Development of an Efficient Protein Recovery System using Liquid Biphasic Flotation

Revathy A/P Sankaran

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

2018

Abstract

Extensive development of biotechnology over the past several decades has induced a great impact in the production of biological products in various industries. To date, major challenges in the biotechnology industry includes the production process of biomolecules with high purity and low cost, whilst retaining functionality. The conventional downstream processing of valuable bioproducts which are widely employed usually involves multi-processing steps, high energy and chemical consumption and often has a large influence on the cost of the finishing product. Therefore, the demand for cost-efficient and simple downstream processes has directed towards an intensive research for exploiting novel separation tool that can achieve high level of product purity with minimum number of processing stages and greener approach. The aim of this thesis is to develop a new separation method that has minimum number of steps, environmentally friendly and with the ability to achieve maximum level of product purity. Liquid biphasic flotation system is a novel technique which incorporates the principles of aqueous two-phase systems and mass transfer mode of solvent sublation. This system has been proposed as an ideal purification technique for separation, purification and concentration of biomolecules. Liquid biphasic flotation have been utilised previously to purify several biomolecules whilst maintaining their functionality. This thesis emphasised on extending the applications, improvising and to diversify liquid biphasic flotation technique as an efficient tool for downstream processing. This thesis has four objectives, in which all the objectives highlight on the usage of liquid biphasic flotation system for maximum biomolecules extraction. The initial part of liquid biphasic flotation application study is to investigate the effect of full and continuous recycling of alcohol and salt phase components in large scale liquid biphasic flotation system for lipase extraction. In this section, main focus was to optimize operating conditions for the recycling of both phase component and to investigate the competence of recycling phase components using liquid biphasic flotation system on a large scale. The liquid biphasic flotation system investigated is composed of 1-propanol and ammonium sulphate whereby both phase components went through complete recycling process. From the results obtained, it is exhibited that by reusing the bottom phase, separation efficiency of lipase was sustained beyond 77.33 % and yield with 80%. This study showed that the recovered phase components could be recycled effectively up to four cycles and able to produce a significantly high yield of lipase. Next study was on a novel approach of liquid biphasic flotation system for lipase recovery utilizing recycling phase components comprising surfactant and sorbitol. This novel method utilized Triton X-100 and xylitol for lipase extraction from *Burkholderia cepacia*. The scope of this study

focuses on eliminating pollution and environmentally friendly process for enzyme extraction via liquid biphasic flotation. This scope is achieved by utilising phase forming components that have recovery and recycling abilities to minimize the use of chemicals for enzyme extraction. A set of optimum conditions were identified which provides a high yield of lipase with 87.49 % and separation efficiency of 86.46%. From the recycling study, it is revealed 97.20% and 98.67% of Triton X-100 and xylitol respectively were recovered after five times of recycling and 75.87% lipase separation efficiency was obtained. Third objective is on the study of the integration process of fermentation and separation of lipase from *Burkholderia cepacia* using liquid biphasic flotation. Integration process exhibited high lipase separation efficiency reaching 92.29% and a yield of 95.73%. This study has proven the diversification of liquid biphasic flotation system in integration of upstream and downstream processes. Since liquid biphasic flotation system can be utilised for various type of biomolecules, the final study was done to examine the integration process of sonication and protein extraction from microalgae using sugaring-out effect. Various operating conditions were assessed for high separation efficiency and yield of protein. Maximum protein separation efficiency of 86.38% and yield with 93.33% were attained from this integration process. This study demonstrated that liquid biphasic flotation system could be integrated with ultrasound for protein separation. This thesis demonstrates the importance and diverse applications of liquid biphasic flotation for biomolecules extraction. This study has led to several novel discoveries of liquid biphasic flotation applications with economic downstream processes on an industrial scale.

Keywords: Downstream processing, liquid biphasic flotation, aqueous two-phase systems, solvent sublation, biomolecules

List of Publications and Achievements

Published journals

R Sankaran., PL Show., JS Chang., (2016). Biodiesel production using immobilized lipase: feasibility and challenges. *Biofuels, Bioproducts and Biorefining.* 10 (6), pp 896-916.
IF: 3.694. Citations: 9

R Sankaran., PL Show., SY Lee., YJ Yap., TC Ling., (2018). Integration process of fermentation and liquid biphasic flotation for lipase separation from *Burkholderia cepacia*. *Bioresource technology.*250, pp 306-316.
IF: 5.65

R Sankaran., PL Show., YJ Yap., HL Lam. TC Ling., GT Pan., TCK Yang., (2018). Sustainable Approach in Recycling of Phase Components of Large Scale Aqueous Two-Phase Flotation for Lipase Recovery. *Journal of Cleaner Production*
IF: 5.715

R Sankaran., PL Show, YJ Yap., Y Tao, TC Ling. (2018).Green Technology of Liquid Biphasic Flotation for Enzyme Recovery Utilising Recycling Surfactant and Sorbitol.*Clean Technologies and Environmental Policy*
IF: 3.331

Journals under review

Author: **Revathy Sankaran**, Sivakumar Manickam, Yee Jiun Yap, Tau Chuan Ling, Jo-Shu Chang, Pau Loke Show.
Year: 2018.
Title: Integration process of sonication and liquid biphasic flotation using sugaring-out effect for protein recovery from microalgae.
Journal: Ultrasonics Sonochemistry
IF: 4.218
(Accepted with minor corrections)

Book chapters

R Sankaran., PL Show., D Nagarajan., JS Chang., (2018). Exploitation and Biorefinery of Microalgae in T Bhaskar., A Pandey., SV Mohan., DJ Lee. & SK Khanal. (Eds), Waste Biorefinery, Elservier Science & Technology.

Conference presentations

R Sankaran., PL Show., and YJ Yap., (2017). Sugaring Out Effect of Lipase via Aqueous Biphasic Sublation (ABS). 4th Postgraduate Colloquium for Environmental Research 2017 (POCER 2017), 25-27 July 2017, Malacca, Malaysia (in proceeding).

R Sankaran., PL Show., and YJ Yap., (2017). Liquid Biphasic Flotation (LBF) System: Lipase Recovery from Burkholderia Cepacia by Incorporating Fermentation and Two-Phase Flotation. International Conference on Science Technology and Management (ICSTM), 5th-6th September, 2017, Melbourne, Australia (in proceeding).

Awards

- Won **People's Choice** Award in UNMC Poster Competition Postgraduate Research Showcase 2016.
- Won **Best Presentation** in 242nd International Conference on Science Technology and Management (ICSTM) in Melbourne, Australia.
- Won **Excellent Paper** in 242nd International Conference on Science Technology and Management (ICSTM) in Melbourne, Australia.

Acknowledgement

I would like to take this opportunity to thank my advisor, Associate Professor Dr. Show Pau Loke, for his guidance involving the direction of the project and teaching that have helped me develop as a researcher. Thank you for allowing me the freedom to work. I would also thank my co-supervisor Associate Professor Dr. Yap Yee Jiun for his help throughout my research. I would thank Professor Sivakumar for his help on borrowing his equipment for my project and also provided his expertise on sonication technique.

I would like to thank my colleagues at University of Nottingham Malaysia Campus for organisational and moral support during my research years. Special thanks to Ricardo, Kalaimani, Harshini, Anand, Tan Jin Yang, Lilian, Saras, Prema, Sutha and many more of my friends for their continuous support and encouragement to complete my studies.

Finally, I would like to express my love and gratitude to my parents, Sankaran and Krishnakumari, my sister, Kausalya, and my brother Ruban who have continuously supported me and provided me with opportunities to achieve success.

Contents

List of Abbreviations and Nomenclature

ACN	Acetonitrile
AG	α-allyl glucoside
AP	Aqueous phase
AOT	sodium bis-(2-ethyl 1-hexyl) sulfo- succinate
API	Active pharmaceutical ingredients
ATPE	Aqueous two phase extraction
ATPS	Aqueous Two Phase System
ATPF	Aqueous Two phase Flotation
BP	Bottom phase
BCL	*Burkholderia cepacia* lipase
BSA	Bovine serum albumin
CALB	Candida *antartica* lipase B
CAP	Chloramphenicol
CB	Cibacron Blue F3GA
CL	Crude lipase
CEX	Cation exchanger
CGA	Colloidal Gas Aphrons
CTAB	Cetyl trimethyl ammonium bromide
DHA	docosahexaenoic acid
DEAE	Diethylaminoethyl
DSP	Downstream Processing
E	Separation Efficiency
EDC	1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride

EOPO	Ethylene oxide and propylene oxide
FT	Flotation time
FAAE	Fatty acid alkyl esters
HIC	Hydrophobic Interaction Chromatography
IL	Ionic Liquid
LBF	Liquid Biphasic Flotation
LBP	Lycium barbarum polysaccharide
LCST	Lower critical solution temperature
MAE	Microwave-assisted extraction
MRM	Mixed reverse micelles
NCA	N-carboxyanhydride
NHS	N-hydroxylsuccin-imide
OD	Optical density
OTC	Oxytetracycline
OVAT	One variable at a time
PAG	Poly(α-allyl glucoside)
PANCMA	Poly(acrylonitrile-*co*-maleic acid)
pCA	para-coumaric acid
PEG	Polyethylene glycol
P_{FT}	Purification factor
pNP	4-nitrophenol
p-NPL	4-nitrophenyl dodecanonate
PPMM	Polypropylene microfiltration membrane
PSLG	Poly(γ-stearyl L-glutamate)
PUFAs	Polyunsaturated fatty acids
Rc	Recycle
RMS	Reverse micelle system

S	Selectivity
SA	Specific activity
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFE	Supercritical fluid extraction
SS	Solvent Sublation
P_{NNB}	Thermo-responsive polymer
TC	Tetracycline
TG	Triglycerides
TP	Top phase
TPP	Three phase partitioning
UAE	Ultrasound assisted extraction
Y	Yield
Y_P	Protein recovery yield

List of Tables

List of Figures

Chapter 1: Introduction

1.0 Introduction

1.1. Research background

With the vast evolution of biotechnology in the last few decades, biological products are extensively exploited in various industries producing food, pharmaceuticals, cosmetic and textile. Due to the development in biotechnology applications, industries are now progressing towards the extraction and purification of protein products derived from biological materials. The major focus of their applications is on the purity of the biological products which makes the downstream processing an essential element in the production process of biomolecules (Carta & Jungbauer, 2010). Downstream processing involves a group of energy demanding steps leading to separation and recovery of purified biological product. The recovery and purification strategies of biomolecules entails a complex and cost demanding process which can reach up to 70% of the product cost of the biological products (Naganagouda & Mulimani, 2008). Biomolecules such as proteins are considered as labile materials, thus they require specific and mild processing conditions so that proteins are not denatured.

Conventional extraction and purification methods such as organic solvents, batch adsorption, extractive distillation, membrane chromatography, crystallisation and others are extensively applied for biomolecules separation. However, these approaches have significant limitations including difficulties in scaling up, expensive production cost and shortage of proper biocompatible solvents. It is reported, due to increasing demand for protein extraction technology, that the isolation and purification process of proteins varies from 30-80% of the production cost (Ghosh, 2003). In light of this, there is a significant interest in the

development of effective and economical downstream processes for the recovery and purification of biomolecules. The present research targets to enhance the prevailing liquid biphasic flotation (LBF) system that leads to efficient recovery of bioproducts partitioning.

LBF system is a special type of liquid-liquid extraction that is composed of the combined principles of aqueous two-phase system (ATPS) and solvent sublation (SS) (Show et al., 2011, 2013a). LBF is a biphasic system predominantly composed of aqueous solution that is able to preserve the native structure of purified biomolecules. In addition, LBF retains the advantages of conventional liquid-liquid extraction such as high productivity, simple operation, short processing time, softer condition, scalability and versatility (P. Y. Bi, Li, & Dong, 2009; Chang, Wei, Bi, & Shao, 2014). This method is suitable for the extraction, separation, concentration and purification of protein since protein is too complex to be purified by other recovery techniques (Show et al., 2011). LBF has been used for the downstream processing of biological compounds such as enzymes (Lin et al., 2015; Show et al., 2011), biopharmaceuticals products (Han et al., 2011; Xia et al., 2016) and other biological products (P. Y. Bi et al., 2013; Chang et al., 2014; Md Sidek et al., 2016). This LBF system has proven its capability for various biomolecules separation. However, its full potential for efficient extraction and purification is yet to be discovered. Extensive exploring of LBF system with different integrated method could open up a new possibility in the bioseparation field.

The exploration for low-cost extraction systems capable for large quantities of protein separation and purification have resulted in the advancement of new technology platforms using diverse extraction methodologies. In this current

project, the diverse application of LBF system for protein and lipase extraction is investigated. In this context, using LBF as the major technology, several improvements and integration method have been applied to seek innovative substitute for the separation technology of biomolecules.

1.2. Problem Statement

Current scenario of protein downstream processing generally demands time consuming processing steps in a typical bioprocess operation. The target biomolecules are often present in low concentration in which a large amount of impurities are naturally present in these complex mixtures. A diverse array of purification strategies are commonly utilised in the downstream processing such as the low-resolution separation techniques for example precipitation, ultrafiltration, centrifugation and microfiltration. These methods are time-consuming and may result in poorer purity, whereas the high-resolution chromatographic methods are expensive, multistep and unsuitable for the large-scale and continuous operation. As a consequence, the demand for cost-effective and mild downstream processes has led to an interest in developing a new separation method that can achieve a desirable level of product purity with the least number of processing steps. The present research targets to improve protein downstream processing.

1.3. Research Aim

The aim of this project is to develop an environmentally friendly, efficient, with minimal procedures and cost effective method for lipase and protein separation. The potential of LBF system as an effective separation technology with respect to their application in downstream processing of protein and enzymes will be studied. This research will focus on the diverse LBF application by using novel phase components, recyclable phase components and different integration process for lipase and protein extraction.

1.4. Objectives

- To recycle phase components comprising alcohol/salt on a large scale using LBF for lipase recovery.
- To develop a sustainable and green LBF lipase extraction method using novel phase components (Triton X-100/xylitol) with recycling principles.
- To exhibit integration performance of LBF system in the lipase fermentation process and subsequent separation process.
- To integrating ultrasonication and LBF system utilising sugaring-out effect for the extraction of protein from microalgae biomass.

1.5. Thesis outline

The aim of this research is to illustrate the various functions of LBF system in biomolecules extraction. This thesis includes the different efforts to extract lipase enzyme and protein using the novel method of LBF. A flow chart at the end of this section summarizes the thesis outline. **Figure 1** displays the flow chart of the thesis outline.

Chapter 1 introduces the background of the problem faced in the downstream processing. It also introduces the potential outcomes of the solution along with the aim and objectives of this thesis. This study demonstrates the diverse application of LBF in biomolecules separation. The first three objectives are focusing on lipase extraction utilizing different approaches of LBF. The last objective is on the protein extraction from microalgae utilizing LBF and ultrasound.

Chapter 2, discusses the background review of the research is discussed. This section covers the challenges in downstream processing, different applications of lipase, different methods of lipase purification and introduction on novel techniques of LBF.

Chapter 3 describes the experimental methods employed for the whole study. All the methods employed in each objective have been compiled in this chapter. Flowchart is included at the beginning of the chapter to give a clear picture of the methods employed in this thesis.

Chapter 4 provides an elaborate investigation on lipase extraction using novel phase components comprising Triton X-100/xylitol with LBF system. In this chapter the recycling ability of the phase components was studied as well. This

chapter on LBF system focuses on the application of different phase components for lipase extraction and the effect of recycling.

Chapter 5 reports on lipase extraction using recycling alcohol/salt in large scale LBF system. In this chapter, the effect of full and continuous recycling of both alcohol and salt phase components in large scale LBF system was investigated.

Chapter 6 discusses the investigation on LBF system where integration of fermentation and separation were done. The diverse use of LBF for integration process was studied. Lipase was fermented and continuous separation was performed to obtain optimized conditions for high yield and separation efficiency.

Chapter 7, discusses the use of LBF for protein extraction from microalgae. In this study, integration of LBF with ultrasound was performed with sugaring-out effect for protein separation. This study has demonstrated the different application of LBF with the combination of ultrasound for different bioproducts separation.

Finally, chapter 8 presents the overall conclusion of the thesis and suggestions on work that can be done in the research field.

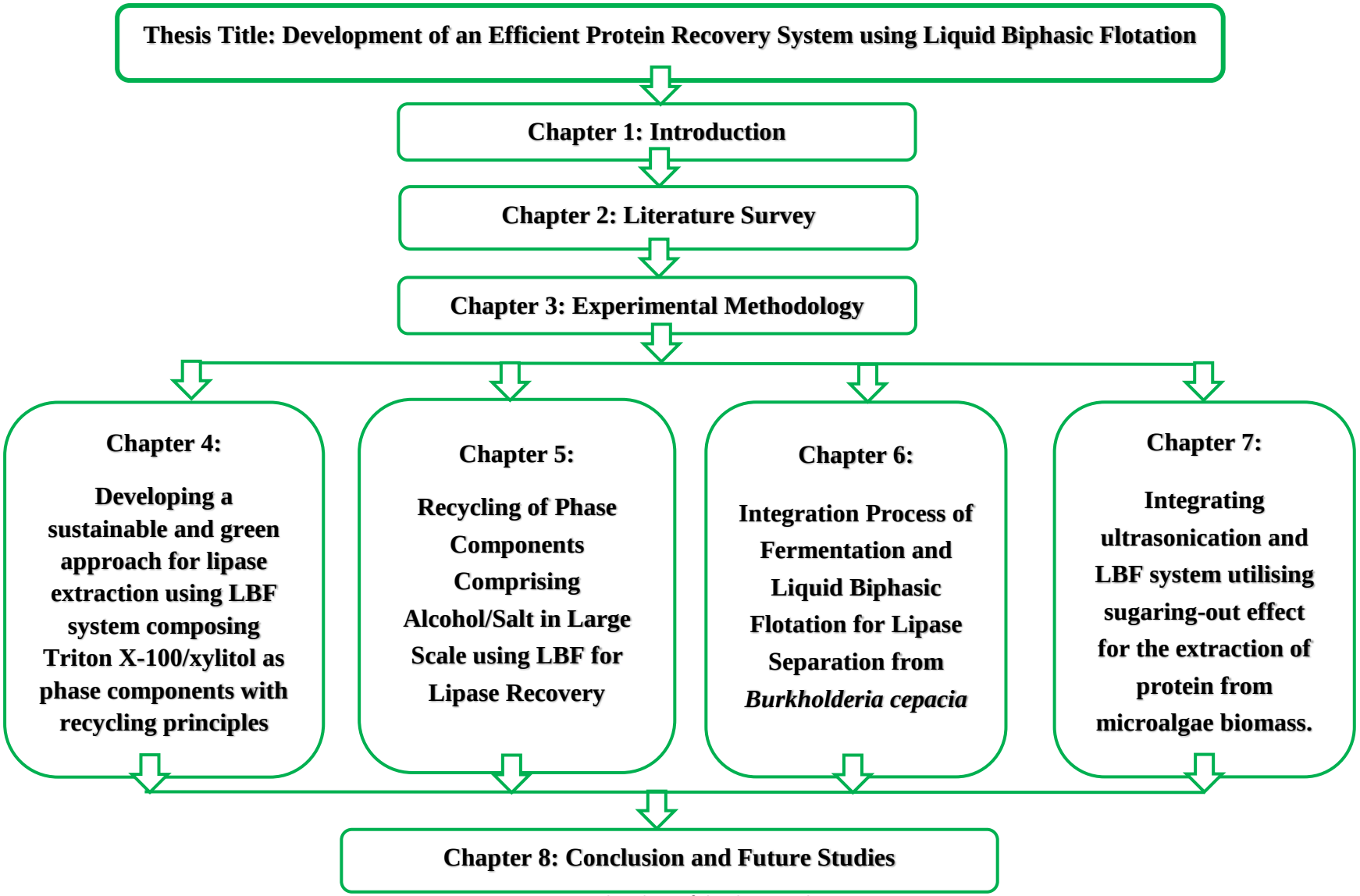


Figure 1: Flowchart of the thesis outline

Chapter 2: Literature Review

2. Literature Review

2.1. Downstream processing

Downstream processing (DSP) can be defined as a cluster of stages that leads to the recovery of highly purified product from either natural sources or fermentation broths. The downstream process normally attains 50-80% of the overall production costs of enzymes (Rodríguez-Durán, Spelzini, Boeris, Aguilar, & Picó, 2013). DSP of proteins began to draw attention in the early 21st century ever since the development of recombinant DNA technology has been possible to produce nearly any protein in a host of choices. Downstream processing of biomolecules can be divided into four main phases: recovery, concentration, purification and polishing. Each of these steps comprises a number of distinct processes subjected to the intracellular or extracellular localization of the product, the required purity as well as the end form of the product.

Recovery step involves the separation of biomolecules (proteins, lipids, metabolites and natural products) from other contaminants. As for protein recovery, it involves the separation of both in nature or denatured type from a mixture containing protein and non-protein. There are two types of products namely extracellular and intracellular. Recovery of these products are achieved either by physical processes such as centrifugation or filtration or by product extracted directly from the cells or cell debris. Common methods of extraction that are being applied are aqueous two-phase (Amid, Shuhaimi, Islam Sarker, & Abdul Manap, 2012; Chow et al., 2013; Y. Jiang, Xia, Yu, Guo, & Liu, 2009; Naganagouda & Mulimani, 2008), organic solvents (Mapari, Meyer, & Thrane, 2006; Velmurugan et al., 2010), batch adsorption (Crini & Badot, 2008),

supercritical fluids (Herrero, Sánchez-Camargo, Cifuentes, & Ibáñez, 2015; Rodríguez-Pérez, Mendiola, Quirantes-Piné, Ibañez, & Segura-Carretero, 2016), and reverse micelles (X. Ding, Cai, & Guo, 2016; Gaikaiwari, Wagh, & Kulkarni, 2012b; Krishna, Srinivas, Raghavarao, & Karanth, 2002; Tonova & Lazarova, 2008).

