Burkholderia sp. ST8 with aqueous micellar two-phase system, *Process Biochemistry, 46,* 1847-1852.

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. (2009a). Direct purification of *Burkholderia Pseudomallei* lipase from fermentation broth using aqueous two-phase systems. *Biotechnology and Bioprocess Engineering, 14***,** 811-818.

Ooi, C. W., Tey, B. T., Hii, S. L., Kamal, S. M. M., Lan, J. C. W., Ariff, A., & Ling, T. C. (2009b). Purification of lipase derived from *Burkholderia pseudomallei* with alcohol/salt-based aqueous two-phase systems. *Process Biochemistry, 44***,** 1083-1087.

Oppermann, S., Stein, F., & Kragl, U. (2011). Ionic liquids for two-phase systems and their application for purification, extraction and biocatalysis. *Applied Microbiology and Biotechnology, 89,* 493-499.

Padilha, G. d. S., Santana, J. C. C., Alegre, R. M., & Tambourgi, E. B. (2012). Extraction of lipase from Burkholderia cepacia by peg/phosphate atps and its biochemical characterization. *Brazilian Archives Biology and Technology, 55*(1), 7-19.

Paiva, A. L., van Rossum,D., & Malcata, F. X. (2002). Kinetics of lipase-mediated synthesis of butyl butyrate in n-hexane. *Biocatalysis and Biotransformation, 20*(1), 43-51.

Pereira, E. B., Zanin, G. M., & Castro, H. F. (2003). Immobilization and catalytic properties of lipase on chitosan for hydrolysis and esterification reactions. *Brazilian Journal of Chemical Engineering, 20*(4), 343-355.

Persson, J., H. O. Johansson & F. Tjerneld (1999). Purification of protein and recycling of polymers in a new aqueous two-phase system using two thermoseparating polymers. *Journal of Chromatography, A, 864***,** 31-48.

Persson, J., Nystrom, L., Ageland, H., & Tjerneld, F. (1998). Purification of recombinant apoliprotein A-1 (Milano) expressed in *Escherichia coli* using aqueous two-phase extraction followed by temperature-induced phase separation. *Journal of Chromatography B, 711*, 97-109.

Petre, M. (2011). *Advances in applied biotechnology*. United Kingdom: InTech.

Pfennig, A., & Schwerin, A. (1995). Analysis of the electrostatic potential difference in aqueous polymer two-phase systems. *Fluid Phase Equilibria, 108*(1-2), 305-315.

Pitt, C. G., Chasalow, F. I., Hibionada, Y. M., Klimas, D. M., & Schindler, A. (1981). Aliphatic polyesters. I. The degradation of poly(epsilon-caprolactone) *in vivo*. *Journal of Applied Polymer Science, 26*, 3779-3787.

Planas, J., Varelas, V., Tjerneld, F., & Hahn-Hagerdal, B. (1998). Amine-based aqueous polymers for the simultaneous titration and extraction of lactic acid in aqueous two-phase systems. *Journal of Chromatography B, 711*, 265–275.

Prasad. N. K. (2010). *Downstream process technology: a new horizon in biotechnology*. New Delhi: PHI Learning.

Prodanović, J. M. & Antov, M. G. (2008). The influence of molecular weight of polyethylene glycol on separation and purification of pectinases from *Penicillium cyclopium* in aqueous two-phase system. *Acta Periodica Technologica* (*APTEFF*), *39*, 193-199.

Qin, W., & Cao, X. J. (2008). Synthesis of a novel pH-sensitive methacrylate amphiphilic polymer and its primary application in aqueous two-phase systems. *Applied Biochemistry and Biotechnology, 150*, 171-183.

Quyen, D.T., Schmidt-Dannert, C., & Schmid, R.D. (1999). High-level formation of active *Pseudomonas cepacia* lipase after heterologous expression of the encoding gene and Its modified chaperone in *Escherichia coli* and rapid in vitro refolding. *Applied and Environmental Microbiology, 65*, 787-794.

Rahman, R. N. Z. R. A., Baharum, S. N., Basri, M., & Salleh, A. B. (2005). High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas sp*. strain S5. *Analytical Biochemistry*, *341*, 267–274.