Recently, there is increasing interest in the usage of surfactants for separation process that leads to the development of the technique known as colloidal gas aphrons (CGA). CGA comprises surfactant-stabilized microbubbles which are produced by intensive stirring of surfactant solution. This technique has characteristics such as high interfacial area, high stability, easy separation from the liquid phase, and eco-friendly processes. CGA method has been used to extract protein (β-Lactoglobulin) from whey (Fuda, Bhatia, Pyle, & Jauregi, 2005), plant pigment from annatto seeds(Alves, Ulson De Souza, Ulson De Souza, & Jauregi, 2006), astaxanthin from fermentation mixture (Zhao L.; Liu, D., 2007), pulp fibers from paper machine backwater (Mukherjee et al., 2015) and newly extraction of red colorants from the fermented broth of *Talaromyces amestolkiae* (Santos-Ebinuma, Teixeira, Pessoa, & Jauregi, 2016).

Products recovered by extraction means requires partial purification depending on selectivity of the method used. Proteins that are attained from the recovery section could be retrieved as dilute or concentrated solution of the desired protein in a blend of contaminants proteins. If the target protein is obtained in diluted solution, it is required to be concentrated before the purification procedure. Purification process involves a series of high- resolution procedures usually employing the various chromatography operations that generates the product to a quantified level of purity.

Among the four steps, purification process itself makes up to 80% of the production cost (Raja, Murty, Thivaharan, Rajasekar, & Ramesh, 2012). Some of the common purification techniques include filtration, precipitation, liquid- liquid two phase separation and chromatography. DSP concerning recovery and purification of biomolecules is complex; this is because the products are highly sensitive to organic solvents, pH, temperatures, low concentration of product and high number of unspecified impurities (Glyk, Scheper, & Beutel, 2015; van Winssen, Merz, Czerwonka, Schembecker, & Dortmund, 2014).

The limitations of the traditional approaches are high production cost due to the conventional purification methods encompassing several steps of unit operations and loss of target biomolecules in each step causing a huge amount of losses. Furthermore, purification process is considered as a bottleneck in the biopharmaceutical production of proteins. Products concerning biopharmaceuticals requires stringent quality requirement by regulatory agencies which involves multiple chromatography steps and are expensive (Fields, Li, O'Mahony, & Lee, 2016; Nfor et al., 2008).

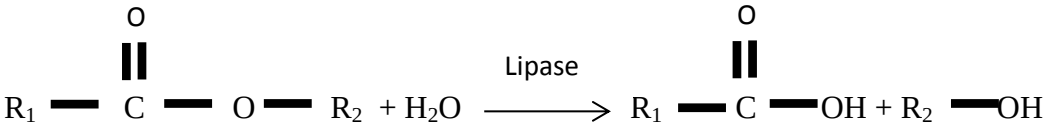
At present, the efficiency of DSP is one of the major barriers for economic success of proteins. Appropriate unit procedures need to be selected and integrated into a single process where the product can be purified in lower cost. This will improve protein purification (Nfor et al., 2008). Demand for alternative methods for separation and purification are required to reduce the number of steps and cost. Hence, in the last few years, there has been an increasing interest in biotechnology for the development of ground-breaking and integrative separation and purification technique that are mild and cost effective to preserve the biomolecules.

Liquid–liquid extraction, also known as aqueous two-phase system (ATPS), is an alternative downstream processing method for the recovery of biomolecules. Numerous works have been dedicated to explore and examine the aqueous rich phase behaviours, the stimulus that exert influence on the partitioning of bioproducts in ATPS as well as modelling of ATPS (Azevedo, Rosa, Ferreira, & Aires-Barros, 2007; Chow et al., 2013, 2014, Reschke, Brandenbusch, & Sadowski, 2014, 2015; Rosa et al., 2009; Alireza Salabat, 2001; Saravanan, Rao, Murugesan, Nair, & Ramasami, 2007). In spite of this, there are several drawbacks of this technique which can be overcome via LBF system.

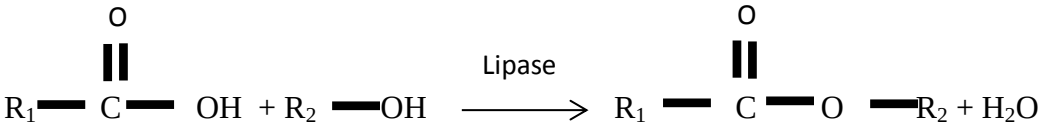
2.2. Lipase

Lipase also known as triacylglcerol acylhydrolases is a ubiquitous enzyme that has substantial physiological importance and industrial potential. The function of lipase is to catalyse the hydrolysis of triacylglycerol into monoglycerides, diglycerides, free fatty acids and glycerols. In aqueous conditions lipase act on carboxyl ester bonds present in triacylglycerol's to release fatty acids and glycerol (Choudhury & Bhunia, 2015). This enzyme carries out the reverse reaction when there is an insufficient water condition. In addition, lipase is able to catalyse extensive range of reactions such as esterification, inter-esterification, alcoholysis, acidolysis and aminolysis (**Figure 2**). Lipase encompasses a common folding pattern that is identified as α/β hydrolase fold. The active site of alpha/beta-hydrolase fold consists of catalytic triad encompassing a nucleophilic residue (serine, cysteine or aspartate), a catalytic acid residue (aspartate or glutamate), and a histidine residue (Ollis et al., 1992).

Hydrolysis

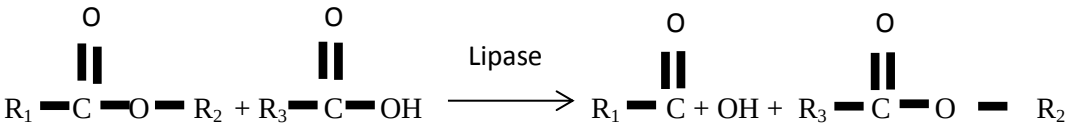


Esterification

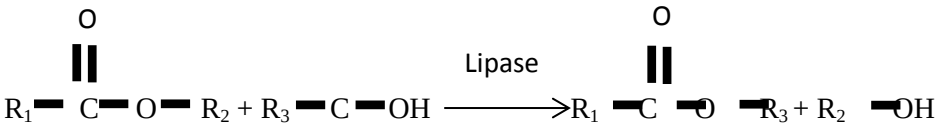


Transesterification

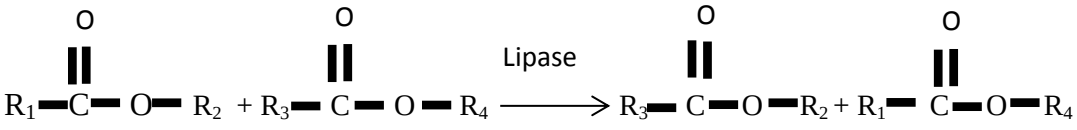
Acidolysis



Alcoholysis



Interesterification



Aminolysis

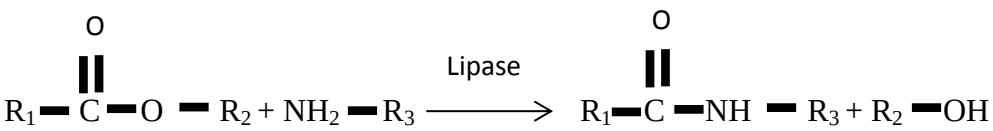


Figure 2: Lipase catalysing reactions

Lipase is produced from numerous species of plants, animals, bacteria, fungi and yeast. Strains that are taxonomically similar may produce diverse types of lipases. Lipases from diverse sources have different enzymological properties and specificities. Lipases extracted from microorganisms dominate a crucial position in a wide range of industrial applications, for example food, detergents, textile, pharmaceutical, cosmetic, and biodiesel production. In addition, lipase is also applied to waste water treatment to remove oil from residual waters of houses, restaurants, and factories (Azhdarpoor, Mortazavi, & Moussavi, 2014; El-gawad, 2014). These versatile industrial potentials of microbial lipases are due to their tolerance towards extreme temperature, pH, organic solvents and ease of mass production (Hasan, Shah, & Hameed, 2006).

Lipase is serine hydrolase and the interfacial activation of lipase is at the lipid-water interface (Angajala, Pavan, & Subashini, 2016). Most bacteria lipases are regarded as extracellular enzyme that are able to carry out various reactions. Lipases are considered as the most commonly utilized biocatalyst for organic reactions accomplished under soft conditions. In the past two decades, researchers have been interested on lipase enzyme due to their growth in industrial applications. The rise of lipase appliance in the industries is due to their unique characteristics of being enantioselective, regioselective and chemoselective (Ray, 2012).

There are several factors that may affect the activity of lipase which includes temperature, nature of acylating agent, solvents and additives. By utilizing lipase in non-aqueous media it is essential to check the temperature of residual water because it could affect the activity of enzyme, thermostability and the

stereoselectivity (Won & Sun Bok Lee, 2001). Lopez demonstrated in a study that with the decrease in temperature from 40°C to -20°C in the ammoniolysis of phenylalanine methyl ester catalysed by the lipase isolated from *Thermomyces lanuginosus* substantively enhanced the enantioselectivity with an enantiomeric ratio of 13 to 84 respectively (López-Serrano, Wegman, Van Rantwijk, & Sheldon, 2001). Esterification reaction catalysed by lipase has limitations related with reversibility of esterification and transesterification. This can be overcome by incorporating acylating agent vinyl acetate or an alternative method by utilizing immobilized lipase. In addition, employing acyl donars different from that of vinyl acetate facilitates the enhancement of the enantioselectivity of transesterification catalysed by lipase (Angajala et al., 2016).

Commercially valuable lipases are generally acquired from microorganisms which are able to produce a large variety of extracellular lipases. Lipases from *Burkholderia spp*. has been well acknowledged in having beneficial characteristics such as highly thermal stable, tolerant towards organic solvent and has a wide range of substrate specificity (J. Yang, Guo, & Yan, 2007). Lipase structure from *Burkholderia cepacia* comprises an alpha/beta-hydrolase fold and a catalytic triad encompassing residues Ser87, His286 and Asp264.

2.2.1. Applications of lipases

The enzymatic properties and substrate specificity of microbial lipases are extensively diversified and this is the reason lipases become very appealing for industrial applications. Based on overall sales volume, lipases are considered the third greatest group succeeding proteases and carbohydrates. The commercial application of lipases that involves broad variability of appliances is a billion-dollar business (Jaeger, Dijkstra, & Reetz, 1999). Lipases have promising uses in biodiesel production, detergent formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, cosmetic production, pharmaceutical applications and manufacturing papers.

2.2.1.1. Biodiesel synthesis

Biodiesel, the monoalkyl fatty acid esters comprising long chain fatty acids derived from vegetable oil or animal fats, is viewed as a potential alternative to fossil fuels. Global biodiesel productions increased by 10.3% in 2015 and biodiesel is a major player in the biofuels market (Sankaran, Show, & Chang, 2016). The global biodiesel market is estimated to reach 37 billion gallon by 2016, with an annual growth rate of 42% (Du, Li, Sun, Chen, & Liu, 2008). The biodiesel production in U.S was about 1,268 million gallons in 2015 and in the European Union, it was 10 million tonnes in 2013 (U.S. Energy information administration, 2015).

The production of biodiesel can be categorised into three different methods: microemulsions, thermochemical decomposition (pyrolysis) and the most common method of transesterification. The transesterification approach is widely used for

large scale biodiesel production due to eco-friendly operation, mild reaction conditions and the extensive viability of the raw materials (Yan et al., 2014). This popular technique, also known as alcoholysis, comprises the catalytic process of converting triglycerides (TG) in oil and animal fat into fatty acid alkyl esters (FAAE) and glycerol with the help of short chain alcohols. Biodiesel synthesis via transesterification can be done either by using chemical or enzyme catalyst.

Microbial lipases are the most common source for the biodiesel production by transesterification, since it can be produced easily via fermentation and straightforward steps of purification(Al-Zuhair, 2007). Moreover, enzymatic catalysis has several advantages including (i) high biodiesel yield, (ii) fewer downstream operations(Yan et al., 2014), (iii) low energy consumption as the catalysis can be done in mild temperature, (iv) high ability to convert both triglycerides and free fatty acids to biodiesel allowing usage of various oil sources including waste cooking oil (v) low cost operation with reusability of immobilized enzyme(Guldhe, Singh, Mutanda, Permaul, & Bux, 2015) (vi) higher purity of the products and (vii) simplicity of biodiesel purification process.

The most widely reported bacterial lipases that is used for transesterification is *Burkholderia cepacia* lipase (BCL), and the yeast enzyme is *Candida antartica* lipase B (CALB). Biodiesel yield varies with different lipase source and feedstock, due to the fact that different types of lipases have distinct characteristics that influence the transesterification process. Lipases from different sources can be used either as a free or immobilized enzyme. However, immobilized lipase are able to attain higher yield (Shah, Sharma, & Gupta, 2004) Not all lipase sources can produce high yield of biodiesel, for example *Porcine pancreatic* lipase (Type II) showed poor transesterification yield of less than 20% due to its

decreased stability (Moreira, Perez, Zanin, & de Castro, 2007). Thus, for effective biodiesel production, the ideal enzyme needs to be selected.

2.2.1.2. Food industry

Lipase enzyme is used widely in food industry for various applications such as for flavour improvement in dairy products, food processing, bakery products and as biosensor for food industry. Amongst all lipases sources, bacterial lipases are widely utilized in food industry for quality enhancement, dairy industry for hydrolysis of milk fat, ripening of cheese, upgrading of aroma in beverages, and applied in health foods for transesterification (S. Sharma & Kanwar, 2014). In the dairy industry lipase is utilized to alter the fatty acid chain lengths and to enrich the flavours of various cheeses. In addition, lipase is able to quicken the ripening of cheese and lipolysis of butter, fats and cream (S. Sharma & Kanwar, 2014). Cocoa butter which is an essential raw material in chocolate is a high value fat containing palmitic acid and stearic acid. These components make cocoa butter hard and brittle at room temperature while in the human mouth it melts completely and produces a desirable cooling sensation. Lipase-based technology that comprises hydrolysis and synthesis reactions is able to enhance some of the less desirable fats to cocoa butter substitutes (Undurraga, Markovits, & Erazo, 2001; H. X. Wang, Wu, Ho, & Weng, 2006). In addition, lipases aids the removal of fat from meat and fish products and are also used in production of fruit juices and vegetable fermentation (Ray, 2012).

In baking industry, lipase is mainly used to enhance the flavour content and also prolong the shelf-life of bakery products. The application of lipases as biosensor is significant in the food industry particularly in fats and oils, beverages,

and soft drinks industries. The fundamental theory of using lipase as biosensor is to generate glycerol from the triacylglycerol present in the analytical sample, and by using chemical or enzymatic method, glycerol released can be quantified. Immobilized lipases are suitable for biosensor application because there are rapid, efficient, precise and cost effective in the quantification and determination of triacylglycerol (Ray, 2012). In a recent study, a potentiometric biosensor utilizing an immobilized lipase in a Nafion membrane on a graphite-epoxy transducer is developed. This device work is based on the detection of changes of pH and it is able to reduce the use of organic solvents without influencing the precision and accuracy of the analysis in complex food samples (Escamilla-mejía, Rodríguez, Álvarez-romero, & Galán-vidal, 2015).

2.2.1.3. Detergent industry

The application of enzymes in detergent formulations is unexceptionally popular. Detergent enzymes comprise around 32% of the entire global industrial enzyme production (Chauhan, Chauhan, & Garlapati, 2013). The first enzymes that were introduced in commercial laundry detergents were proteases. Currently, many laundry detergent products encompass cocktails of enzymes containing proteases, amylases, cellulases, and lipases. Lipases are able to remove difficult fats and oils stains from textiles and hard surfaces either by the decomposition of fatty materials into more hydrophilic substances or by the release of surface active molecules that is capable of altering the properties of the soil or water interfaces (Jurado, Garcia-Roman, Luzon, Altmajer-Vaz, & Jimenez-Perez, 2011). There is increasing demand of alkaline lipases as a detergent additive and this is primarily owing to its affiliation with the non-phosphate detergents (Cherif, Mnif, Hadrich, Abdelkafi, &

Sayadi, 2011). In addition, detergent lipases are becoming popular as their escalating utilization in washing machine, where it impart softness and resiliency to fabrics, are easily dispersible in water and mild to eyes and skin (Bajpai & Tyagi, 2007).

The first commercial lipase created to be applied as a detergent additive was Lipolase which was developed by Novozymes, and the lipase was originated from *Thermomyces lanuginosus* a kind of fungus (Jurado et al., 2011). Lipases with essential requirements of broad substrate specificity, activity and stability at high temperature and alkaline pH and compatibility with different components in detergents including metal ions, surfactants and oxidants are needed as they can to be utilized in detergent formulation (Hemlata, Uzma, & Tukaram, 2016). Lately, researches are exploring new sources of lipases that are able to remain stable and active under harsh conditions including in the presence of diverse detergents components, alkaline pH, and wide range of temperatures (Chauhan et al., 2013; Cherif et al., 2011; X. L. Li et al., 2014; Romdhane, Fendri, Gargouri, Gargouri, & Belghith, 2010).

2.2.1.4. Pharmaceutical industry

Lipases are also utilized in the medical and pharmaceutical industry. Selective acylation and deacylation reactions via enantioselective interesterification and transesterification reactions in pharmaceutical industry are aided by lipases (STINSON, 1995). Lipases originating from microbial are utilized to enrich polyunsaturated fatty acids (PUFAs) from animal and plant lipids. In addition, their mono and diacylglycerides are exploited to generate a variety of pharmaceuticals (Ray, 2012). Liposomes are applied in the medical field to improve the action of

drugs by means of transporting them to target areas. This facilitates the avoidance of drug waste inactivation and anatomical barriers (Linko & Wu, 1996). In addition, lipases can be used to resolve the racemic mixtures and to produce chiral building blocks for pharmaceuticals. There are a few lipases that are able to tolerate and retain their activity in nonpolar organic solvent. These types of lipases can be utilised in the hydrolysis of water-insoluble esters, such as in the resolution of racemic mixtures through stereospecific hydrolysis (Hasan et al., 2006).

Along with other lipase, applications, the synthesis of enantiomerically pure active pharmaceutical ingredients (APIs) and their intermediates using lipase is a focus of interest of researchers. Efficiency of many drugs depends on the chirality, therefore the production of single enantiomers of drug intermediates turns out to be significant in the pharmaceutical industry. Researchers are interested in the vast potential of microorganisms and enzymes for the transformation of synthetic chemicals with high chemo-, regio- and enantioselectivity (Patel, 2002). In a study, lipase from *Candida antarctica* (Novozym® 435) was employed for the kinetic resolution of racemic flurbiprofen using the method of enantioselective esterification with alcohols (H. Y. Zhang, Wang, Ching, & Wu, 2005). Azole subunit is part of the structure of a number of drugs such as econazole, fluconazole, ketoconazole, miconazole, ravuconazole and voriconazole. The presence of this subunit discloses noteworthy properties contributing to numerous possibilities in both organocatalysis and medicinal chemistry. In a recent study, Daniel and his colleagues prepared a new family of racemic azole derivatives and submitted to a kinetic resolution through transesterification using lipase from *Pseudomonas cepacia* (Méndez-Sánchez, Ríos-Lombardía, Gotor, & Gotor-Fernández, 2015).

2.2.2. Lipase purification method

In the past two decades, lipase has become vital for various industrial applications. There are different strategies for lipases purification that has been explored which includes conventional methods such as precipitation techniques, chromatographic and membrane processes. Novel methods comprise reverse micelle system (RMS), immunopurification, aqueous two-phase system (ATPS) and liquid biphasic floatation (LBF).

While commonly produced by fermentation process, lipases derived from microorganisms are generally found extracellularly. Following fermentation, cells and other insoluble particles from culture broth will be removed by centrifugation or filtration. In order to concentrate the supernatant, ultrafiltration is applied and as for the extraction process, precipitation with ammonium sulphate or extraction using organic solvents is usually employed (C. H. Tan et al., 2015). Almost 80 % of purification techniques involve the precipitation method (Yagmurov, Kozlov, & Pushkarev, 2017). Protein precipitation via ammonium sulphate is the most commonly applied method as it accounts for 60 %, while the remaining precipitation method accounts for 35 % utilizes ethanol, acetone or hydrochloric acid (Saxena, Sheoran, Giri, & Davidson, 2003). Precipitation process is usually considered as the preliminary stage of purification procedure which will be followed by a chromatographic separation. It is reported that purification process involving precipitation method gives higher yield roughly 87 % in contrast to other approaches which give lesser yields of about 60–70% (Yagmurov et al., 2017).

2.2.2.1. Chromatography method

In order to obtain high purity of lipase, a series of chromatography steps is required. Purification using the chromatography technique is based on several properties, for instance size, protein form, total charge, hydrophobic groups surrounding the surface and the capability of binding with the stationary phase (Coskun, 2016). There are two major components that form the base of chromatography namely the stationary phase comprised of solid phase as well as a layer of a liquid adsorbed on the surface a solid. Next is the mobile phase composed of liquid or a gaseous component. Target proteins will be separated from the stationary phase while moving with the help of a mobile phase.

There are three main chromatography methods available for protein extraction, namely, (i) ion exchange chromatography, (ii) gel filtration and (iii) affinity chromatography. The most popular chromatography method is ionic exchange using core anion and cation exchangers such as diethylaminoethyl (DEAE) group and carboxymethyl. It is discovered that, for lipase purification, strong ion exchangers based on triethylaminoethyl groups and Q-Sepharose are widely utilized (Iftikhar, Niaz, Jabeen, & ul Haq, 2011). The next position of most employed chromatography is gel filtration chromatography. Due to limited capacity for protein loading, this technique is normally employed in the final stage of polishing. As for affinity chromatography, hydrophobic based integration of chromatography is the most common type utilised for lipase purification. This method is popular for lipase because the active sites of lipase are surrounded with hydrophobic surface favouring their separation (Saxena et al., 2003). Adsorption chromatography is another type of affinity chromatography considered to be

expensive method. Although it is costly, this technique is able to give a high purification factor from 2-10 in each step it is used. With the continuous development of downstream process, there is constant research on chromatography methods for novel breakthroughs. An example is the purification of lipase from porcine pancreatic using the affinity precipitation technique. In this study, by synthesising a new thermo-responsive polymer (P_{NNB}) with Cibacron Blue F3GA (CB) conjugation, lipase was able to be separated from its crude material with 16 fold of purification factor (Z. Ding, Zheng, & Cao, 2014). In another study, biomimetic affinity chromatography was used to purify lipase B from *Candida*. By connecting synthetic ligands to Sepharose, 4B lipase was recovered with 73% of yield (Z. Ding et al., 2014). **Table 1** presents recent comprehensive description of the purification strategies adopted for various types of lipases comprising the chromatography method.

Despite the extended application of this technique in various biomolecules purification, there are several limitations needed to be considered for large scale production such as the cost for large scale process, amount of steps in the separation train, complications packing large-scale columns, column lifetime limitations, flow rate limitations and floor space conditions (Thömmes & Etzel, 2007).

Table 1: Recent purification strategies for lipases using various types of chromatography techniques

Strain	Purification technique	Fold increases/yield (%)	References
	Bacteria		
A.calcoaceticus LP009	Ultrafiltration, gel filtration on Sephadex G-100 chromatography	NA	(Pratuangdejkul & Dharmsthiti, 2000)
Acinetobacter sp. RAG-1	MonoQcolumn, butyl Sepharose column and elution with Triton X-100	10/22 %	(Snellman, Sullivan, & Colwell, 2002)
B. licheniformis KM12	80% ammonium sulphate precipitation and Q-Sepharose FF column	15.63/34.2%	(Malekabadi, Badoei-dalfard, & Karami, 2018)
Burkholderia ubonensis SL-4	80% ammonium sulphate precipitation, Q Sepharose FF anion exchange and Superdex 75 gel filtration chromatography	68.5/13.34%	(W. Yang, He, Xu, Zhang, & Yan, 2016)
Bacillus sp	80% ammonium sulphate and phenyl sepharose CL-4B column	5.1/10.5%	(Sivaramakrishnan & Incharoensakdi, 2016)

Bacillus methylotrophicus PS3	Ammonium sulphate precipitation and Sephadex G-100 gel column chromatography	2.90/24.1%	(P. Sharma, Sharma, Pathania, & Handa, 2017)
Cohnella sp. A01	Ion-exchange chromatography (DE52 resin)	13.35/38%	(Golaki et al., 2015)
Pseudomonas aeruginosa BUP2	Ammonium sulphate precipitation and sephadex G-100 gel filtration	36/20%	(Unni, Priji, Sajith, Faisal, & Benjamin, 2016)
Yersinia enterocolitica	Ni-affinity chromatography	34.67/73%	(Ji, Li, et al., 2015)
Xanthomonas oryzae pv. Oryzae YB103	Ammonium sulphate precipitation and phenyl sepharose chromatography	101.1/15.7%	(Mo, Liu, Guo, Zhang, & Li, 2016)
Bacillus pumilus	Ammonium sulphate precipitation, Sephacryl S-200 gel filtration and Mono S chromatography	210/36%	(Laachari et al., 2015)
Geobacillusstearothermophilus	DEAE-cellulose, sephadex G-150 and sephadex G-25 chromatography	18.3/19.7%	(Ekinci, D'Inçer, Baltaş, & Adigüzel, 2016)
Yersinia enterocolitica KM1	Ultrafiltration, 80% ammonium sulphate precipitation, sephacry™ HRS-100 gel filtration, superdex G-75 gel filtration chromatography	26/10.3%	(Ji, Chen, Zhang, Lin, & Wei, 2015)
Streptomyces sp. OC119-7	80% ammonium sulphate precipitation and Sephacryl S100 gel filtration	5.52/3.43%	(Ayaz, Ugur, & Boran, 2015)
	Fungi/Algae		
Aspergillus niger NCIM 1207	90% ammonium sulphate precipitation and Sephacryl S-100 column chromatography	149/54 %	(Mhetras, Bastawde, & Gokhale, 2009)

Aspergillus niger MYA 135	DEAE-sepharose CL-6B column	16.6/53.4%	(Romero et al., 2012)
Botryococcus sudeticus (UTEX 2629)	15–65% ammonium sulphate precipitation and size exclusion column chromatography.	2.88/NA	(Yong, Lim, Saleh, & Tey, 2016)
Candida viswanathii	Hydrophobic octyl column (Hiprep™ 16/10 Octyl Sepharose FF fast flow) and elution with Triton X-100	8.7/78.4%	(Almeida, Terrasan, Terrone, Tauk-Tornisielo, & Carmona, 2017)
Mucor hiemalis f. corticola	75% ammonium sulphate precipitation, Sephadex G75 and Q-Sepharose fast flow 3560	12.63/27.7%	(Ülker & Karaoĝlu, 2012)
S. aureus strain ALA1 (SAL4)	65% ammonium sulphate precipitation and C18 column for RP-HPLC	81.4/51.7%	(Ben Bacha, Al-Assaf, Moubayed, & Abid, 2016)
Talaromyces thermophilus	Ammonium sulphate precipitation, gel filtration using Sephacryl S-200 and anion exchange using Mono-Q Sepharose	105/29%	(Romdhane et al., 2010)
	Others		
Carica papaya	HisTrap FF- affinity chromatography	7.18/70%	(Rivera, Robles, Mateos-Díaz, Gutierrez-Ortega, & Sandoval, 2017)
*Lates calcarifer (*liver of seabass)	40-60% ammonium sulphate precipitation, DEAE-sepharose anion exchange column and Sephadex G-75 gel filtration	11.06,42.39/4.06%, 0.81%	(Sae-leaw & Benjakul, 2018)
Hexaplex trunculus (Marine snails hepatopan- creas)	60% ammonium sulphate precipitation, sephacryl S-200 column gel filtration, mono-Q sepharose column and octyl-sepharose column	158.10/15%	(Zarai et al., 2012)

Crab hepatopancreas (crab digestive lipase)	60% ammonium sulphate precipitation, Sephacryl S-200 and Mono-Qcolumn	165/10%	(Cherif et al., 2007)
*Scorpion hepatopancreas (*scorpion digestive lipase)	(DEAE) column, 60% ammonium sulphate precipitation, Sephadex G-100 gel filtration, CM-Sephadex and FPLC column Superdex 75	506/16.7%	(Zouari, Miled, Cherif, Mejdoub, & Gargouri, 2005)

2.2.2.2. Membrane processes

In the last few decades, the application of membranes in the purification process has gradually increased. The advantages of membrane systems include a high surface area of membrane as well as selectivity. In addition, their capability to function at low temperatures and pressure ranges from no changes of phases able to reduced denaturation and deactivation of protein (Yagmurov et al., 2017). The most popular membrane separation processes are ultrafiltration and microfiltration including filtration of fermentation media, purification of buffers and proteins. Ultrafiltration is a membrane technique which has the capability to isolate and concentrate target biomolecules. In a study on ultrafiltration, a dual-layer biomimetic membrane was developed by binding chitosan onto the surface of poly(acrylonitrile-*co*-maleic acid) (PANCMA) ultrafiltration hollow fiber membrane in the presence of 1-ethyl-3- (dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxylsuccin-imide (NHS). This fabricated dual-layer biomimetic membrane successfully purified lipase from *Candida rugosa* in the existence of glutaraldehyde attached on the membrane (Ye, Xu, Che, Wu, & Seta, 2005). Tao et al. has recently discovered a novel ultrafiltration approach combined with high- performance liquid chromatography and quadrupole-time-of- flight mass spectrometry to screen lipase binders in *D. officinale*. In this study, the proposed approach was applied to identify antiobesity agents from the extracts of medicinal plants and they successfully identified ligands that interacted with the lipase from different extracts of *D. officinale* (Tao, Cai, Li, & Cai, 2015).

Another advanced development on lipase purification involves the combination of two strategies. Lipase from *Staphylococcus carnosus* was purified

by using Sartobind-phenyl membrane as the first approach and cation exchanger (CEX) mixed-mode prototype as second approach. This approach involves the combination electrostatic (sulfonic acid groups) with hydrophobic interaction chromatography (HICs). By using HIC–phenyl (Sartobind-phenyl) membrane, the lipase yield obtained was 89%, residual activity 92% with a purification factor 3.2. Whereas for the mix-mode CEX membrane prototype, the yield obtained was 93% and the residual activity was 98% while purification factor of 7.8 (Beutel, Villain, & Scheper, 2016). An alternative to ultrafiltration are the microfiltration membrane processes. Polypropylene microfiltration membrane (PPMM) is considered as a hydrophobic membrane with potential in the industries due to fine structured porosity and chemical inertness. Deng et al. modified PPMM by grafting sugar-containing polymer onto membrane surface with the plasma-induced polymerization of α-allyl glucoside (AG) and combining poly(γ-stearyl L-glutamate) (PSLG) membrane surface through the ring opening polymerization of N-carboxyanhydride (NCA) of γ-stearyl l-glutamate. Attaching Poly(α-allyl glucoside) (PAG) and PSLG onto PPMM enhances the bio- compatibility of the membrane. PAG-modified PPMMs was reported at 60.7 U/mg of specific activity and 49.9% of activity retention, which is considered low. The poor result indicated that the sections of the membrane with high hydrophilicity inhibited lipase adsorption. As for PSLG-modified PPMM, they recorded high specific lipase activity with 88.1 U/mg and 72.4% of activity retention owing to long stearyl chains that is distributed through the membrane, therefore a greater retention of activity was obtained (Deng et al., 2004).

In a recent study, Reinehr et al. utilized successive tangential filtration with membranes for lipase separation and concentration from solid-state fermentation of

agro-industrial residues by *Aspergillus niger*. In this study, a series of tangential filtration that includes microfiltration and ultrafiltration with different pore sizes were used to concentrate lipase. Based on this study it is found that the sequential approach is able to provide concentrated extracts with higher hydrolytic activities (60.72 U/mL) than those in the initial extract (20.24 U/mL) (Reinehr et al., 2017).

2.2.2.3. Reverse micelle system (RMS)

Reverse micelle system (RMS) for protein extraction is considered a fascinating liquid-liquid extraction technique in downstream process. Generally, reverse micelle develops in solvent which is non-polar. The hydrocarbon tails of surfactant molecules are facing the organic solvent whereas the polar head cluster is facing inwards. RMS is able to support an internal hub of water molecules which facilitates the dissolution of hydrophilic proteins and enzymes. The formation of reverse micelles is influenced by the head group charge. As the head group charge increases, the formation of reverse micelles will decrease as a result of the electrostatic interactions (Shin & Vera, 2002). The factors that could influence the performance of RMS includes pH, concentration of target proteins, temperature, ionic strength, types and concentration of surfactant used and time of process (Yagmurov et al., 2017). The application of surfactant in RMS plays a major role in the solubility of organic compound. Interfacial area of solvent is increased in the presence of surfactant for large formation of micelle and enzyme recovery. This enables a higher yield of product, greater purity of enzyme and amplified specific enzyme activity (C. H. Tan et al., 2015).

The RMS recovery process consist of two key steps: the first step that involves a forward extraction process comprising of the entrapment of proteins from a bulk aqueous solvent encompassing the target enzyme into the pool of water of reverse micelles in an inorganic solvent, and the second step involving the back extraction process in which the proteins are shifted from the reverse micelles into another aqueous solvent for the recovery. **Table 2** displays a summary of several recent studies on the RMS method for lipase purification. RMS serves as a valuable and economical approach for enzyme purification. However; the application of this technique is limited due to a few reasons. The separation and recovery of protein seems to be difficult at higher surfactant concentrations; and the effective selection of organic solvents for the technique functions is restricted by the constraint to circumvent the denaturation of protein while permitting protein solubilisation (Basheer & Thenmozhi, 2010).

Table 2: Recent purification strategies of lipases using reverse micellar system

Strain	RMS purification scheme	Fold increases/yield (%)	References
Thermomyces lanuginosa	100 mM AOT/Isooctane	92%	(Fernandes et al., 2004)
Aspergillus niger	Forward extraction 0.10M Tris buffer, pH 9.0 containing 0.10M NaCl in aqueous phase and 0.20M CTAB in organic phase consisting of isooctane/butanol/ hexanol in the ratio of 75/15/10 (v/v/v). Backward extraction 0.05M potassium phosphate buffer pH 7.0, containing 1.0M KCl.	4.09/82.72%	(Nandini & Rastogi, 2009)
Aspergillus niger	Forward extraction 0.104 M salt, 0.204 M CTAB in organic phase comprising (isooctane 75%, hexanol 10 % and butanol 15 % at pH 9. Backward extraction 0.8M salt and pH at 7.23.	4/78%	(Nandini & Rastogi, 2010)
Aspergillus niger	Reverse micelles-mediated transport in liquid emulsion membrane (LEM). 0.1 M CTAB and 0.18 M Span 80 and cosolvents (isooctane and paraffin light oil ratio 1:1) 0.1 M NaCl at pH 9.	3.14/78.6%	(Bhavya, Priyanka, & Rastogi, 2012)
Pseudomonas sp. CSD3 isolate	RMS (50 mM AOT/Isooctane) at 25°C , pH 6.5, 15 min and back extracted with 0.25M NaCl and 15% isopropanol at 25°C/pH 7 for 30 min.	15/80%	(Gaikaiwari, Wagh, & Kulkarni, 2012a)

Aspergillus niger	Mixed reverse micelles (MRM) consisting of cationic and non-ionic surfactants in LEM. 0.075 M CTAB, 0.012M Tween 80, internal aqueous phase pH 7, feed pH 9, 1M KCl, 0.1 M NaCl and ratio of membrane emulsion to feed volume 1:1.	17/100%	(Bhowal, Priyanka, & Rastogi, 2014)

2.2.2.4. Immunopurification

Immunopurification also known as immunoaffinity chromatography is a technique of protein purification that utilizes an antibody-antigen principle. This technique is able to purify biomolecule that is difficult to be separated by other methods. Immunopurification is considered as a powerful method because of its high selectivity for particular proteins and it provides a wide ranging purification factor of 1000 to 10000 fold in a single step (Yagmurov et al., 2017). Generally, in most of the protein immunopurifications approaches are comprised of two types of antibodies, namely, monoclonal and affinity-purified polyclonal. Two key factors must be taken into account when selecting the antibody: the availability of appropriate monoclonal antibody for desired protein as well as identifying contaminants present in the sample and this includes the composition and concentration of the impurities (Nandini & Rastogi, 2009).

Immunopurification technique is considered expensive due to the application of monoclonal antibodies. Nevertheless, with the recent development in biotechnology, cheaper methods of mass production of antibody were discovered leading to reduction in cost of immunopurification. Recently, a simple and inexpensive technique was discovered which utilizes previously existing antibodies and antigens represented by means of combination of the target protein and a specific tag (Wood, 2014). In a study on lipase purification, genetic engineering of *Candida antarctica* of lipase B (CalB) is conducted to attach a free cysteine thru histone (His) tag to generate CalB-HisGGC and CalB- C311A-His. A spacer consists of heterobifuntional NHS-PEG-maleimide was employed for the

immobilization of the CalB variants. By using the maleimide-activated glass support, lipase was purified with 10, 000 fold in a single step (Blank, Morfill, & Gaub, 2006).

In another study, lipases lip11 and lip12 from *Yarrowia lipolytica* MSR80 was attained by integrating lipases with IgG tag. Novel lipases lip 2, lip11 and lip12 were cloned and expressed with IgG tag in *E. coli* HB101 pEZZ18 system. The recombinant enzymes were purified by affinity chromatography using IgG-Sepharose column and it was found that the specific activity obtained from this study was 314, 352 and 198 U/mg for Lip2, Lip11 and Lip12 respectively. The study on the effect of metal ion on lipases was done and it wass found that Lip2 and Lip12 were activated by $Ca2^{+}$ whereas Lip11 was by $Mg2^{+}$ (Kumari, Verma, & Gupta, 2012). In another study on affinity tags, Rivera et al. functionally expressed Carica papaya lipase 1 sequence (CpLip1) in Pichia pastoris. The recombinant protein contained a C-terminal extension including a His6 tag which was purified by HisTrap affinity chromatography. From this study, a purification factor of 7 and lipase activity of 25 U/mg was achieved (Rivera et al., 2017).

2.2.2.5. Aqueous two phase system (ATPS)

Aqueous two phase system (ATPS) is an alternative technique for biomolecules separation involving liquid-liquid fractionation utilizing two aqueous phases. It was first proposed in the 20^{th} century as a powerful technique for the separation and purification of biological materials (Baskir, Hatton, & Suter, 1989). This extraction of biomolecules which is also recognized as aqueous two phase extraction (ATPE) is grounded on the separation of biomolecules between the two aqueous phases. ATPE has been extensively employed for biomolecules separation

that includes proteins, antibiotics, chemical compounds (Z. Yang, Tan, Li, & Li, 2016) and enzymes (Rodríguez-Durán et al., 2013).

The principles of ATPS is based on incompatibility of two immiscible hydrophilic compounds, commonly involving salt/polymer, polymer/polymer, an ionic liquid (IL)/salt, and a low molecular weight alcohol/salt system in water exceeding certain concentrations. The separation of biomolecules is grounded on the selective partitioning of the target products between the two phases (Rosa, Ferreira, Azevedo, & Aires-Barros, 2010). The most soluble matter will separate to the lower phase which is more polarised, while the biomolecules of interest such as proteins will move to the top phase which is less polarised and highly hydrophobic. Partitioning via a two-phase system is a mild technique for protein purification. In addition, biological activities of the biomolecules are preserved and denaturation is not usually seen. This is because the system has low interfacial tension and high water content that is able to protect the proteins. In addition, in the polymer system they may have a stabilizing effect that sustains the protein (Asenjo & Andrews, 2011).

The advantages of ATPS that instigates much attention are (a) straightforward and simple method, (b) high efficiency, (c) carried out in mild condition, (d) scale up is easy, and (e) reduced protein denaturation. There are abundant studies on ATPE of protein that has been done including casein proteins from cow milk samples (A. S. Lopes et al., 2007), serine protease (Amid et al., 2012), lipase from *Burkholderia Cepacia* (Show et al., 2012), fibrinolytic proteases (de Medeiros e Silva et al., 2013; Nascimento et al., 2016), collagen and its hydrolysate from a homogenate (Selvakumar, Ling, Covington, & Lyddiatt, 2012) and many more. This method is also applicable to separate and purify recombinant

proteins (Aguilar, Albiter, Serrano-Carreón, & Rito-Palomares, 2006; Guan, Lilley, Treffry, Zhou, & Wilkinson, 1996; Harris, Andrews, Wright, Pyle, & Asenjo, 1998). In a latest study, researchers have investigated a new application of an aqueous two phase system deproteinization (Y. Wang et al., 2016). In this study, by using thermosensitive triblock copolymer (EOPOEO) and inorganic salt (NaH2PO4) ATPS combined with dialysis membrane, lycium barbarum polysaccharide (LBP) was purified and impurities including protein and pigments were removed.

The most traditional types of ATPS composed of two or more polymers such as (i) polyethylene glycol (PEG) and dextran (Ooi, Hii, Kamal, Ariff, & Ling, 2011), (ii) a polymer and salt (M. Antov & Omorjan, 2009) and non-ionic surfactant micellar system (Xing & Li, 2012). Partition of biomolecules from the system can be achieved through the manipulation of the partition coefficient, by modifying the experimental conditions of the medium such as molecular weight of polymers, types of ions in system, and ionic strength (presence of hydrophobic groups) (Zaslavsky, Boris, 1994).

As mentioned earlier, ATPS has been utilised for lipase separation. ATPS composing of PEG/phosphate has been used to extract lipases from *Enterococcus faecium* MTCC5695. Lipase was successfully partitioned to the bottom phase of PEG8000/sodium dihydrogen phosphate with the optimum conditions for purification at 41.24% TLL and 0.25 phase volume ratio. The integration of ATPS and ultrafiltration of MTCC5695 lipase led to a purification factor of 5.99 and recovery of 82.09% (Ramakrishnan et al., 2016). A recent study demonstrated combined phase partitioning systems for recovery of lipase from Pacific white shrimp hepatopancreas. In this study, three phase partitioning (TPP) system with

ATPS was combined to enhance lipase recovery. The initial step of separation using TPP was done with CE/*t*-butanol ratio of 1:1 in the presence of 50% (w/v) $(NH_4)_2SO_4$ and followed with ATPS comprising 25% PEG1000-15% $MgSO_4$ at pH 5.0. From this study lipase was separated into the top phase with 5.19 of purification factor and 78.46 % of yield. This research demonstrated a novel combination technique for high lipase recovery (Kuepethkaew, Sangkharak, Benjakul, & Klomklao, 2017).