Rai, B., Teoh, S. H., Ho, K. H., Hutmacher, D. W., Cao, T., Chen, F., & Yacob, K. (2004). The effect of rhBMP-2 on canine osteoblasts seeded onto 3D bioactive polycaprolactone scaffolds. *Biomaterials, 25*(24), 5499-5506.

Raja, S., & Murty, V. R. (2013). Optimization of aqueous two-phase systems for the recovery of soluble proteins from tannery wastewater using response surface methodology. *Journal of Engineering*, 2013.

Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V., & Ramesh, V. (2011). Aqueous two phase systems for recovery of biomolecules: A review. *Science and Technology, 1*(1), 7-16.

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*, 315-324.

Ramjit, H. G., & Sedgwick, R. D. (1976). The kinetics of ester-ester exchange reactions by mass spectrometry. *Journal of Macromolecular Science: Part A-Chemistry, 10*(5), 815-824.

Ramyadevi, D., Subathira, A., & Saravanan, S. (2012a). Aqueous two-phase poly (ethylene glycol) – Maltodextrin system for protein partitioning from shrimp waste : Influence of molecular weight and pH. *International Journal of Environmental Sciences, 2*(4), 2462–2469.

Ramyadevi, D., Subathira, A., & Saravanan, S. (2012b). Use of response surface methodology to evaluate the extraction of protein from shrimp waste by aqueous two-phase system polyethylene glycol and ammonium citrate). Journal of Environmental Research and Development, 6(4), 1012-1018.

Ratanapongleka, K. (2010). Recovery of Biological Products in Aqueous Two Phase Systems. *International Journal of Chemical Engineering and Applications, 1*(2), 191–198.

Rathi, P., Bradoo, S., Saxena, R. K., & Gupta, R. (2000). A hyper-thermostable, alkaline lipase from *Pseudomonas* sp. with the property of thermal activation. *Biotechnology Letters, 22,* 495-498.

Rathi, P., Saxena, R. K., & Gupta, R. (2001). A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. *Process Biochemistry, 37*, 187-192.

Rathnasamy, S. & Kumaresan, R. (2013). Integration and investigation of metal ligands in aqueous two phase extraction and its partition coefficient studies in papain extraction. *Indian Journal of Biotechnology*, 12, 225-230.

Regalado, C., Asenjo, J. A., & Pyle, D. L. (1996). Studies on the purification of peroxidase from horseradish roots using reverse micelles. *Enzyme and Microbial Technology, 18,* 332-339.

Regupathi, I,. Srikanth, C. K., & Sindhu, N. (2011). Liquid-liquid equilibrium of poly(ethylene glycol) 2000 + diammonium hydrogen citrate + water system at different temperatures. *Journal of Chemical & Engineering Data*, 56(9), 3643-3650.

Regupathi, I., Murugesan, S., Govindarajan, R., Amaresh, S. P., & Thanapalan, M. (2009). Liquid-Liquid equilibrium of poly(ethylene glycol) 6000 + triammonium citrate + water systems at different temperatures. *Journal of Chemical & Engineering Data, 54*(3), 1094-1097.

Renard, D., Lefeb*v*re, J., Griffin, M. C. A., Griffin, W. G. (1998). Effects of pH and salt environment on the association of β-lactoglobulin revealed by intrinsic fluorescence studies. *International Journal of Biological Macromolecules, 22*, 41-49.

Rito-Palomares, M. (2004). Practical application of aqueous two-phase partition to process development for the recovery of biological products. *Journal of Chromatography. B, 807,* 3-11.

Rito-Palomares, M., & Lyddiatt, A. (2002). Process integration using aqueous two-phase partition for the recovery of intracellular proteins. *Chemical Engineering Journal, 87,* 313-319.

Rito-Palomares, M., Negrete, A., Galindo, E., and Serrano-Carreon, L. (2000). Aroma compounds recovery from mycelial cultures in aqueous two-phase processes. *Journal of Chromatography B, 743*, 403-408.