Apart from ATPS based on polymer/salt, ionic liquid (IL) grounded ATPS has generated abundant attention recently due to IL exclusive characteristics, for example non-flammable and extreme chemical stability (Baranyai, Deacon, MacFarlane, Pringle, & Scott, 2004; Wasserscheid & Keim, 2000). In a study concerning IL based ATPS, lipase A derived from *Candida antarctica* was extracted by combining an imidazolium based IL with an alkylsulfate anion. In this study, it was reported that 1-ethyl-3-methylimidazolium butyl sulphate $[C_2MIM][C_4SO_4]$ combined with ammonium sulphate, attained a lipase recovery of 99%. Several enhancements were discovered utilizing this technique involving shorter processing time (approximately an hour), simple operation conditions, small consumption of energy, high enzyme recovery (> 99%) and finally the potential usage of IL as medium for bio-catalytic transformations (Deive, Rodríguez, Rebelo, & Marrucho, 2012). Lee et al., incorporated centrifugation and IL/polymer ATPS methods for lipase recovery from *Burkholderia cepacia*. In this study, ATPS composed of [Ch][BES] and PPG 400 was utilized and lipase was successfully recovered from the fermentation broth with a purification factor of 17.96 and 99.30 % yield. In addition, recycling of phase-forming components was conducted and results indicated that there was no major change in the purification

and lipase recovery compared to the systems using fresh chemicals (Lee, Khoiroh, Ling, & Show, 2017a).

The disadvantage of ATPS system is slow separation of two-phase in polymer/polymer ATPS. This process is time demanding due to phase separation under gravity and the separation time is influenced by the rheological properties of the aqueous rich phase as it is dependent on the properties (Asenjo & Andrews, 2012). Besides, there are problems related to the recycling of phase-forming components (Show et al., 2013a). In addition, up to now all the reported enhancements in ATPS are comprised of their chemical composition modification, and essentially by altering the traditional mass transfer mode, one is able to increase the separation efficiency and decrease the cost (P. Y. Bi et al., 2009).

2.3. Liquid Biphasic Flotation (LBF)

Liquid biphasic flotation (LBF) also known as aqueous two-phase flotation (ATPF) is a novel separation, extraction and purification technique resulting from the integration of SS and ATPE system (Show et al., 2011). This method was first introduced by Bi and his colleague in separating penicillin G from fermentation broth (P. Y. Bi et al., 2009). LBF is a type of adsorptive gas bubble partition method that involves the absorption of surface-active sites proteins from the salt rich aqueous phase on the bubble surfaces of an ascending gas stream and the dissolution in an organic layer on top of the aqueous phase when the bubbles rupture. This method is efficiently utilized for the retrieval of biological products comprising hydrophobic/surface active site for instance phenyl or alkyl group.

LBF is achieved by combining the ideologies of ATPS and SS involving the creation of two immiscible mixed liquid phases in equilibrium resulting from

the dissolution of two immiscible hydrophobic liquid phases with each other above critical conditions and combining bubble attachments of the target bioproducts transmitting from aqueous rich phase (bottom) to less denser (top) solvent phase (Lee, Khoiroh, Ling, & Show, 2016). LBF works by combining the mode of mass transfer of (SS) and ATPS. SS was firstly introduced by Sebba as an enhanced technique for the ion flotation that involves an adsorptive gas bubble separation method without foam formation (Sebba, 1962). In this technique, the surface active compound which is hydrophobic present in aqueous phase will be adsorbed on the surface of the gas bubble of an ascending gas stream which then will be accumulated in a small layer of organic solution above the aqueous phase (P. yu Bi, Dong, & Dong, 2010; Kislik, 2012). This process involves the passage of apolar air bubbles accompanying surface active molecules in the interface of air and water which is produced from the gas-diffuser through a narrow column known as sublation column and finally circulated to organic phase (Valsaraj, Thoma, Thibodeaux, & Wilson, 1991). **Figure 3** displays the complete mass transfer mechanism that is applied among the phases in SS method. The mass transfer process of SS is similar to LBF and it will be discussed further in the later part of this thesis.

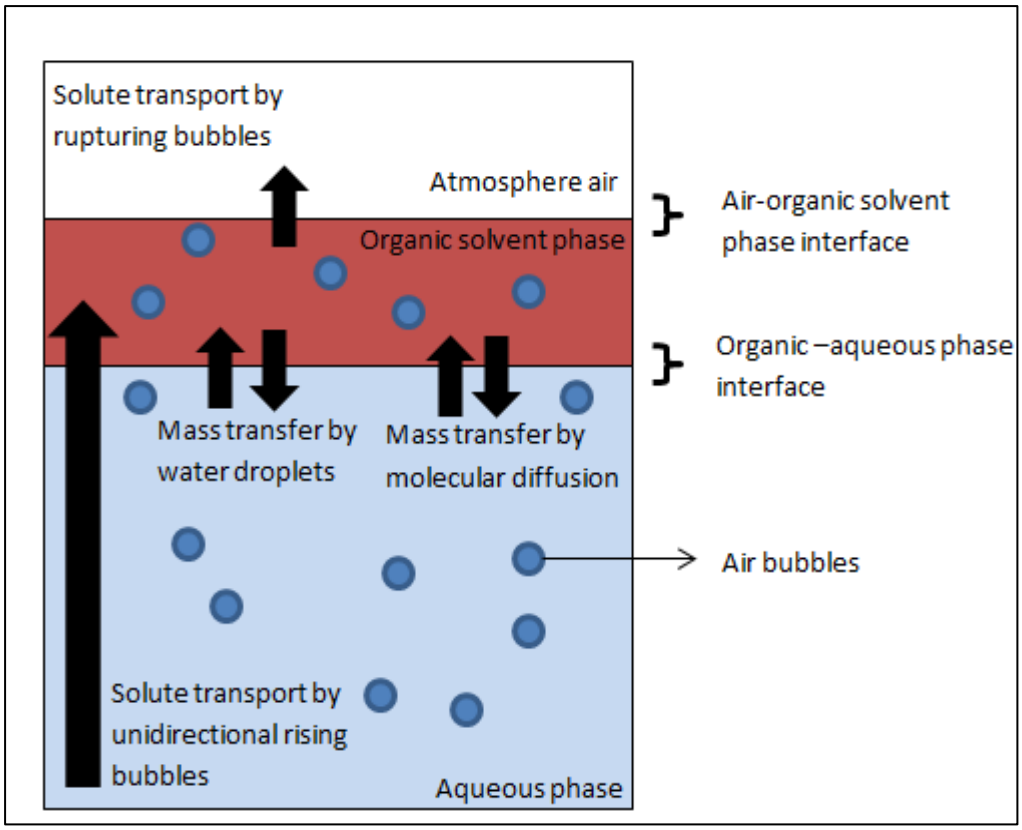


Figure 3: Graphic illustrating the mass transfer mechanism involved among the phases in SS method

In LBF method, usually two non-miscible aqueous phases that has greater ratio of volume (salt:polymer) (or additional new element) is applied. Compared to polymer, salts are desired because high usage of polymer results in high cost damaging environmental impact. Normally in LBF techniques, polymer develops the upper rich phase that functions as biomolecule accumulator and hence, a small volume of polymer is cost effective. Show et al. reported a thin coating of polymer phase concealing the aqueous phase rich in salt which is adequate for effective biomolecules extraction (Show et al., 2011). There are three major types of LBF, namely, hydrophilic organic solvent/salt LBF (Show et al., 2013b), polymer/salt LBF (P. Y. Bi et al., 2013) and ionic liquid/salt LBF (Z. Tan et al., 2013). All the

three types of LBF is based on the same theory: Firstly, the crude feedstock comprising biomolecules is mixed with the bottom salt aqueous phase. This mixed suspension is then moved into the flotation column where a layer of polymer solution will be poured on top of the mixed solution where the salt solution and the polymer are in constant equilibrium as two non-miscible parts. Stream of air bubbles are introduced at the bottom of the flotation column via a sintered glass disk in the low aqueous salt-phase. The bubbles move upwards, heading to the polymer rich top phase.

During the floating of bubbles, there will be interaction between the biomolecules and gas bubbles. The hydrophobic non-polar tails of the biomolecules will have the tendency to move towards the bubble and remain inside of the air bubbles while their polar heads which is hydrophilic will interrelate with the outer layer of the gas bubbles which is salt-rich aqueous solution. In the ascending one-way of air bubbles, the biomolecules are transported and accumulated at the top aqueous polymer-phase. There are two means of the accumulation of the biomolecules at the top phase; either by rupturing of air bubbles at the interface of atmosphere (air-aqueous) or by direct dissolution of the biomolecules within the passage of gas bubbles across the top phase. Since the target compound is discharged into the top phase through the dissolution of the bubbles, efficient separation could be attained. **Figure 4** elucidates the simple activity that takes place within the LBF system.

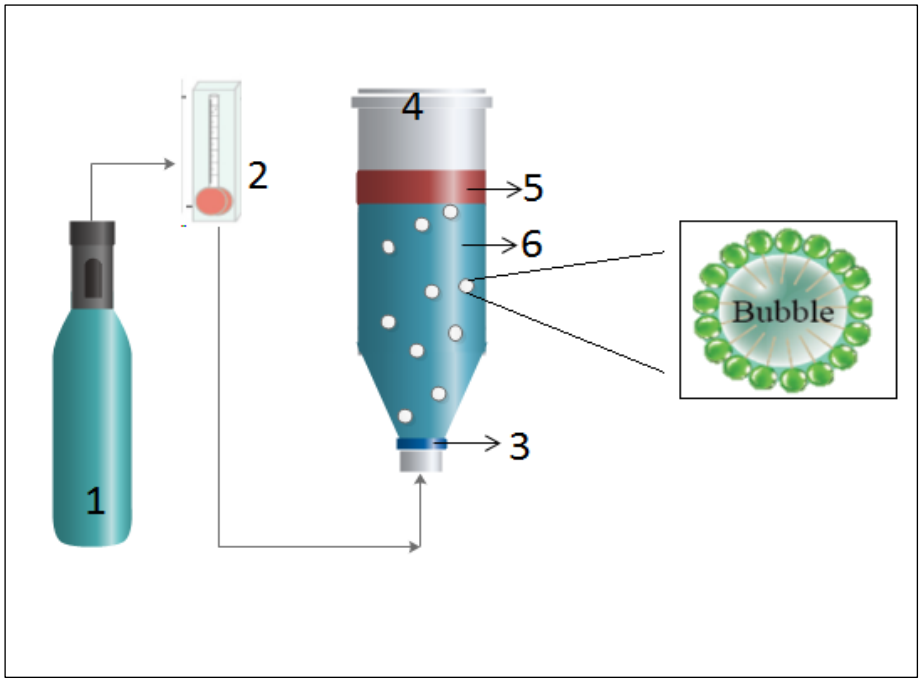


Figure 4: The schematic diagram of LBF apparatus. (1) gas cylinder; (2) flowmeter; (3) sintered glass disk; (4) flotation column; (5) organic phase; (6) aqueous phase

In the LBF system there are two basic processes for mass transport of the target compounds from aqueous bottom phase to top organic phase. The main and vital mass transport is via air bubbles. There are three major components entailed in the gas bubbles: (i) polar gas phase, (ii) interfacial shell and (iii) polar exterior fluid enclosing the shell (Parmar & Majumder, 2013). The shell of gas bubbles is the most essential section in offering interfacial area for surface active adsorption of targeted components. Hence, gas bubbles play a crucial role in facilitating mass transfer from lower to upper phase and overcoming the interfacial tension among aqueous and polymer phases. Next mass transport route is at the interface of aqueous two-phase that occurs via diffusive molecular mass transfer. This process is normally stimulated by the hydrophobic difference between immiscible aqueous two phases. Another mass transfer process that is applied in the LBF is the water sheath drag by the air bubbles (Han et al., 2011). This mass transfer process is also applied in SS system of loaded solute among organic and aqueous solution. The air bubbles will draw a thin layer of water towards the upper organic phase and in the

end depletes as water droplets which is low in solute skink and return to the lower aqueous solution via the force of gravity (P. Y. Bi, Dong, & Wang, 2008). In contrast to SS, the gas bubbles of LBF at the salt rich aqueous solution will also be surrounded with liquid film similar to the bubble shell. However, once the bubbles infiltrate and burst in the top organic solution, the water film may disperse immediately in the organic phase. This is because the top phase of the solute accumulator utilizes water soluble phase elements whereas in the usual SS, the top phase uses hydrophobic insoluble organic solvent (Lee et al., 2016).

In the LBF column, the gas bubbles are produced by the sparging of gas through the pores of sintered glass disk which is positioned bottom of the flotation column as shown in **Figure 4**. Normally, G4 porosity (5∼10 μm pore size) of sintered glass disk is utilized in the LBF system as it is able to produce improved separation effectiveness of biomolecules in contrast to bigger glass disk G3 porosity with a pore size of 20–30 μm or smaller sintered disk of G5 with pore size of 1–4 μm (Show et al., 2011, 2013b). For the bubbling of the flotation column, any inert gas will be able to generate bubbles but nitrogen gas is favoured as it is cheaper and easily obtainable. The efficiency of LBF is dependent on a few variables such as the type of phase forming components, molecular weight and concentration of phase forming components, size of gas bubbles, gas flow rate, flotation time and pH.

The type component for phase formation in LBF is similar to ATPS which is usually a two-polymer system or polymer/salt system. For ATPS system, the most frequently used components is PEG and dextran (Asenjo & Andrews, 2011; Ng et al., 2013). PEG is popular in ATPS system due to it being cheap, easily obtainable and the capability to form biphasic system with another polymer or salts

(Andrews, Head, Dunthorne, & Asenjo, 1990). A few LBF experiments were done utilizing PEG as the top phase for the target compound to be dissolved (P. Y. Bi, Dong, & Yuan, 2010; P. Y. Bi et al., 2009; M. Li & Dong, 2010; Md Sidek et al., 2016). The PEG molar mass is vital for LBF, as different molecular weight will have an effect on the concentration of aqueous salt phase which then will influence the two phase system. In an LBF study based on polyethylene glycol (PEG) and sodium citrate, it has been demonstrated that with the increase of PEG molecular weight the concentration ratio increased while the separation efficiency showed the opposite trend (Md Sidek et al., 2016). LBF is different from ATPS system because the volume of aqueous phase is more than that of PEG. Thus, the concentration of inorganic salt is the major aspect to maintain an immiscible two-phase system via a salting-out effect (P. Y. Bi et al., 2009).

Albeit with most LBF experiments done utilizing PEG as the top phase, it is not suitable to be used for large scale due to the high viscosity and it cannot be recycled and reused in downstream processing. As a result of these drawbacks of PEG, researchers are now focusing on using thermo separating copolymer of ethylene oxide and propylene oxide (EOPO) as polymer for phase formation. EOPO is a kind of important thermosensitive polymer and are water soluble. This copolymer can be thermoseparated into two phases at a temperature that is above a lower critical solution temperature (LCST) (Tubio, Nerli, Pic, Venncio, & Teixeira, 2009). Show et al. studied the recycling capability of phase components in LBF system using EOPO and ammonium sulphate salt for lipase separation from fermentation broth of *Burkholderia cepacia* strains ST8. They were able to recover EOPO up to 70% and achieve the average separation efficiency and purification fold of 76% and 13%, respectively. In addition, they demonstrated that there was

no major variance in purification fold, separation efficiency and recovery yield of lipase enzyme attained between LBF utilizing new and recycled chemicals (Show et al., 2011).

In addition to polymer/salt phase components, Show and colleagues investigated alcohol/salt LBF system. They used hydrophilic alcohol/salt consisting of 2-propanol and potassium phosphate to extract lipase from *Burkholderia*. From this study, they have obtained a separation efficiency of 90.2%, purification factor (P_{FT}) of 14.4 and 99.2% yield of lipase at the optimized process condition (Show et al., 2013b). The advantage of using alcohol/salt is that these phase forming components can be effortlessly recovered by removing aliphatic alcohols via straightforward evaporation method and furthermore, the evaporated alcohol can be recycled, thereby saving costs. Lately, researchers are interested to work with ionic liquid (IL) as it demonstrates its potential as a green substitute solvent for numerous enzymatic reactions (Van Rantwijk, Lau, & Sheldon, 2003). ILs have several beneficial properties such as having elevated thermal stability, high conductivity of electricity, high ionic conductivity, low vapour pressure, non-flammable, non-volatile, ability to tune immiscibility and has extreme range of liquid temperature (Kislik, 2012; S. Zhang, Sun, He, Lu, & Zhang, 2006). In the latest study, Han et al. utilized IL and organic salt in LBF system to separate and determined the concentration of chloramphenicol (CAP) in environment and food samples. They used imidazolium ionic liquid (1-butyl-3-methylimidazolium chloride, [C_4mim]Cl) and inorganic salt (K_2HPO_4) in their LBF system and achieved recovery of CAP with 97.1–101.9% from aqueous samples of environmental and food samples (Han et al., 2011). In another research of IL,

Wang and colleagues used [Bmim]BF4–(NH4)2SO4 to separate tetracycline (TC) and oxytetracycline (OTC) (Y. Wang, Xu, Han, & Yan, 2011).

Table 4 displays the applications of LBF. Most of the LBF applications utilize PEG as their polymer for compound extraction and a few used ionic liquid as it is environmentally friendly. In addition to biomolecules separation, LBF has also been applied to chiral separation (Zhuang, Yang, Chen, & Jiao, 2014). LBF is used for the H_2A enantioseparation composing PEG2000/$(NH_4)_2SO_4$ and achieved separation factor (α) and enantiomer excess (e.e.) of 1.99 and 23.49 %, respectively. LBF is new and constantly displays great potential in the biotechnology field. Various LBF systems have been successfully applied to deal with surface-active compounds in bioseparation and bioengineering. LBF contains many advantages, including better separation efficiency and enrichment coefficient, soft condition, simple operation, reduced dosage of organic and minimal impact on environment.

2.4. Sugaring-out effect

Sugaring-out is considered as a novel and less explored phase separation technique. It is similar to the salting-out effect where instead of salt, sugar is used for the partitioning in a two-phase system. Sugaring-out technique was first discovered by Wang et al., where they found an immiscible phase formation of acetonitrile (ACN) when monomeric sugar or disaccharide as a mass separating agent is added into an ACN–water solution (B. Wang, Ezejias, Feng, & Blaschek, 2008). From the study conducted, ACN is found to be partitioned to the upper phase while sugar to the bottom phase. The study concerning sugaring-out

comprises several system that includes acetonitrile/sugar (Dhamole et al., 2016; Dhamole, Mahajan, & Feng, 2010b; Tsai et al., 2010), propylene glycol/sugar (Tubtimdee & Shotipruk, 2011) and ethanol/sugar (Tubtimdee & Shotipruk, 2011).

Since this technique is relatively new there are few studies on sugaring-out method. This technique has been utilised in the recovery of proteins (Dhamole et al., 2010b), metal ions (C. Zhang, Huang, Yu, & Liu, 2012), antibiotics (Dhamole, Mahajan, & Feng, 2011), vanillin (De Brito Cardoso et al., 2013), sulphonamides (Tsai et al., 2010), podophyllotoxins and flavones (L. Zhang, Wang, & Wu, 2017), phycocyanin (Bachchhav, Kulkarni, & Ingale, 2017), para-coumaric acid (pCA) (Dhamole et al., 2016) and acetoin (Dai, Ma, Wang, Guan, & Xiu, 2016).

The sugaring-out method provides several advantages compared to salting-out including: (i) no alteration in the pH even with high concentration of where it could avoid protein denaturation and damage to product which cannot be avoided in high salt concentration, (ii) circumventing unwanted reaction which can be seen in salting-out (Jones, Prabel, Glennon, Copeland, & Kavlock, 1993) and (iii) preventing equipment corrosion (Leinonen, 1996). To date, sugaring-out extraction has only been utilised in ATPS, and the potential use of this technique in the integration with other method, for instance LBF is yet to be discovered. Given that sugaring-out is comparatively a new emerging technique that possesses many benefits compared to the conventional method, the former has a great potential to be explored intensely as an enhanced separation method. In general, LBF system utilizes the concept of salting-out effect for the separation. In this work, the concept of sugaring-out has been introduced for the extraction of protein from microalgae using LBF system.

2.5. Ultrasonication Assisted Extraction

Sonication is a process of utilizing sound energy to agitate particles in a sample. This method has been used for numerous purposes for example extraction of various compounds from plants, microalgae and seaweeds. Generally, ultrasonic frequencies waves of >20kHz will result in the agitation of a liquid sample (Ravanfar, Tamadon, & Niakousari, 2015). It is found that this ultrasound process which gives low frequency and high intensity is able to produce strong shear and mechanical forces. Ultrasound can be used in various applications by tuning the frequency.

Sonication with a frequency range from 20-40 kHz has been used widely for applications such as generating oil-in-water emulsions, molecular weight reduction in polymers, extraction of bioactive compounds and cleaning (Ashokkumar, 2015). Meanwhile, high frequency ultrasound ranging from 200 kHz to 1 MHz is frequently used in applications where a high amount of redox radicals is necessary (Ashokkumar, 2015). Recently, power ultrasound with 20-40 kHz that could provide acoustic cavitation has been established as a standard method in microbiology for cell disruption to release contents (Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017). Due to this, there is a great demand for using sonication process for appropriate extraction methods for extra efficient extraction. Chemat and colleagues have described that ultrasonication technology is a major breakthrough in accomplishing the aim of sustainable ‘‘green" chemistry and extraction (Chemat et al., 2017).