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

Rogers, R. D., Willauer, H. D., Griffin, S. T., & Huddleston, J. G. (1998). Partitioning of small organic molecules in aqueous biphasic systems. *Journal of Chromatography B: Biomedical Sciences and Applications, 711*, 255-263.

Roque, A. C. A. & Lowe, C. R. (2006). Advances and applications of de novo designed affinity ligands in proteomics, *Biotechnology Advances, 24*, 17-26.

Rosa, P. A. J., Azevedo, A. M., & Aires-Barros, M. R. (2007). Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. *Journal of Chromatography A, 1141*, 50-60.

Rosa, P. A. J., Ferreira, I. F., Azevedo, A. M., & Aires-Barros, M. R. (2010). Aqueous two-phase systems: a viable platform in the manufacturing of biopharmaceuticals. *Journal of Chromatography A, 1217*, 2296-2305.

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*, 65-73.

Saeki, S., Kuwahara, N., Nakata, M., &Kaneko, M. (1976). Upper and lower critical solution temperatures in poly (ethylene glycol) solutions. *Polymer, 17*(8), 685-689.

Saitoh, T., Tani, H. M., Kamidate, T., & Watanabe, H. (1995). Phase separation in aqueous micellar solutions of nonionic surfactants for protein separation. *Trends in Analytical Chemistry, 14,* 213-217.

Salameh, M. A., & Wiegel, J. (2010). Effects of detergents on activity, thermostability and aggregation of two alkalithermophilic lipases from *Thermosyntropha lipolytica*. *The Open Biochemistry Journal*, 4, 22-28.

Sanchez-Ferrer, A., & Garcia-Carmona, F. (1994). Biocatalysis in reverse self-assembling structures: Reverse micelles and reverse vesicles. *Enzyme and Microbial Technology, 16,* 409-415.

Sanchez-Ferrer, A., Bru, R., & Garcia-Carmona, F. (1989). Novel procedure for extraction of a latent grape polyphenoloxidase using temperature-induced phase separation in Trition-X 114. *Plant Physiology, 91*, 1481-1487.

Saravanan, S., Rao, J. R., Murugesan, T., Nair, B. U., & Ramasami, T. (2006). Recovery of value-added globular proteins from tannery wastewaters using PEG-salt aqueous two-phase systems. *Journal of Chemical Technology and Biotechnology,* 81, 1814-1819.

Saravanan, S., Rao, J. R., Murugesan, T., Nair, B. U., & Ramasami, T. (2007). Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)-magnesium sulfate systems: Effects of molecular weights and pH. *Chemical Engineering Science, 62*(4), 969–978.

Saxena, R. K., Sheoran, A., Giri, B., & Davidson, W. S. (2003). Purification strategies for microbial lipases. *Journal of Microbiological Methods*, *52*(1), 1–18.

Schmidt, A. S., Ventom, A. M., Asenjo, J. A., (1994), Partitioning and purification of α-amylase in aqueous two-phase systems. *Enzyme and microbial technology, 16*, 131- 142.

Selber, K., Tjerneld, F., Collen, A., Hyytia, T., Nakari-Setala, T., Bailey, M., Fagerstrom, R., Kan, J., Van Der Laan, J., Penttila, M., & Kula, M. R. (2004). Large scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. *Process Biochemistry, 39*, 889-896.

Shady, S. F., Gaines, P., Garhwal, R., Leahy, C., Ellis, E., Crawford, K., Schmidt, D. F., & McCarthy, S. P. (2013). Synthesis and characterization of pullulan-polycaprolactone core-shell nanospheres encapsulated with ciprofloxacin. *Journal of Biomedical Nanotechnology*, *9*(9), 1644-1655.

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and application of lipases. *Biotechnology Advances, 19*, 627-662.

Show, P. L., Tan, C. P., Shamsul, A. M., Ariff, A., Yusof, Y. A., Chen, S. K., & Ling, S. K. (2012). Extractive fermentation for improved production and recovery of lipase derived from Burkholderia cepacia using a thermoseparating polymer in aqueous two-phase systems. *Bioresource Technology, 116*, 226-233.

Sin, M. C. Gan, S. N., Annuar, M. S. M., & Tan, I. K. P. (2010). Thermodegradation of medium-chain-length poly(3-hydroxyalkanoates produced by *Pseudomonas putida* from oleic acid. *Polymer Degradation and Stability, 95*, 2334-2342.

Sin, M. C., Tan, I. K. P., Annuar, M. S. M., & Gan, S. N. (2011). Characterization of oligomeric hydroxyalkanoic acids from thermal decomposition of palm kernel oil-based biopolyester. *International Journal of Polymer Analysis and Characterization, 16*, 337-347.

Silvars, U., Abramson, J., Iwata, S., & Tjerneld, F. (2000). Affinity partitioning of a poly(histidine)-tagged integral membrane protein, cytochrome bo3 ubiquinol oxidase, in a detergent-polymer aqueous two phase system containing metal-chelating polymer. *Journal of Chromatography B, 743*, 307-316.

Singh, R. K., Tiwari, M. K., Singh, R., & Lee, J. K. (2013). From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes. *International Journal of Molecular Science, 14*(1), 1232-1277.

Singh, S., Singh, A. N., Verma, A., & Dubey, V. K. (2013). Biodegradable polycaprolactone (PCL) nanosphere encapsulating superoxide dismutase and catalase enzymes. *Applied Biochemistry and Biotechnology, 171*(7):1545-1558.

Sinha, R., & Khare, S. K. (2014). Effect of organic solvents on the structure and activity of moderately halophilic *Bacillus* sp. EMB9 protease. *Extremophiles*. Advance online publication. doi: 10.1007/s00792-014-0683-4.

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.

Spelzini, D., Farruggia, B., & Picó, G. (2005). Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol-phosphate: chymosin and pepsin. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 821(1), 60–66.

Sperling, L. H. (2005). *Introduction to physical polymer science* (4th ed.). New York: Wiley-Interscience.

Srinivas, N. D., Barhate, R. S., & Raghavarao, K. S. M. S. (2002). Aqueous two-phase extraction in combination with ultrafiltration for downstream processing of Ipomoea peroxidase. *Journal of Food Engineering*, 54(1), 1–6.

Srinivas, N.D. (2000). *Aqueous two-phase extraction for the downstream processing of enzymes* (unpublished doctoral dissertation). University of Mysore, India.

Su, C. K. & Chiang, B. H. (2006). Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system. *Process Biochemistry, 41*, 257-263.

Subathra, K., Jeevitha, G. C., & Deepa, R. (2012). Aqueous two phase extraction of protease from neem leaves [Azadirachta indica]. *International Journal of Chemical Sciences and Applications, 3*(3), 346-351.

Sun, H., Mei, L., Song, C., Cui, X., & Wang, P. (2006). The *in vivo* degradation, absorption and excretion of PCL-based implant. *Biomaterials*, *27*, 1735-1740.

Svendsen, A. (1994). Sequence comparisons within the lipase family. In: Woolley, P., Petersen, S.B. (Eds), *Lipases: Their Structure, Biochemistry and Application* (pp. 1-21). Cambridge: Cambridge University Press.

Tanuja, S., Srinivas, N. D., Raghava Rao, K. S. M. S., & Gowthaman, M. K. (1997). Aqueous two-phase extraction for downstream processing of amyloglucosidase. *Process Biochemistry, 32*, 635-641.

Thakur, G., Kumar, N., Raghu, H. V., & Malik, R. K. (2010). Development of off-line enzyme substrate based assay for monitoring enterococci in milk. *NDRI Newsletters, 2*(1), 2–3.

Tjerneld, F., Persson, I., Albertsson, P. Å., & Hahn-Hagerdal, B. (1985). Enzymatic hydrolysis of cellulose in aqueous two-phase systems. I. partition of cellulases from *Trichoderma reesei*. *Biotechnology and Bioengineering, 27*, 1036-1043.