Currently, ultrasonication has been acknowledged extensively by the researchers as an effective technique for cell disruption and many studies have

been performed using this technique (Gerde, Montalbo-Lomboy, Yao, Grewell, & Wang, 2012; M. Wang, Yuan, Jiang, Jing, & Wang, 2014; Wu, Joyce, & Mason, 2011). The challenges with the existing protein extraction techniques include: they are laborious, time consuming and are economically unviable protein extraction processes especially for large-scale applications. High shear forces that are produced from cavitation bubbles of ultrasound help in the cell wall disruption for protein extraction (Dey & Rathod, 2013).

In the ultrasound assisted extraction method, acoustic cavitation is used to produce cavitation bubbles. This bubbles will implodes and contributes high shear forces that help in the cell wall disruption (Dey & Rathod, 2013). It is discovered that the probe system gives better result compared to ultrasonic bath. This is because probe system is able to distribute the ultrasonic intensity through a smaller surface compared to ultrasonic bath. Besides, probe ultrasound gives direct delivery of ultrasound in the media with minimum loss of ultrasonic energy (Chemat et al., 2017). There have been many efforts to develop low cost process with high concentration of extracted bioactive compounds. There have been studies reported of integration of ultrasound and soxhlet extraction. Soxhlet extraction applies leaching technique and is considered as conventional method and is mostly used for more than one century (Luque de Castro & García-Ayuso, 1998). Sono-Soxhlet method which consist the combination of sonication and soxhelt was developed by Luque de Castro and Chemat (Luque-García & Luque De Castro, 2004; Luque de Castro & García-Ayuso, 1998). This integration study shows advantages of enhanced mass transfer which reduces the time of extraction and accurate extraction of the samples.

In another separate study, the integration of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) was reported for the extraction of oils from vegetable sources; viz., soybean germ and a cultivated seaweed rich in docosahexaenoic acid (DHA) (Cravotto & Binello, 2010). It is found that this combination process is economical technique for fast sample preparation and an innovative strategy for process intensification. However, there is limited research on this integration process and the great potential of this method has not yet been effectively exploited (Chemat et al., 2017).

Balachandran and colleagues investigated the combination technique of ultrasound with supercritical fluid extraction (SFE) for soluble essences from Ginger. This integration technique enhances the mass transfer of the species of interest from the solid phase to the solvent used for extraction (Balachandran, Kentish, Mawson, & Ashokkumar, 2006). In spite of increased yield, there are several disadvantages of this technique to perform in large scale that includes the need for expensive equipment of SFE and the concern of extracting polar molecules without adding modifiers to CO_2 (Chemat et al., 2017). Ultrasound method is known to be sustainable, economical and environmentally friendly method. The design of an efficient ultrasound-assisted large-scale extraction requires process intensification and energy consumption reduction. The combination with the conventional methods such as microwave, soxhlet extraction and supercritical fluid are restricted for industrial scale mainly due to long operation time, complication in the equipment set-up for large scale, high process intensification and energy consumption and finally on the overall cost.

The choice of which method has to be used to execute extraction of a desired metabolite from a specific plant has to be a result of a compromise between the

efficiency and reproducibility of extraction, ease of procedure, together with considerations of cost, time, safety and degree of automation. In this study, the integration of ultrasound with the simple, environmentally friendly method with high separation efficiency of LBF has been proposed for the protein extraction. The ultrasound assisted extraction with this novel LBF method has not been studied. Due to the advantages of LBF method, this combination process could enhance the extraction process for improved downstream processing.

2.6. Microalgae

Algae also defined as thallophytes, are simple photosynthetic aquatic organisms that discovered in two different forms either in unicellular or multi-cellular forms. Previously, algae were considered as "aquatic plants" however, they are then categorized separately as they do not have stems, roots, embryos and vascular system as higher plants. Microalgae found abundantly in the natural environment due to its high survival and growth rate. In addition, they can even grow under harsh and inhospitable conditions. Through photosynthesis process, microalgae convert CO_2, water and sunlight to generate lipids, carbohydrates and proteins which make microalgae as primary producers for life on the earth (Ozkurt, 2009).

The market for microalgae biomass is expanding with the advent of new technologies in cultivating, handling and utilization of microalgae biomass. The major product of the microalgae biomass found as sun-dried, spray-dried powder or in the pastilles type to be directly use as nutraceuticals. Microalgae are also applied in wastewater treatment, biofuel production (including biodiesel, bioethanol, biobutanol, biohydrogen, biogas), food additives, feed and high value chemicals,

such as carotenoids, polyunsaturated fatty acids, oils, natural dyes, sugars and antioxidants (Borowitzka, 2013).

Microalgae are considered as prokaryotic photosynthetic microorganisms and they have been acknowledged as sustainable alternative protein sources due to the high protein content. Owing to their simple cell structure, they able to survive and grow rapidly in harsh environments (Dos Santos, Moreira, Kunigami, Aranda, & Teixeira, 2015). It has been found that the microalgae proteins contain various bio-functionalities that provide positive impacts not only to health but offer various functional applications in pharmaceuticals, cosmetic industry and nutraceuticals (Samarakoon & Jeon, 2012). It has been reported that protein is one of the main components in the biomass of microalgae other than carbohydrate and lipid (Blackburn & Volkman, 2012). The fact that microalgae can be cultivated both in outdoors or indoors and many research investigations have been focused on mass cultivation of microalgae for the extraction of protein. The major challenge in mass cultivation for the extraction of product is the downstream processing, which includes cell disruption and protein extraction (Phong, Le, Show, Chang, & Ling, 2016).

Cell disruption is considered as the most important process for higher protein extraction efficiency. There are two categories of cell disruption methods which are non-mechanical and mechanical. Non mechanical method comprises the addition of chemicals or enzymatic degradation, whereas mechanical approach includes ultrasonication, high-pressure homogenization, bead milling and others (Lee, Show, Ling, & Chang, 2017). Sonication method has several advantages over other techniques as mentioned earlier such as it requires less energy, does not require the

addition of beads, scalable to larger volume, able to operate continuously and could be able to disrupt a diverse range of algal species (M. Wang et al., 2014).

Since UAE has the advantage of efficient extraction technique due to shorter extraction time they have been used in several microalgae studies. Hadiyanto and partner utilized ultrasound to extract phycocyanin form microalgae *Spirulina platensis* (Hadiyanto & Suttrisnorhadi, 2016). They demonstrated with ultrasound assisted extraction, high yield of phycocyanin with 13.61% was obtained compared to conventional method with only 11.13%. In a latest study of microalgae protein, ultrasound was used for protein extraction from *Spirulina platensis.* It was found the optimized conditioned were temperature 45°C, pH 7.46 and time 120 min gave extraction yield (29.05%), total phenolic content (3.52 mg caffeic acid equivalent/g dw), antioxidant activity and in vitro protein digestibility (99.36%) (Aysun, Oznur, Ceren, Fatih, & Beraat, 2018). In another study, ultrasonication and aqueous solutions of ionic liquids (ILs) as extractive solvents was utilized as single process to extract protein from *Chlorella vulgaris* (Lee, Show, et al., 2017). The results show high protein recovery with (95.0 ± 1.9%) and demonstrated this technology as an efficient alternative for microalgae cell disruption and protein recovery in a single-step.

Despite the use of ultrasound is found to have significant enhancement in the extraction efficiency. However, till now, the ultrasound process is combined with the conventional methods which have disadvantages such as constraint for industrial scale mainly due to long operation time, complication in the equipment set-up for large scale, high process intensification and energy consumption and finally expensive overall cost. Therefore, further improvement with efficient process integration could enhance the protein extraction. In this study, the

application of ultrasound assisted LBF system with sugaring out effect is experimented for the enhancement in protein extraction from microalgae.

Chapter 3: Materials & Methodology

3. Experimental Methodology

This chapter will address on the methodologies employed in the project. This study focuses on the different applications of LBF for biomolecules separation. The mutual connection in this thesis is the approach of using LBF system. Hence, in this chapter four different applications of LBF will be comprehensively discussed. This LBF study is divided into four objectives in which the first three objectives are related to the extraction of lipase while the fourth is on protein extraction.

First objective is on the extraction of lipase using recycling alcohol/salt in large scale. The details on the materials and approach used are explained comprehensively. Since the first three objectives are related to lipase extraction the methodology of bacteria fermentation for lipase production is similar and will be reflected in this section.

Second objective is on the methodology of lipase extraction using surfactant/xylitol and the methods for the recycling study. Third objective is on the procedures for integration study of fermentation and separation of lipase. Materials and methods for the combined process and the optimizing study has been explained in detailed. Fourth & the last objective is on the methods employed for protein separation from microalgae using ultrasonication assisted LBF system.

Flow chart in **Figure 5** gives a brief illustration of the methods applied in each objective. Materials and methodology used in each objective will be discussed separately.

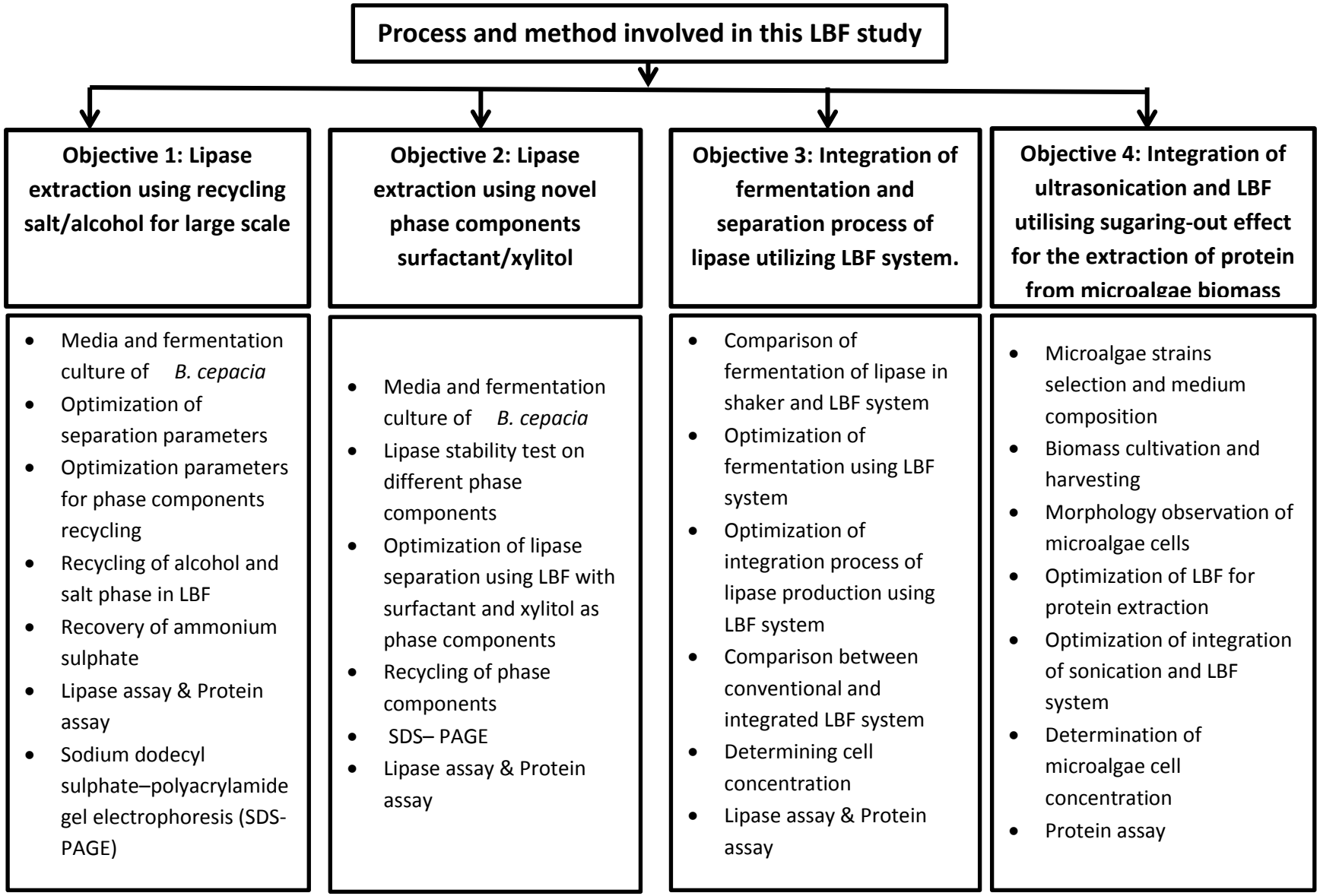


Figure 5: Flow chart describing the methods involve in each objective for this LBF stu

3.1. Objective 1: Lipase extraction using recycling salt/alcohol for large scale

This section will discuss on details on the apparatus, materials and methodology employed for the first objective of the thesis which is on the lipase extraction using recycling salt/alcohol for large scale. **Figure 6** shows a brief flowchart on the methodology used for this particular study.

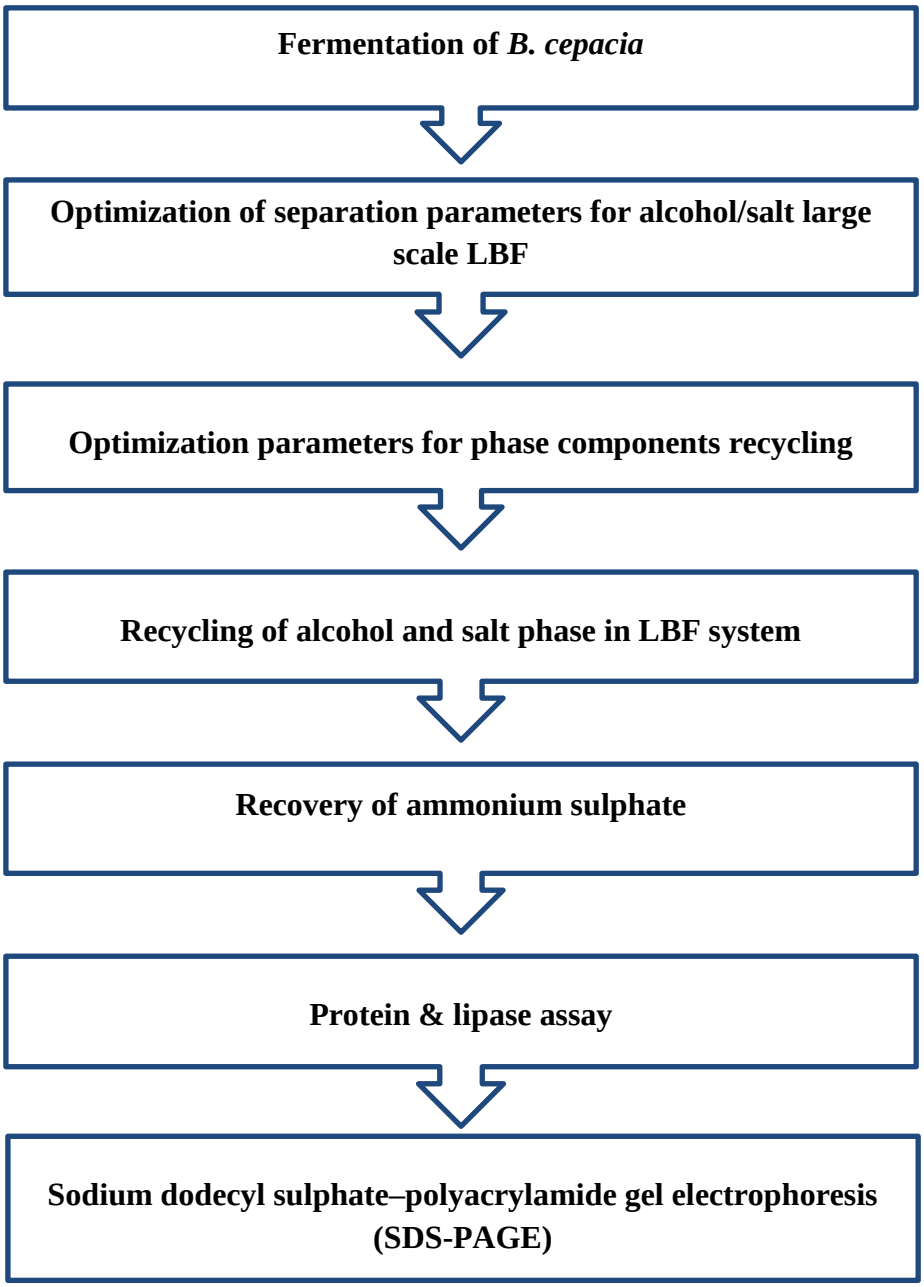


Figure 6: Flowchart illustrating the overall methodology for lipase extraction using recycling salt/alcohol for large scale.

3.1.1. Apparatus

In this study LBF glass column with largest size with a volume capacity of 2500 mL as shown in **Figure 7** were used. Based on previous literature of LBF design, preliminary test was conducted to obtain appropriate height and internal diameter for consistence bubbling effect (Mathiazakan et al., 2016). The design of the LBF apparatus was selected based on the preliminary test study that gave the best bubbling effects. The height of the glass column is 0.25 m and the internal diameter is 0.12 m. The bottom of the column is equipped with a sintered disk of G4 porosity (pore size approximately10 μm) attached to a compressed air system that is used to generate the air bubbles to the column. Based on literature, G4 porosity has demonstrated better separation efficiency of biomolecules as compared to sintered glass disk with bigger G3 (20–30 μm pore size) or smaller G5 (1–4 μm) porosity, correlated to the size of gas bubbles produced (Lee et al., 2016). Due to this G4 porosity is selected for this study.

The flowmeter used to measure the air flowrate and it is a rotameter (model: RMA-26-SSV) with a range of 0.5-5 LPM (Dwyer, USA) which is connected to the compressor after a pressure regulator set. The flow meter is fixed based on the capacity of the LBF system for producing air bubble. Preliminary study was done to obtain the ranges of flow meter to suit the LBF system.

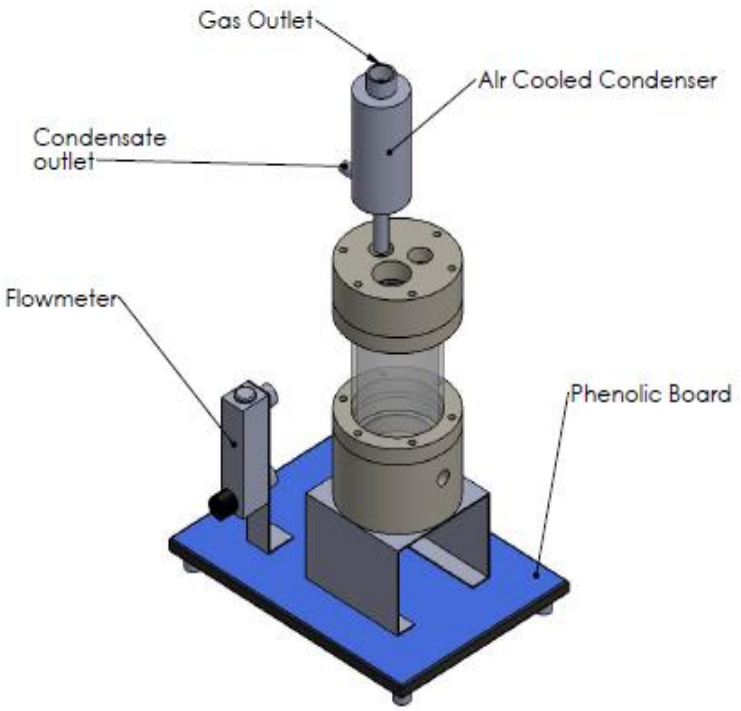


Figure 7: Schematic diagram of the large scale LBF system with 2500mL working volume capacity.

3.1.2. Materials

Nutrient broth, glucose, calcium chloride ($CaCl_2$), olive oil, and gum Arabic that serve as the fermentation medium were purchased from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide for the pH regulator, sodium carbonate (Na_2CO_3), with 4-nitrophenol (pNP) and 4-nitrophenyl dodecanonate (p-NPL) for lipase analysis were obtained from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA) and Bradford reagent for protein assay and standard curve was purchased from Sigma-Aldrich (St. Louis, USA). Ammonium sulphate [(NH4)2SO4], ethanol, methanol, 1-propanol, 2-propanol, butanol for the phase components study was bought from R&M Chemicals (United Kingdom).

3.1.3. Methodology

3.1.3.1. Media and fermentation culture of B. cepacia

B. cepacia strain ST8 which is a gram negative bacterium was used in this research for producing lipase. This strain is selected for this study due to advantages characteristics of lipase from this strain that are high thermal stability, high tolerance towards organic solvents and aliphatic alcohol which makes them suitable for this study (Mathiazakan et al., 2016). B. cepacia strains ST8 (a kind gift from Prof. Ling Tau Chan) obtained from Laboratory of Biological Science, Science Faculty, University of Malaya was used in this research for producing lipase.