Tubío, G., Nerli, B., & Picó, G. (2007). Partitioning features of bovine trypsin and alpha-chymotrypsin in polyethyleneglycol-sodium citrate aqueous two-phase systems. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 852*(1-2), 244–249.

van Berlo, M., Luyben, K. C. A. M., & Van der Wielen, L. A. M. (1998). Poly(ethylene glycol)-salt aqueous two-phase systems with easily recyclable volatile salts. *Journal of Chromatography B: Biomedical Sciences and Applications, 711*, 61-68.

Vernau, J., & Kula, M. R. (1990). Extraction of proteins from biological raw material using aqueous polyethylene glycol-citrate phase systems. *Biotechnology and Applied Biochemistry, 12*, 397-404.

Vieille, C., & Zeikus, G. J. (2001). Hyperthermophilic enzymes: Sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews*, 65(1), 1-43.

Walter, H., Brooks, D. E., & Fisher, D. (1985). *Partitioning in aqueous two-phase systems: Theory, methods, uses, and applications to biotechnology*. Academic Press, Orlando.

Wang Y., Mao Y., Han J., Liu Y., & Yan Y. (2010). Liquid-liquid equilibrium of potassium phosphate/potassium citrate/sodium citrate + ethanol aqueous two-phase systems at (298.15 and 313.15) K and correlation. *Journal of Chemical & Engineering Data*, 55(12), 5621-5626.

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*, 171-176.

Waziri, S. M., Abu-Sharkah, B. F., & Ali, S. A. (2003). The effect of pH and salt concentration on the coexistence curves of aqueous two-phase systems containing a pH responsive copolymer and polyethylene glycol. *Fluid Phase Equilibria, 205*, 275-290.

Wijethunga, P. A. L., & Moon, H. (2012). *A study of on-chip aqueous two phase system formation and its applications*. Proceedings from ASME 2012 Third International Conference on Micro/Nanoscale Heat and Mass Transfer. Atlanta, Georgia, USA.

Wolfenden, R., Snider, M., Ridgway, C., & Mille, B. (1999). The temperature dependence of enzyme rate enhancements. *Journal of the American Chemical Society, 121*, 7419-7420.

Woodruff, M. A., & Hutmacher, D. W. (2010). The return of a forgotten polymer: Polycaprolactone in the 21st century. *Progress in Polymer Science, 35*(10), 1217-1256.

Woodward, S. C., Brewer, P. S., Moatamed, F., Schindler, A., & Pitt, C. G. (1985). The intracellular degradation of poly-epsilon-caprolactone. *Journal of Biomedical Materials Research*, 19, 437-444.

Wu, J., Liao, C., Wang, Z., Cheng, W., Zhou, N., Wang, S., & Wan, Y. (2011). Chitosan-polycaprolactone microspheres as carriers for delivering glial cell line-derived neurotrophic factor. *Reactive and Functional Polymers*, *71*(9), 925-932.

Wu, Y. T., Zhu, Z. Q., & Mei, L. H. (1996). Interfacial Tension of Poly (ethylene glycol) + Salt + Water Systems. *Journal of Chemical & Engineering Data*, *9568*(96), 1032–1035.

Xavier, L., Freire, M. S., Vidal-Tato, I., & González-Álvarez, J. (2013). Aqueous two-phase systems for the extraction of phenolic compounds from eucalyptus (*Eucalyptus globulus*) wood industrial wastes. *Journal of Chemical Technology and Biotechnology*. Advance online publication. doi: 10.1002/jctb.4260.

Xia, E. Q., Wang, B. W., Xu, X. R., Zhu, L., Song, Y., & Li, H. B. (2011). Microwave-assisted extraction of oleanolic acid and ursolic acid from *Ligustrum lucidum* ait. *International Journal of Molecular Sciences, 12*(8), 5319-5329.

Xia, J., Dubin, P. L., & Kokufuta, E. (1993). Dynamics and electrophoretic light scattering of a water-soluble complex formed between pepsin and poly(ethylene glycol). *Macromolecules, 26*, 6688-6690.

Yang, Z., Niu, X., Fang, X., Chen, G., Zhang, H., Yue, H., Wang, L., Zhao, D., & Wang, Z. (2013). Enantioselective esterification of ibuprofen under microwave irradiation. *Molecules, 18*, 5472-5481.