For the inoculum, the bacteria were incubated in a nutrient broth 1 % (w/v) for 16 h. Fermentation was conducted in a 250 ml shake flask that consisted 150 ml of medium. Fermentation medium comprised of nutrient broth, 0.325 % (w/v); glucose, 1 % (w/v); CaCl2, 0.1 % (w/v); olive oil, 1 % (v/v); and gum arabic, 1 % (w/v). The pH of the solution is regulated to 7.0 by adding NaOH. The compositions of the medium are obtained from literature (Lee, Khoiroh, Ling, & Show, 2017b). Olive oil is added in the medium as a carbon source as it able to boost the lipase production (R. Sharma, Chisti, & Chand, 2001), whereas the addition of CaCl2 can increase and prolong the enzymatic activity for 30 days (Padilha, Santana, Alegre, & Tambourgi, 2012). 5 % (v/v) of the pre-grown inoculum was added to the fermentation medium and the culture was subjected to a shaking water bath with an agitation speed of 200 rpm at 37 °C. The agitation speed and fermentation temperature were chosen based from literature (Lee, Khoiroh, et al., 2017b). Water bath is used for the fermentation as it is able to give

uniform and constant temperature for the bacteria to grow. The fermented culture was collected for the LBF experiments after 72 h of incubation. Optimization study was conducted to obtain the optimized conditions for maximum lipase recovery.

3.1.3.2. Optimization of separation parameters for alcohol/salt large scale LBF

Following fermentation, crude lipase feedstock that was obtained will be transferred into the LBF system for the separation process. The research was performed in a series of batches. In this study, one variable at a time (OVAT) method was employed to explore the influences of the parameters on the retrieval of lipase from fermentation broth. OVAT was chosen in this study because it is the best method evaluates each parameter individually on the lipase partitioning. Besides, this is the first study on recycling using large scale, this study could serve as the basis for further inter and combined variable study.

Optimization of large scale LBF for lipase recovery was done previously (Mathiazakan et al., 2016). This study focused on recycling effects of alcohol/salt. Thus, initial study was done to obtain the optimized concentration of crude lipase feedstock and types of salt for maximum lipase recovery. After obtaining the optimized parameters, recycling study based on that will be conducted. The initial total volume of aqueous phase were 1500 mL, 50 % (w/w) of 1-propanol, 250 g/L ammonium sulphate [(NH4)2SO4], pH 6, 300 mL of alcohol phase, 20 % (w/w) of CL, 20 min of flotation time (FT) and 1.5 cc/min of flowrate. All the experiments were carried out thrice at room temperature.

3.1.3.3. Optimization parameters for phase components recycling

The parameters that are optimized for the recycling of phase components in order to obtain maximum lipase extraction are volume of crude lipase added in successive recycling, concentration of alcohol and types of alcohol. For each parameter, three cycles of extraction were done by reusing the same bottom aqueous phase while for the top phase after recycling for three times fresh alcohol was used. For the recycling study, initial conditions were 20 % of crude feedstock and 300 ml of 50% of 1-propanol of top phase.

Table 3 shows the experiments design for the recycling of phase components using LBF. After obtaining the optimized parameters, the salt from bottom phase was recovered from previous experiments. **Figure 8** illustrates better clarity of the recycling process that was carried out. Flowchart in **Figure 9**, briefly describes the methodology that was carried out. The flowchart contains all the factors and measurement that was used for the recycling.

Table 3: Arrangement order for the recycling of phase components using LBF

Parameters		1st Extraction	Recycling 1	Recycling 2
Volume of crude lipase added (mL)	25	Tp: F	Tp: R_c	Tp: R_c
		Bp: F	Bp: R_u	Bp: R_u
	50	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	75	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	100	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
Concentration of alcohol % (w/w)	50	Tp: F	Tp: R_c	Tp: R_c
		Bp: F	Bp: R_u	Bp: R_u
	75	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	85	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	100	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u

Types of alcohol	1-propanol	Tp: F	Tp: R_c	Tp: R_c
		Bp: F	Bp: R_u	Bp: R_u
	2-propanol	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	Ethanol	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u
	Methanol	Tp: F	Tp: R_c	Tp: R_c
		Bp: R_{u*}	Bp: R_u	Bp: R_u

Tp Top phase; Bp Bottom phase; F Fresh; R_c : Recycle from previous extraction system; R_{u*}: Reuse from previous extraction with addition of pure crude; R_u: Reuse from previous extraction system. The system was made with 1.5 L bottom phase with 300mL of initial volume of crude and 300ml of top phase. The initial concentration of alcohol 1-propanol is 50%. The flotation time for the experiment was 20 minutes with 1.5 cc/min flow rate of air.

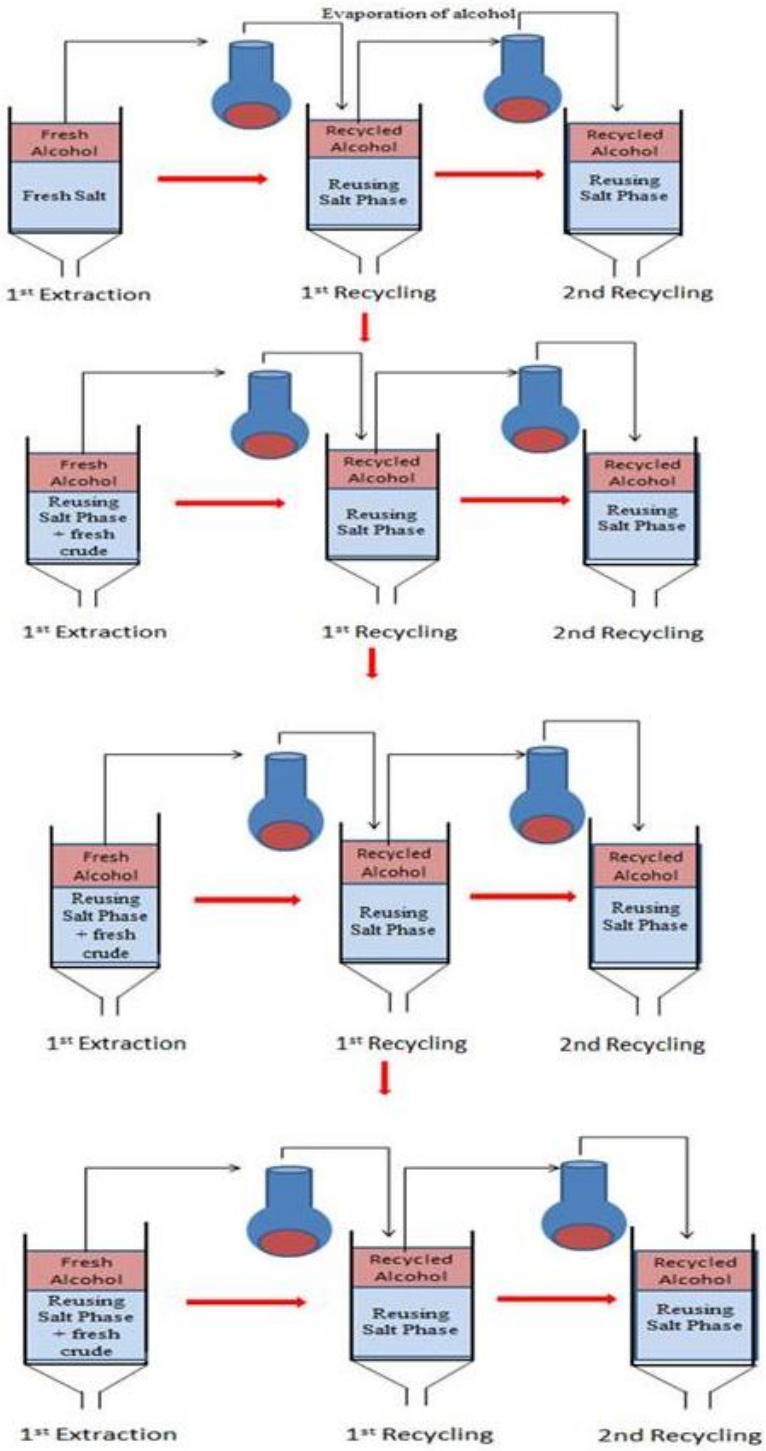
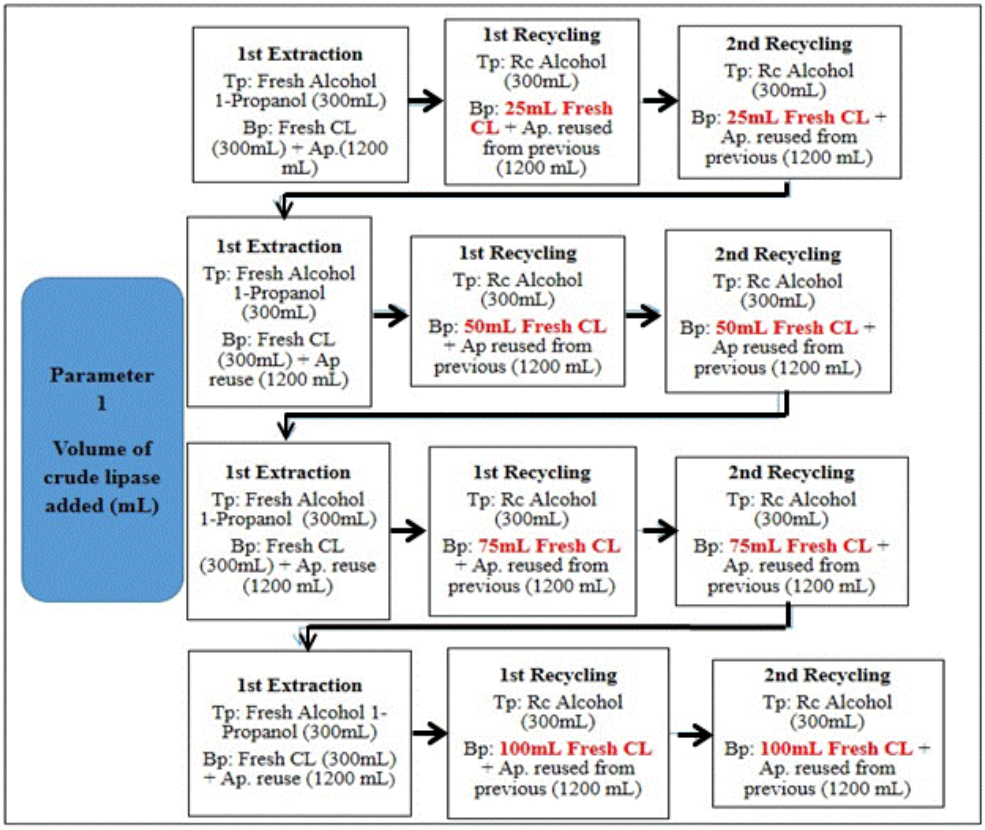


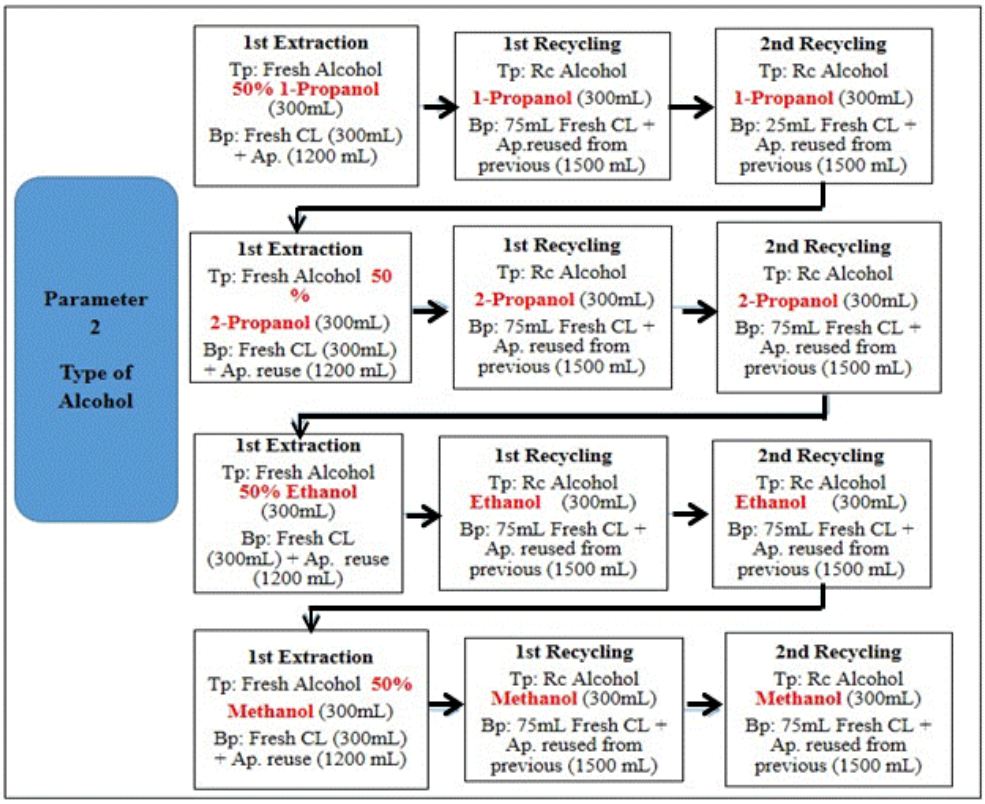
Figure 8: Figure above illustrates the experiment process for the recycling of phase components.

The salt phase was reused by maintaining the concentration using conductivity meter. Alcohol is recycled using evaporation method. The working volume of LBF

involved 1.5 L of bottom phase while for alcohol phase 300 ml was used. Each time for first extraction, the volume of salt is adjusted to 1.5 L and 20 % w/w of fresh crude feedstock will be added into the salt phase obtained from previous extraction system for recycling. The 1st row indicates for first value of the parameter for instance amount of crude lipase 25 mL, the 2nd line is for the next value which is 50 mL, 3rd row is for 75 mL and the 4th is for 100 mL. For each parameter, 4 sets of recycling will be done whereby 11 times of recycling of the bottom phase is carried out.

Figure 9 displays the flowchart of the experiment design for recycling phase components. This flowchart describes briefly the parameters and measurement applied in each step of the process. For each parameter, the aqueous phase was recycled 11 times, while the alcohol top phase was recycled twice for each set of value. The system was made with 1.5 L bottom phase with 300mL of initial volume of crude and 300ml of top phase. The initial concentration of alcohol used is 1-propanol and the concentration used is 50% w/w. The flotation time for the experiment was 20 minutes with 1.5 cc/min flow rate of air. Following the separation process, collected sample from top and bottom phase will be used for protein and lipase assay.


Parameter 1
Volume of crude lipase added (mL)
1st Extraction
Tp: Fresh Alcohol 1-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap.(1200 mL)
1st Recycling
Tp: Rc Alcohol (300mL)
Bp: 25mL Fresh CL + Ap. reused from previous (1200 mL)
2nd Recycling
Tp: Rc Alcohol (300mL)
Bp: 25mL Fresh CL + Ap. reused from previous (1200 mL)
1st Extraction
Tp: Fresh Alcohol 1-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol (300mL)
Bp: 50mL Fresh CL + Ap reused from previous (1200 mL)
2nd Recycling
Tp: Rc Alcohol (300mL)
Bp: 50mL Fresh CL + Ap reused from previous (1200 mL)
1st Extraction
Tp: Fresh Alcohol 1-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap. reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1200 mL)
2nd Recycling
Tp: Rc Alcohol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1200 mL)
1st Extraction
Tp: Fresh Alcohol 1-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap. reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol (300mL)
Bp: 100mL Fresh CL + Ap. reused from previous (1200 mL)
2nd Recycling
Tp: Rc Alcohol (300mL)
Bp: 100mL Fresh CL + Ap. reused from previous (1200 mL)


Parameter 2
Type of Alcohol
1st Extraction
Tp: Fresh Alcohol 50% 1-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap. (1200 mL)
1st Recycling
Tp: Rc Alcohol 1-Propanol (300mL)
Bp: 75mL Fresh CL + Ap.reused from previous (1500 mL)
2nd Recycling
Tp: Rc Alcohol 1-Propanol (300mL)
Bp: 25mL Fresh CL + Ap. reused from previous (1500 mL)
1st Extraction
Tp: Fresh Alcohol 50 % 2-Propanol (300mL)
Bp: Fresh CL (300mL) + Ap. reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol 2-Propanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)
2nd Recycling
Tp: Rc Alcohol 2-Propanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)
1st Extraction
Tp: Fresh Alcohol 50% Ethanol (300mL)
Bp: Fresh CL (300mL) + Ap. reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol Ethanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)
2nd Recycling
Tp: Rc Alcohol Ethanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)
1st Extraction
Tp: Fresh Alcohol 50% Methanol (300mL)
Bp: Fresh CL (300mL) + Ap. reuse (1200 mL)
1st Recycling
Tp: Rc Alcohol Methanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)
2nd Recycling
Tp: Rc Alcohol Methanol (300mL)
Bp: 75mL Fresh CL + Ap. reused from previous (1500 mL)

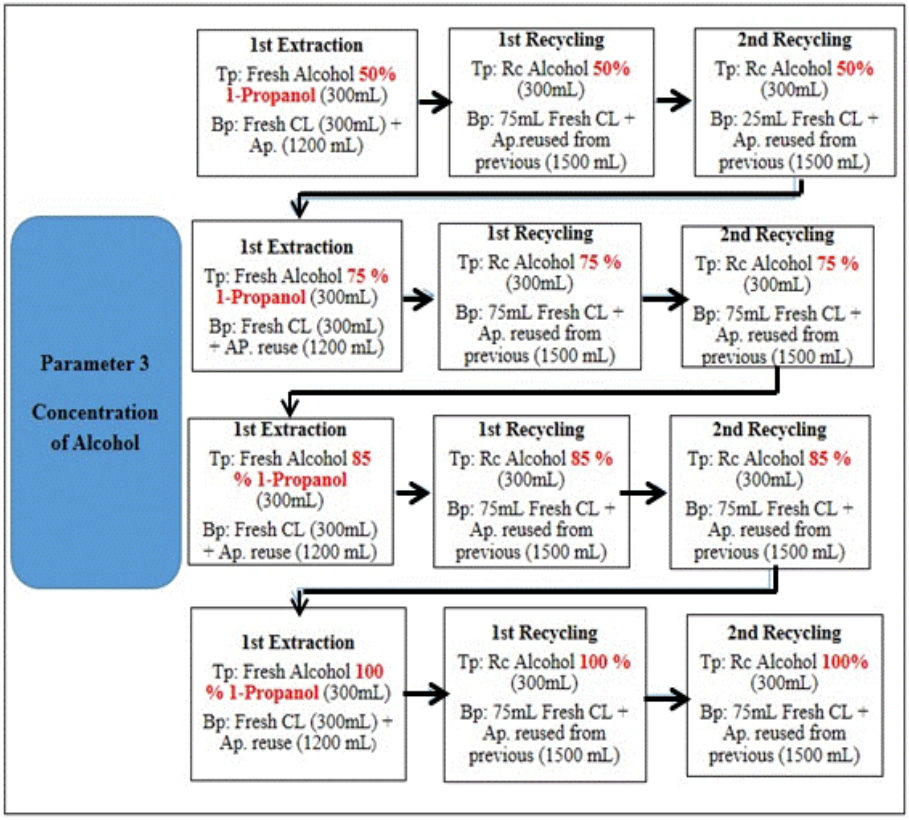


Figure 9: Flowchart of the recycling process. Tp: Top phase; Bp: Bottom phase; Rc: Recycle from previous extraction system; Ap: Aqueous phase

3.1.3.4. Recycling of alcohol and salt phase in LBF

For the first extraction, primary system was made up of 50 % (w/w) 1-propanol, 20 % (w/w) C_L and 250 g/L of ammonium sulphate [(NH4)2SO4]. After the first extraction of LBF, the top hydrophilic organic solvent-rich phase was separated from LBF and was subjected to evaporation in vacuum rotary evaporator for 30 min (200 rpm) at 60 °C. The next two extractions of lipase were conducted by using the evaporated alcohol while the bottom phase was reused from the previous systems. The concentration of salt in bottom aqueous phase was measured by using a conductivity meter and was maintained by adding appropriate amount of crude lipase and salts to sustain the concentration for two phase formation.

3.1.3.5. Recovery of ammonium sulphate

Ammonium sulphate from the bottom phase was recovered by adapting method described by (Z. Li, Teng, & Xiu, 2010). After extraction of lipase using LBF system containing 250 g/L of ammonium sulphate in bottom phase, methanol (2 times the volume of bottom phase) was added to the bottom phase to precipitate the ammonium sulphate. This was then subjected for filtration to recover the crystallized ammonium sulphate. The crystallised salt was then reused for the LBF system as the bottom phase for lipase extraction.

3.1.3.6. Protein assay

Sample that was collected from top and bottom phase were analysed for protein and lipase assay. The concentration of soluble proteins was determined by Bradford method using BSA as the standard protein (Bradford, 1976). A volume of 0.25 ml of protein sample extracted was mixed with 2.5 ml of Bradford reagent. The reading was determined at a wavelength of 595 nm using the UV-Vis spectrophotometer. The absorbance reading obtained was converted to protein concentration by using a standard calibration curve that was established using a standard protein, bovine serum albumin (BSA).

3.1.3.7. Lipase assay

Lipase activity assay was performed by applying spectrophotometric method described by (Ooi et al., 2011) with minor modifications. The activity of lipase was measured using a UV-vis spectrophotometer (model UV-1800, Shimadzu, Japan) at room temperature and the absorbance was taken at 405 nm after 30 min of reaction time. The quantity of 4-nitrophenol (p-NP) emitted from

the 4-nitrophenyl dodecanonate (p-NPL) hydrolysis was calculated via the standard curve of p-NP prepared previously. One unit (U) of lipase activity denotes to the volume of lipase needed for releasing 1 μmol of p-NP per min.