Yong, H. S., Tey, B. T., Hii, S. L., Kamal, S. M. M., Ariff, A., & Ling, T. C. (2010). Application of a high density adsorbent in expanded bed adsorption of lipase from *Burkholderia pseudomallei*. *African Journal of Biotechnology*, *9*(2), 203-216.

Yu, D., Wu, H., Zhang, A., Tian, L., Liu, L., Wang, C., & Fang, X. (2011). Microwave irradiation-assisted isomerization of glucose to fructose by immobilized glucose isomerase. *Process Biochemistry, 46*(2), 599-603.

Yücekan, İ., & Önal, S. (2011). Partitioning of invertase from tomato in poly(ethylene glycol)/sodium sulfate aqueous two-phase systems. *Process Biochemistry*, *46*(1), 226–232.

Zafarani-Moattar, M. T., Sadeghi, R., & Hamidi, A. A. (2004). Liquid-liquid equilibria of an aqueous two-phase system containing polyethylene glycol and sodium citrate: Experiment and correlaction. *Fluid Phase Equilibria, 219*, 149-155.

Zaslavsky, B. Y. (1995). *Aqueous two-phase partitioning: Physical chemistry and bioanalytical applications*. New York: Marcel Dekker, Inc.

Zhai, S. L., Luo, G. S., & Liu, J. G. (2001a). Aqueous two-phase electrophoresis for separation of amino acids. *Separation and Purification Technology*, *21*(3), 197-203.

Zhai, S. L., Luo, G. S., & Liu, J. G. (2001b). Selective recovery of amino acids by aqueous two-phase electrophoresis. *Chemical Engineering Journal, 83*(1), 55-59.

Zhang, S., Cao, X., Chu, J., Qian, J., & Zhuang, Y. (2010). Bioreactors and bioseparation. In G. T. Tsao, P. Ouyang & J Chen (Eds.), *Biotechnology in China II: Chemicals, Energy and Environment* (pp.105-150). Heidelberg, Germany: Springer-Verlag.

Zhao, L., Budge, S. M., Ghaly, A. E., Brooks, M. S., & Dave, D. (2013). Partition of Pepsinogen from the stomach of red perch (sebastes marinus) by aqueous two phase systems: effects of peg molecular weight and concentration. *Food Processing and Technology, 2*(1), 1–13.

Zhu, J. H., Wei, D. Z., Cao, X. J., Liu, Y. Q., & Yuan, Z. Y. (2001). Partitioning behaviour of cephalexin and 7-aminodeacetoxicephalosporanic acid in PEG/ammonium sulfate aqueous two-phase systems. *Journal of Chemical Technology & Biotechnology, 76*(11), 1194–1200.

Zhu, Z. Q., Li, M., Mei, L. H., Han, Z. X. (1996). Measurement and correlation of partition coefficients of urokinase in aqueous two-phase peg-potassium phosphate system. *Chinese Journal of Chemical Engineering, 4*(1), 19-27.

Zijlstra, G. M. (1998). *Animal-cell culture in aqueous two-phase system*. Groningen Netherland: DijkhuizenVan Zanten.

Zijlstra, G. M., Gooijer, C. D., & Tramper, J. (1998). Extractive bioconversions in aqueous two-phase systems. *Current Opinion in. Biotechnolology, 9*(2), 171-176.

List of Publication

Chew, P. L., Annuar, M. S. M., & Ling, T. C. (2014). Screening of the optimized conditions for extractive bioconversion of polycaprolactone in an aqueous two-phase system. *Proceedings of the UTAR National Postgraduate Fundamental and Applied Sciences Seminar*; 2014 Jun 14-15; Kampar, Perak, Malaysia. p. 57. (Poster presentation).

Chew, P. L., Annuar, M. S. M., Show, P. L., & Ling, T. C. (2015). Extractive bioconversion of poly-ε-caprolactone by *Burkholderia cepacia* lipase in an aqueous two-phase system. *Biochemical Engineering Journal, 101*, 9-17.