3.1.3.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS– PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was conducted using 4.5% stacking gel and 12% resolving gel. SDS PAGE was used to analyse samples from crude extract, samples extracted using recycled phase components top and bottom phases in the LBF. Stacking gel consist of 0.63mL 1M Tris pH 6.8, 0.05mL 20% SDS, 0.083mL 40% Acrylamide, 3.4mL distilled water, 50 μL 10% Ammonium persulfate and 5 μL TEMED. Resolving gel of 12 % comprises of 3.75mL 1M Tris pH 8.8, 0.05mL 20% SDS, 3mL 40% Acrylamide, 3.2mL distilled water, 100μL 10% Ammonium persulfate and 10μL TEMED. Gel plate was set up before the gel solution was mixed. The resolving gel was poured into the plate followed with 1mL of 70% ethanol. Ethanol was added to create a smooth top of the gel and to prevent oxygen from interfering with the polymerization reaction. The gel was allowed to polymerize for 30-60 minutes. Once the resolving gel polymerises, stacking gel mixture was poured gently on top of the resolving gel (using a pasteur pipette). Comb was inserted into the plate and was left to solidify. Once solidified, the binding clips were removed from the plate and the comb was removed carefully from gel. The glass plate was then placed sandwiched into electrophoresis core. 1X Electrophoresis buffer was added to the core. Buffer should be added to the top of the assembly. 1 – 2 inches of 1X Electrophoresis buffer was added to the running tank.

For the protein sample preparation, protein samples were thawed rapidly in room temperature water bath. About 200 µL of 6X Sample Buffer was added into protein samples and the samples were heated for 5-10 minutes in the 95^{O}C dry bath. 25 µL of sample was loaded into wells using 200 µL pipet tip. Electrodes were attached so that proteins will move towards the anode (+ or red lead). The gel was run at 100-200 V until dye front reaches the bottom of the gel. For the staining procedure, electrodes were disconnected, electrophoresis buffer was poured out and sandwiched gel was removed from tank. Staining solution was poured into pipet tip box. The plate with gel was inverted into the staining solution and gently allowed the gel to "float" off of plate into solution. Gently the gel was agitated on gel rocker for 15-30 minutes. Staining solution was removed and gel was rinsed with distilled water to remove excess stain. Water was added to the pipet tip box and gently washed over gel surface. Distilled water was used for distaining and the gel was agitated on gel rocker for 10-15 minutes. Protein bands were visualized by imaging systems. The SDS–PAGE technique has been explained in the past (Show et al., 2011).

3.1.3.9. Analytical methods: Calculation of separation efficiency (E), purification factor (P_{FT}), selectivity (S), lipase yield (Y), and recovery of solvent (alcohol) (R_{solv}).

There are several formulas that were used to calculate the separation efficiency, purification factor, selectivity, lipase yield and recovery of alcohol. This section will introduce the formulas that were used in this study.

The separation efficiency (E) which defines the concentration of lipase/protein being recovered in alcohol phase was calculated using Eq (1):

$$E = \left[1 - \frac{C_w}{C_{wi}}\right] \times 100\ \% \quad (1)$$

where C_w signifies the concentration of lipase/protein in the bottom phase at time t, while C_{wi} represents the initial concentration of lipase/protein in aqueous phase.

Purification factor (P_{FT}) was defined as the ratio of specific activity (SA) of lipase in the top alcohol phase to the lipase in the crude feedstock, as in Eq. (2):

$$P_{FT} = \frac{SA\ of\ top\ phase}{SA\ crude\ feedtock} \quad (2)$$

SA represents the ratio of enzyme activity of lipase to the protein concentration as shown in Eq. (3)

$$SA = \frac{Enzyme\ activity\ (U)}{Protein\ (mg)} \quad (3)$$

Selectivity (S) was expressed by using the ratio of the enzyme partition coefficient (K_e) to the protein partition coefficient (K_p) as in the Eq. (4)

$$S = \frac{K_e}{K_p} = \frac{E_T}{E_B} \times \frac{P_B}{P_T} \quad (4)$$

P_T and P_B denote the protein concentrations (mg/mL) at the top and bottom phases, correspondingly whereas E_T and E_B signify the lipase concentrations (U) at the top and bottom phases, respectively.

The yield of lipase in the top organic phase after extraction was calculated using Eq. (5)

$$Y\ (\%) = \frac{Lipase\ activity\ in\ top\ phase}{Lipase\ activity\ of\ crude\ feedstock} \times 100\ \% \quad (5)$$

Recovery of solvent (alcohol) (R_{solv}) is expressed as Eq. (6):

$$R_{solv} = \left[\frac{V_{solv}}{V_{isolv}}\right] \times 100\ \% \qquad (6)$$

where V_{solv} is the volume of alcohol recovered from the evaporation process, V_{isolv} is the total volume of alcohol used in the hydrophilic organic solvent phase of LBF.

3.2. Objective 2: Lipase extraction using novel phase components surfactant/xylitol

This section will discuss on details on the apparatus, materials and methodology employed for the second objective of the thesis. This objective is on the lipase extraction using novel phase components comprising surfactant and xylitol and studying on the recycling ability. **Figure 10** shows a brief flowchart on the methodology used for this particular study.

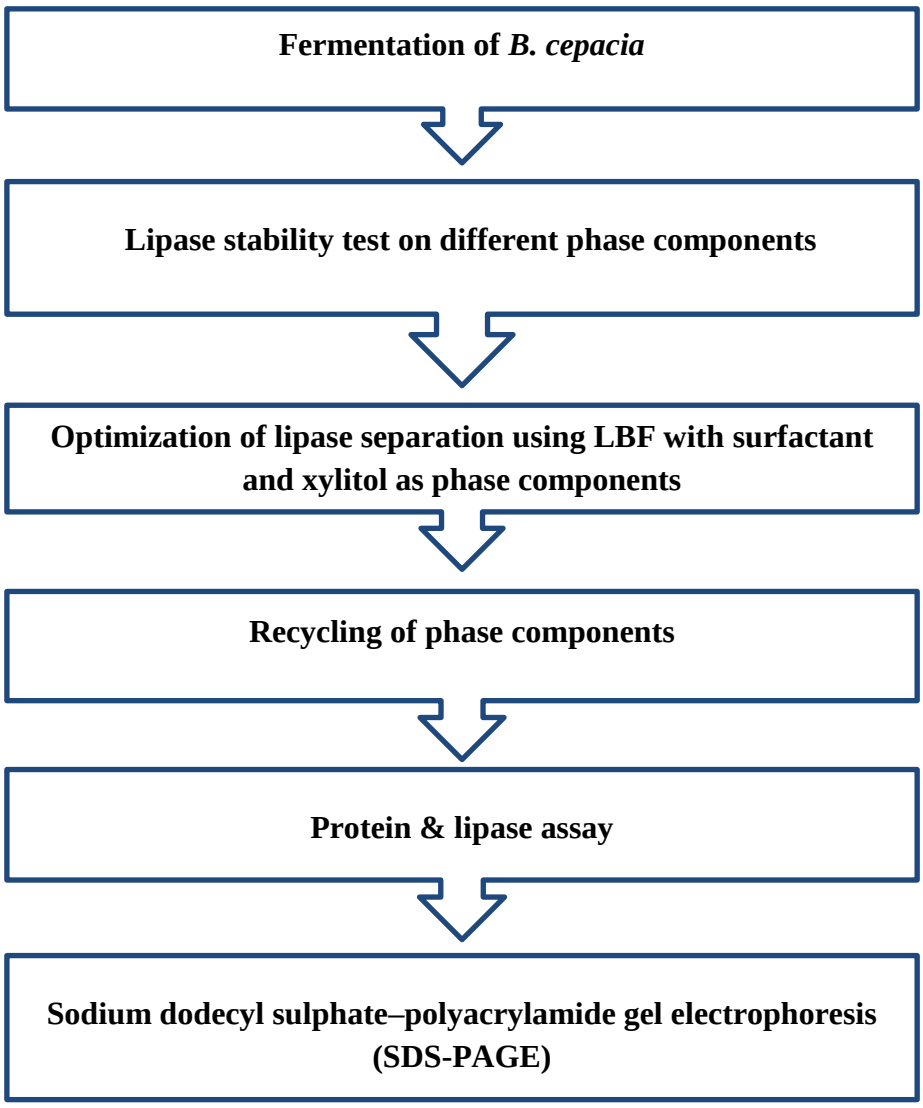


Figure 10: Flowchart describing the methodology used in the lipase extraction using novel phase components surfactant/xylitol

3.2.1. Apparatus

In this study two different LBF glass column with varying sizes were used. The design of the LBF apparatus was selected based on the preliminary test study that gave the best bubbling effects. The smallest LBF system consists of a glass column of with thickness of 0.28 cm, 2.4cm inside diameter, 25 cm height and volume capacity of 50 mL as shown in **Figure 11**. The medium size LBF glass column is consisting of 7 cm inside diameter, 15 cm height and volume capacity of 500 mL as shown in **Figure 12**. The schematic set-up of the system is shown in **Figure 13**.

There are two different flowmeters used to measure the air flowrate and it is a rotameter (model: RMA-26-SSV) with a range of 10-50 cc/min, 25-250 cc/min and 0.5-5 LPM (Dwyer, USA) which is connected to the compressor after a pressure regulator set. The flow meter is fixed based on the capacity of the LBF system for producing air bubble. Preliminary study was done to obtain the ranges of flow meter to suit each of the LBF system. Small LBF system requires small range flow meter with 10-50 cc/min whereas bigger LBF system requires larger range flow meter 25-250 cc/min.

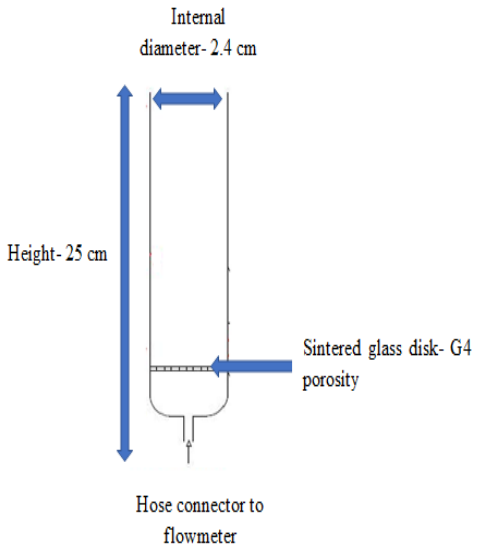


Figure 11: Schematic diagram of small scale LBF column.

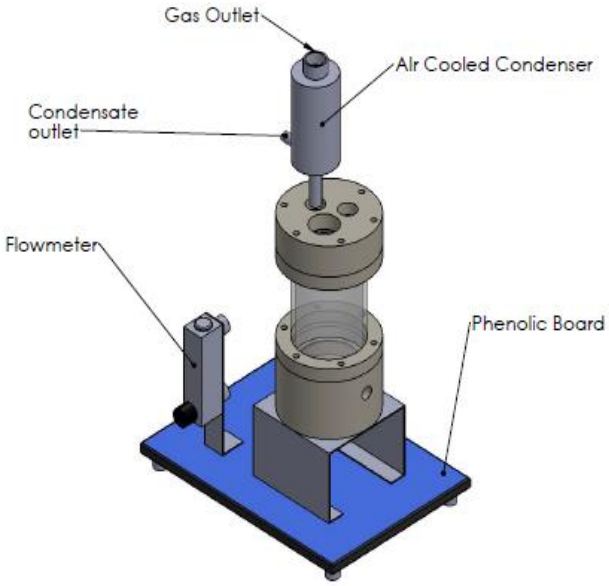


Figure 12: Schematic diagram of the medium scale LBF system with 500mL working volume capacity.

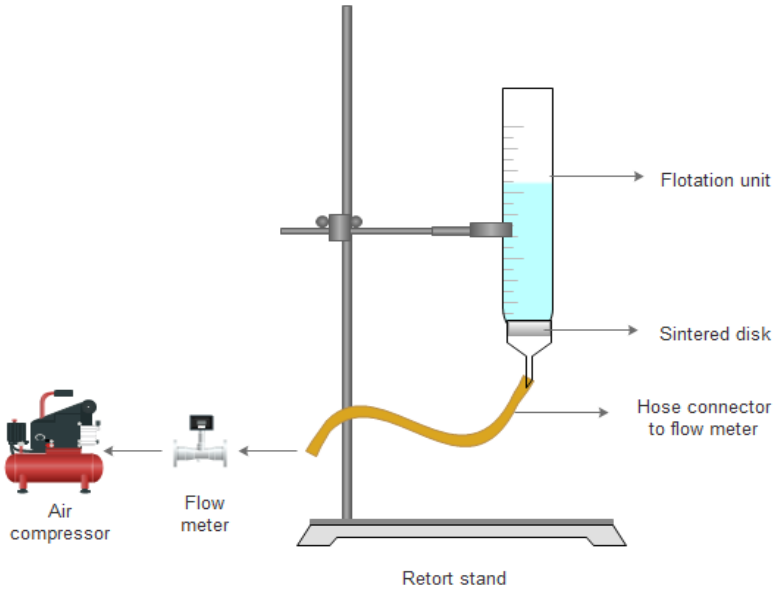


Figure 13: The schematic diagram of the set- up of LBF apparatus.

3.2.2. Materials

Nutrient broth, glucose, calcium chloride (CaCl2), olive oil, and gum Arabic as the fermentation medium were purchased from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide for the pH regulator, sodium carbonate (Na2CO3), with 4-nitrophenol (pNP) and 4-nitrophenyl dodecanonate (p-NPL) for lipase study were obtained from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA) and Bradford reagent for protein assay and standard curve was purchased from Sigma-Aldrich (St. Louis, USA). Triton X-100, Tween-20, Tween 80, Sodium dodecyl sulphate (SDS) and xylitol which are the phase components for the separation process were obtained from R&M Chemicals (United Kingdom).

3.2.3. Methodology

The methodology begins with the fermentation of *B cepacia* for lipase production. This method is similar to the previous objective and has been explained previously

in section 3.1.3.1. Succeeding the lipase fermentation, lipase stability test with the phase components were conducted.

3.2.3.1. Lipase stability test on different phase components

This is to study on the influence of phase components which are Triton X-100, Tween-20, Tween 80, SDS and Xylitol on the lipase stability. 1 mL of crude lipase was mixed with 1 mL of phase components at different concentration. The mixture was incubated for 1 h at room temperature. Control was run under similar conditions without the presence of phase components. The residual lipase activity in each sample was calculated with respect to control as 100%.

3.2.3.2. Optimization of lipase separation using LBF with surfactant and xylitol as phase components

This study was performed in a series of batches. The LBF parameters [concentration of xylitol, type of surfactant, concentration of surfactant, concentration of feedstock, volume of top phase, volume of bottom phase, pH and time of flotation] were optimized for highest lipase separation. In this research, one variable at a time (OVAT) method was employed to discover the influences of the factors on the lipase retrieval from fermentation broth. The initial total volume of the system was 33 mL which comprised of 30 mL bottom phase, 15% (w/w) xylitol, 3 mL of 30% (w/w) triton X-100 and 100% (w/w) of crude feedstock. This value was chosen based on the preliminary study done. From the preliminary study, the best conditions were selected for maximum lipase yield. The initial pH of the medium is 4. As for the large scale, total volume used was 165 mL in which bottom phase comprised of 150 mL and top phase with 15 mL of the optimized conditions. The experimentation was operated at room temperature and the initial

timing for LBF run was 15 minutes. Air flow rate was initially set at 25 cc/min. The flow rate for the large scale was fixed at 100 cc/min. The compressed air supply system was maintained at 0.5 bar and the air was filtered using a sterile air filter with pore size of 0.2 micron and was installed at the neck of the flotation tank. The experiments were expressed as the average of triplicate readings.

3.2.3.3. Recycling of phase components

The study on the recycling effect was done by recycling phase forming components. Xylitol which is from bottom phase of primary LBF was collected and recycled in successive LBF study. The concentration of top and bottom phase from initial and subsequent LBF was measured and maintained in each cycle of LBF. The top phase surfactant rich primary cycle of LBF was collected and diluted with distilled water using ratio 1:1. Diluted top phase was then placed in water bath for 30 min at a temperature of 65 °C to induce thermoseparation. After 30 min incubation, the top phase water contains anticipated enzyme and concentrated surfactant observed in bottom phase (Amid, Manap, Hussin, & Mustafa, 2015).

Top phase containing desired enzyme was utilized for analysing total protein concentration and lipase assay. Whereas for bottom phase containing concentrated surfactant, the volume of surfactant recollected was evaluated and recorded. Subsequent stage was to measure the bottom phase of LBF system which comprises xylitol. Volume of xylitol recovered was recorded and calculated. The recycling steps were reiterated for 5 times. Further recycling showed decreased in the lipase yield and separation efficiency. Thus, up to five times of recycling was conducted. By using a refractometer, refractive index of surfactant concentration recovered and xylitol concentration were measured.

Samples from the top and bottom phase were collected and was analysed for protein and lipase concentration. These methods were explained in the previous section 3.1.3.6 and 3.1.3.7 and will not be repeated in this section. Next step after protein and lipase assay is on the confirmation test of the extracted lipase. SDS-PAGE was conducted to confirm the lipase is extracted and present in the top phase of the LBF system. This method is also has been explained in the earlier section 3.1.3.8.

3.2.3.4. Analytical method: Calculation of separation efficiency (E), purification factor (PF), selectivity (S), lipase yield (Y), recovery of surfactant (R_{surf}) and recovery of xylitol ($R_{xylitol}$).

The calculation for lipase separation efficiency, purification factor, selectivity and yield has been described earlier in section 3.1.3.9. In this section the formula for surfactant recovery and xylitol recovery will be explained.

Formula to calculate recovered surfactant is shown in Equation (1) below:

$$R_{surf} = \left[\frac{V_{surf}}{V_{isurf}}\right] \times 100\ \% \qquad (6)$$

where V_{surf} is the volume of surfactant recovered from recycling process, V_{isurf} is the total volume of surfactant used in the initial process of LBF.

Xylitol recovery was defined as:

$$R_{xylitol} = \left[\frac{V_{xylitol}}{V_{ixylitol}}\right] \times 100\ \% \qquad (7)$$

where $V_{xylitol}$ is the volume of xylitol recovered from recycling process, whereas $V_{ixylitol}$ is the total volume of xylitol used in the initial process.

3.3. Objective 3: Integration of fermentation and separation process of lipase utilizing LBF system

This segment discusses on the third application of LBF system. The method involves for integration of fermentation and separation using LBF for lipase will be covered in this part. Flow chart below elucidates an overall depiction of the processes involved in this objective.

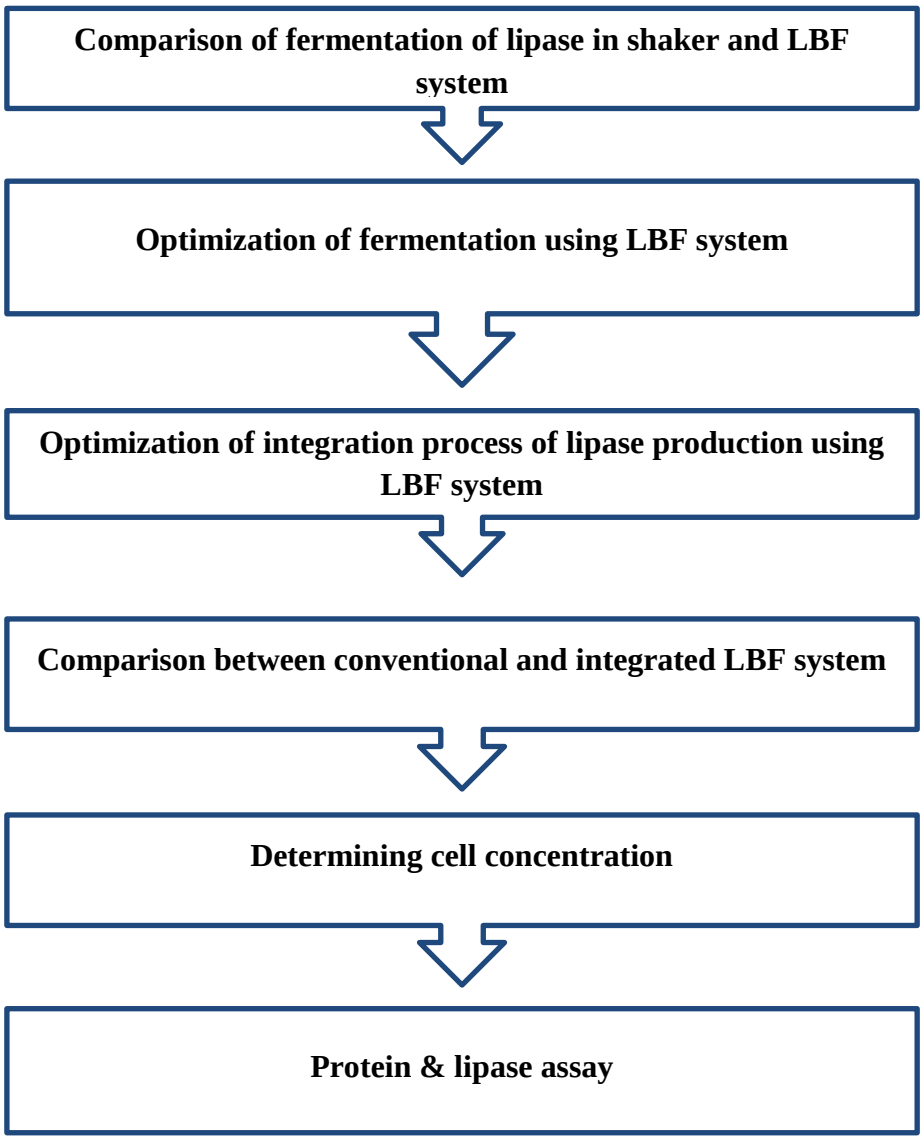


Figure 14: Flow chart describing the methodology employed in the integration study for lipase recovery.