Appendix

Appendix A: Standard curve of PEG 6000, PEG 8000 and potassium phosphate salt conductivity

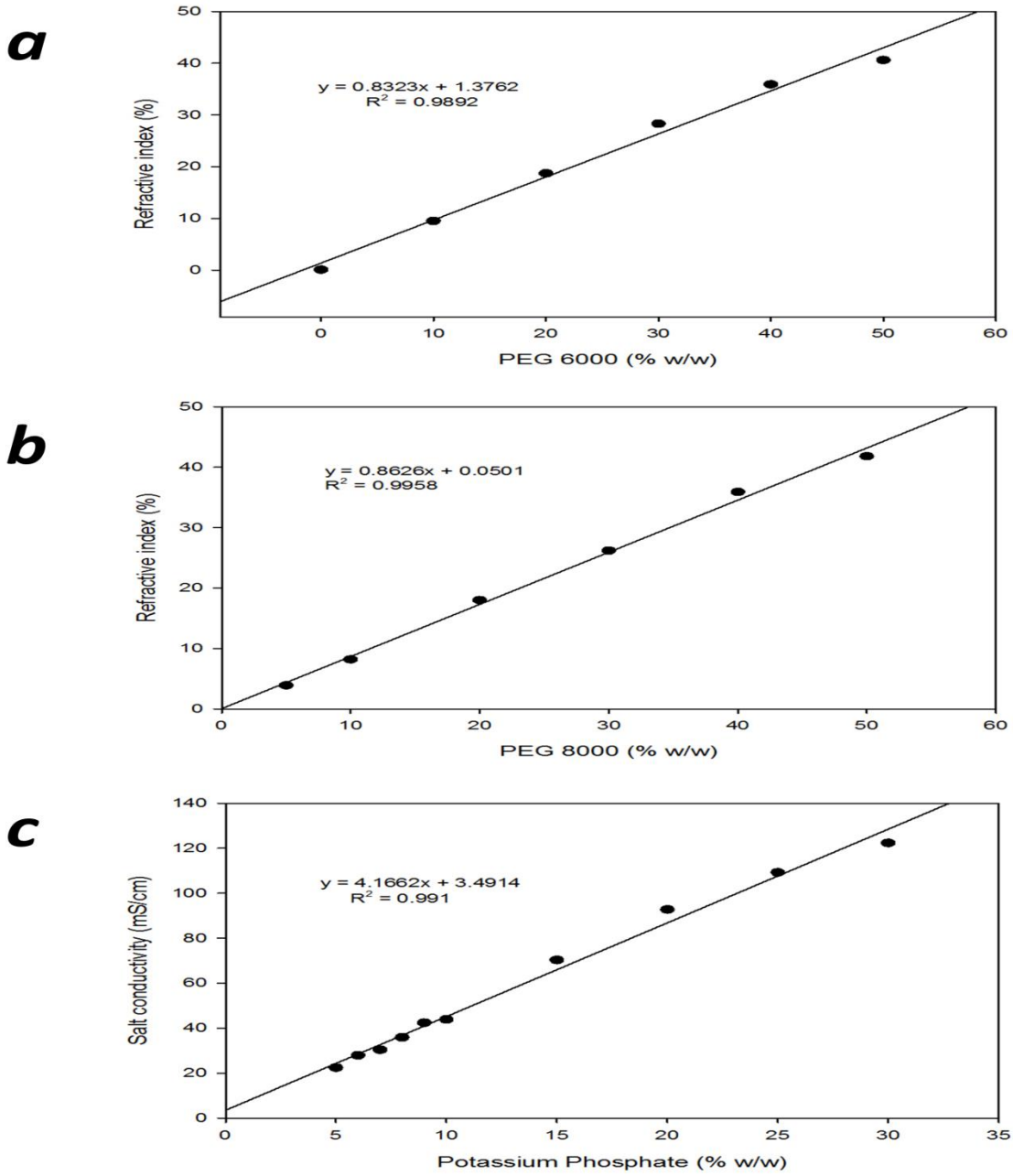


Figure A.1: Standard curve of (*a*) PEG 6000, (*b*) PEG 8000 and (*c*) potassium phosphate salt conductivity