3.3.1. Apparatus

In this study two different LBF glass column with varying sizes were used. The medium size of LBF with the working volume of 500 mL and the largest size of LBF with the working volume of 2.5L were utilized in this study. The medium size LBF glass column is consisting of 7 cm inside diameter, 15 cm height and volume capacity of 500 mL as shown in **Figure 12**. The optimization study was conducted using the medium scale LBF. The large scale LBF shown in **Figure 7** was used to study on the consistency and the reliability of the integration process. Two different flow meter were used (model: RMA-26-SSV) with a range of 25-250 cc/min and 0.5-5 LPM (Dwyer, USA). The integration of the fermentation and separation was done in this single LBF system.

3.3.2. Materials

Nutrient broth, glucose, calcium chloride (CaCl2), olive oil, and gum Arabic as the fermentation medium were purchased from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide for the pH regulator, sodium carbonate (Na2CO3), with 4-nitrophenol (pNP) and 4-nitrophenyl dodecanonate (p-NPL) for lipase study were obtained from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA) and Bradford reagent for protein assay and standard curve was purchased from Sigma-Aldrich (St. Louis, USA). Salts that were used as the phase components such ammonium sulphate [(NH4)2SO4], trisodium citrate [$Na_3C_6H_5O_7$], magnesium sulphate [$MgSO_4$], di-potassium hydrogen phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4), and the alcohols that were used for the top phase components such as ethanol, methanol, 1-propanol, 2-propanol, butanol, acetonitrile were purchased from R &M Chemicals (United Kigdom).

3.3.3. Methodology

3.3.3.1. Comparison of fermentation of lipase in shaker and LBF system

This study compares the fermentation process done between two different systems: the conventional method using shaker and the novel method of using LBF system. Fermentation medium for this study is similar to the previously described method in the section 3.1.3.1. Shake flask fermentation was subjected to a shaking water bath with an agitation speed of 200 rpm at room temperature for 96 h (Lee, Khoiroh, et al., 2017b). As for fermentation in LBF system, the culture was subjected to bubble flotation with the air flowrate 100 cc/min at room temperature for 96 h. The air flow rate with 100 cc/min was selected based on the preliminary study done. From the study, it was exhibited that 100 cc/min gave maximum growth of bacteria and lipase production. Samples were collected from both systems at appropriate time interval (6 hours and 12 hours) to obtain cell growth and lipase activity. The cell growth and lipase production obtained from the fermentation in conventional shaker and LBF system were compared.

3.3.3.2. Optimization of fermentation using LBF system

This study was done in a series of batches. One variable at a time (OVAT) method was employed to explore the influences of the parameters on the bacteria growth and lipase production. Optimization of glucose concentration and air flow rate of the flotation system were performed. The initial volume of fermentation medium was 150 mL, glucose concentration of 1 % and the air flow rate was initially set at 100 cc/min. The compressed air supply system was maintained at 0.5 bar and the air was filtered using a sterile air filter with standard pore size of 0.2

micron and was fit to the neck of the flotation tank. 0.5 bar of compressed air gave the best flow rate of air with suitable size of bubbles for the fermentation. Higher air supply causes high flow rate of air which then causes irregular bubble size and vigorous aeration in the system that eventually resulting foam formation in the LBF system.

3.3.3.3. Optimization of integration process of lipase production using LBF system

Fermentation and extraction of lipase was conducted as a single and continuous process. Instantaneously after 96 h of fermentation in the flotation unit, salt and alcohol were added into the system for two-phase formation for lipase extraction. Salt was added to the flotation system and mixed, subsequently alcohol was added to form two-phases. The LBF parameters, including the concentration of crude feedstock, the type of salt, the concentration of salt, the type of alcohol, the concentration of alcohol, the volume of bottom and top phase, the air flow rate and the flotation time, were optimized for highest separation and purification of lipase. The total volume of phase separation process was 290 mL, aqueous phase was 250 mL, 40 mL of alcohol phase, 50% (w/w) of 1-propanol, and 250 g/L ammonium sulphate was used as the initial parameters. The initial concentration of crude lipase (CL) was 40% (w/w) and 50 cc/min of air flowrate was used for the separation process. The initial conditions were selected based on the preliminary studies done for maximum lipase separation. The experiment was carried out at room temperature and the time interval for lipase extraction using flotation was set at 15 min. The samples from top and bottom phases were collected at the end of the experiment. The recovered lipase was found at the top alcohol phase, the alcohol can be easily removed from the solution by rotary evaporation.

3.3.3.4. Comparison between conventional and integrated LBF system

For the comparison study, conventional method of fermentation was performed in shaker and after 24 h the crude was introduced in the LBF system for separation process. While for the integration method, fermentation was performed in the LBF system for 24 h and subsequent separation process was done as continuous process. Both systems used similar parameters for separation process. Both systems were compared using optimized conditions which were 40 % (w/w) crude lipase concentration, 300 mL of 300g/L of ammonium sulphate, 30 mL of 80 % 1-propanol and 100 cc/min of flow rate with 30 min of flotation.

3.3.3.5. Cell concentration measurement

Cell concentration was quantified by measuring an absorbance at 600 nm using a UV-vis spectrophotometer (model UV-1800, Shimadzu, Japan) at room temperature. The results were expressed as a mean of triplicate readings.

Lipase that was collected from the LBF system was then analysed for the protein and lipase concentration. The methods for protein and lipase assay have been described in the section 3.1.3.6 and 3.1.3.7.

3.3.3.6. Analytical methods: Calculation of separation efficiency (E), purification factor (PF), selectivity (S), lipase yield (Y)

The calculation for lipase separation efficiency, purification factor, selectivity and yield has been described earlier in section 3.1.3.9.

3.4. Objective 4: Integration of ultrasonication and LBF system utilising sugaring-out effect for the extraction of protein from microalgae biomass

This segment discusses on the fourth application of LBF system. This section will be on the LBF application for the protein extraction from microalgae. Sugaring-out effect is used in this study and the integration of sonication with LBF will be explored. **Figure 15** shows the flow chart that elucidates an overall depiction of the processes involved in this last objective.

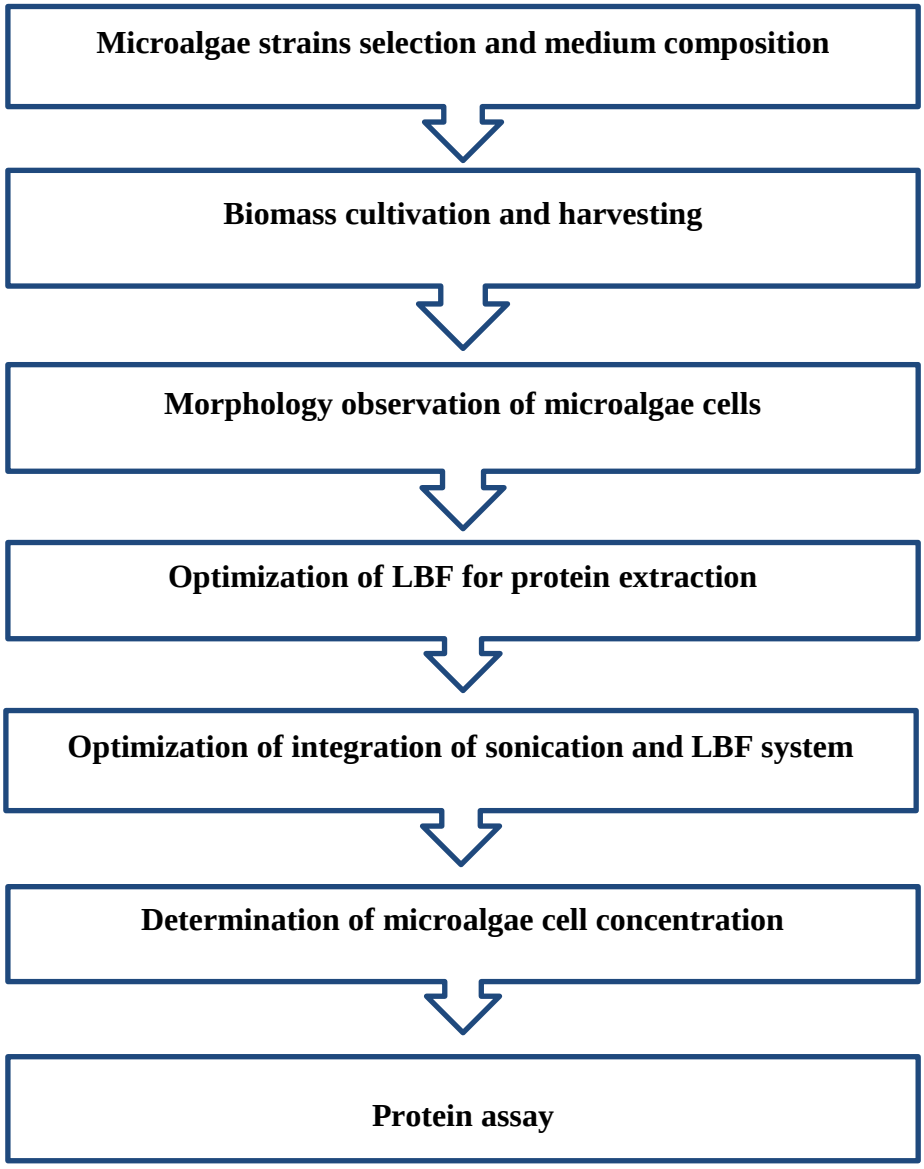


Figure 15: Flow chart describing methodology employed in the integration study of sonication and LBF system for protein extraction from microalgae

3.4.1. Apparatus

3.4.1.1. LBF system

In this study two different LBF glass column with varying sizes were used. The medium size of LBF with the working volume of 500 mL and the largest size of LBF with the working volume of 2.5L were utilized in this study. The medium size LBF glass column is consisting of 7 cm inside diameter, 15 cm height and volume capacity of 500 mL as shown in **Figure 12**. The optimization study was conducted using the medium scale LBF. The large scale LBF shown in **Figure 7** was used to study on the consistency and the reliability of the integration process. Two different flow meter were used (model: RMA-26-SSV) with a range of 25-250 cc/min and 0.5-5 LPM (Dwyer, USA). The integration of the sonication and separation was done in this single LBF system.

3.4.1.2. Ultrasonication

A Bandelin Sonoplus (UW 2200, 20 kHz, 200 W, Germany) with a titanium horn sonotrode (TT 13/FZ) was employed to introduce ultrasound.

3.4.2. Materials

Alcohols (acetonitrile, methanol, ethanol, 1-propanol and 2-propanol), sugars (glucose, maltose, sucrose and fructose) which were used as the phase components and salts (dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium sulphate, magnesium sulphate, calcium chloride, potassium nitrate, manganese chloride, zinc sulphate and iron sulphate) which were used for microalgae cultivation were purchased from R&M Chemicals (United Kingdom). All of the chemicals are of analytical grade. Additionally, Bradford reagent and

BSA for the protein assay was also purchased from R&M Chemicals (United Kingdom).

3.4.3. Methodology

3.4.3.1. Microalgae strains selection and medium composition

In this investigation, Chlorella *vulgaris* FSP-E microalgae strain was used, which was obtained from National Cheng Kung University, Taiwan. . This specific strain was selected for this study due to its advantages of fast growing and high protein content. The microalga was cultivated using a laboratory-scale photobioreactor (PBR) and for the pre-culture Basal medium was used. Pre-culture of microalgae was accomplished for a week with a continuous supply of carbon dioxide (2.5%). The following composition of Basal medium was utilised: (g/L), KNO_3, 1.25 g/L; KH_2PO_4, 1.25; $MgSO_4.7H_2O$, 1; $CaCl_2.2H_2O$, 0.1106; $FeSO_4.7H_2O$, 0.0498; EDTA.2Na, 0.5; H_3BO_3, 0.1142; $ZnSO_4.7H_2O$, 0.0882; $MnCl_2.4H_2O$, 0.0144; $Na_2MoO_4.2H_2O$, 0.0119; $CuSO_4.5H_2O$, 0.0157; and $Co(NO_3)_2.6H_2O$, 0.0049 (Phong et al., 2017). To avoid contamination the preliminary pre-culturing of microalgae was prepared within a laminar flow chamber.

3.4.3.2. Biomass cultivation and harvesting

Microalgae cultivation was carried out by utilizing a laboratory scale PBR with a glass vessel of working volume of 1 litre. **Figure 16** illustrates the schematic diagram of the PBR. Both sides of PBR were mounted with external LED lights to continuously illuminate them (light intensity: 200 μmol/m^2/s). The temperature was maintained at 25 ± 1 ºC (Phong et al., 2017). As for the inoculation of pre-culture, an inoculum with a size of 0.06 g/L was inoculated

into PBR and the agitation speed was fixed at 450 rpm (Lee, Show, et al., 2017). The culture broth was aerated constantly with a mixture of air and CO_2 at a rate of 400 mL/min (0.1 vvm, volume gas per volume media per min) to achieve a concentration of 2.0% (Lee, Show, et al., 2017). An air filter with the size of approximately 0.22 mm was fixed at the tube opening to filter the air (C. H. Tan et al., 2016). Cultivation of microalgae was carried out up to 10 days. Harvesting of microalgae was done when nitrogen starvation begins (a consumption of 90% nitrogen in the medium). The implementation of this method is very promising for achieving a maximum protein content since accumulation of protein increases and a minimum lipid and carbohydrate are found out during nitrogen shortage phase (Chen et al., 2015; Ho, Chen, & Chang, 2012; Yeh & Chang, 2011). Harvested biomass was collected from the culture broth by centrifugation at 6000 rpm (Eppendorf, 5430) for 5 min. The obtained supernatant was removed, where the biomass paste remains. Experiments were carried out using microalgae on wet-basis. The paste was stored at 4 ◦C and was employed for the study within 5 days after harvesting.

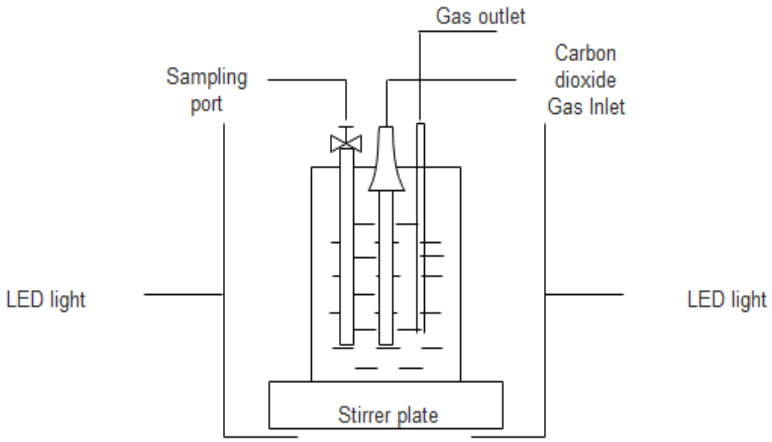


Figure 16: Schematic diagram of lab scale of microalgae photobioreactor

3.4.3.3. Morphology observation of microalgae cells

Microalgae cells obtained before and after centrifugation were subjected to the integrated processes of sonication and protein extraction and were then resuspended for microscopy observations. Microscope (VanGuard, 1113AMH) with the magnification of 1000x was used to observe the cell morphologies. The image of the microalgae morphology will be discussed in the results and discussion section later. Oil immersion was placed in the slide together with the microalgae cells for the observations. Images of cell morphologies with and without sonication were captured.

3.4.3.4. Optimization of LBF for protein extraction

For the LBF study, in order to prepare aqueous solutions, an appropriate amount of biomass paste (1g) was resuspended in water and mixed with glucose. One variable at a time (OVAT) method was employed to explore the influences of various parameters on the protein extraction using sugaring-out effect and sonication. The initial working volume of the medium was 150 mL of aqueous solution and 150 mL of acetonitrile, 0.6% of biomass concentration, 150 g/L of sugar solution, 10 min of flotation time with an initial air flow rate was at 75 ml/min. The initial working conditions were selected based on the preliminary study that was done. The compressed air supply system was maintained at 0.5 bar and the air was filtered using a sterile air filter with a pore size of 0.2 micron and was fit to the neck of the flotation tank (Phong et al., 2017).

As for the ultrasonication, ultrasound was introduced at 20% amplitude (a maximal power of 200 W), and in a pulse mode (5s ON /5s OFF) at ambient temperature (Lee, Show, et al., 2017). Ultrasonication probe was placed at the

bottom phase of the aqueous solution. The sonication time was initially fixed at 15 min. Higher sonication time may increase the temperature of the solution that eventually resulting in protein denaturation. Thus, 15 min was selected as the initial condition. Initial study was performed by comparing the experiments both in presence and absence of sonication. In the absence of sonication, the solution will only undergo flotation whereby bubbles were introduced into the system. As for without flotation system, the solution was subjected only to sonication without introducing any bubbles into the system.

3.4.3.5. Optimization of integration of sonication and LBF system

Ultrasonication and protein extraction via flotation was conducted as a single continuous process. **Figure 17** illustrates the schematic diagram and **Figure 18** shows the experimental setup of the integration process of sonication and LBF system. Sugar solution and acetonitrile were prepared in the flotation system and ultrasonication was introduced into the system simultaneously for protein extraction. In the presence of sonication, microalgae cell could be easily and effectively disrupted and thereby the protein could be easily released. These proteins will be extracted continuously from the bottom aqueous solution to the top acetonitrile phase by using a two-phase partitioning. The parameters studied include the concentration of microalgae biomass, the location/position of sonication probe, the pulse mode of sonication, sonication time (continuous without any resting period), sonication resting mode (varying the OFF time of sonication), the concentration of glucose, the type of sugars, the concentration of acetonitrile and the air flow. After obtaining the optimized conditions, a large-scale study was also subjected to.

The temperature of the sonication system was fixed to not exceed 40^{O}C. If the temperature rises above 40^{O}C, the system will stop operating. However, in this entire experiment such occurrence did not happen. Thus, in this experiment the operation temperature was below 40^{O}C and it does not affect the extraction process. In addition, since pulse mode was introduced in the system, temperature rising was controlled. The optimum conditions for maximum protein extraction and yield were 5 min of 5s ON/10s OFF pulse mode, this indicating short sonication process with short interval of operation period. Thus, the temperature rise is under control.

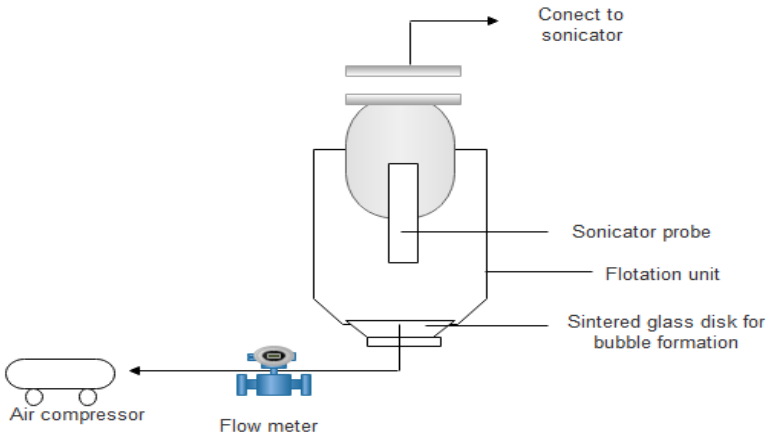


Figure 17: Schematic diagram of the integration process of sonication and LBF

Figure 18: Image of experimental setup of the integration process for protein extraction via sonication and LBF system

3.4.3.6. Determination of microalgae cell concentration and protein content

Microalgae cell concentration was measured using a UV–Vis spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). A wavelength of 688 nm was used to determine the algae concentration (Helena, Rodrigues, Arenzon, Raya-rodriguez, & Fontoura, 2011).

Following the cell concentration measurement, protein assay will be done for the collected samples. The procedure for protein assay has been mentioned earlier in the section 3.1.3.6.

3.4.3.7. Analytical methods: Calculation for separation efficiency of protein and total recovery yield (Y_P) of protein.

In this section the formula for protein separation efficiency and total protein recovered (Y_P) will be explained.

The separation efficiency (E) which defines the concentration of protein being extracted was calculated using the following Eq (8):

$$E = \left[1 - \frac{C}{C_i}\right] \times 100\ \% \qquad (8)$$

Where c signifies the protein concentration in the bottom phase at the end of experiment and C_i is the original protein concentration in the crude feedstock.

As for the total recovery yield (Y_P) of protein it was calculated by using the following Eq (9):

$$Y_P = \left[\frac{\text{Total protein recovered in top phase}}{\text{Total protein content initial}}\right] \times 100\ \% \qquad (9)$$

4. Chapter 4: Results and Discussion

4. Recycling of Phase Components Comprising Alcohol/Salt in Large Scale using LBF for Lipase Recovery.

4.1. Introduction

This chapter discusses the first objective of the research that is to recycle phase components comprising alcohol/salt on a large scale using LBF for lipase recovery. The use of recyclable phase components in the LBF technique could be an essential key in developing an efficient and cost effective separation technique. In this chapter the recycling ability of alcohol/salt in larger scale using LBF system for the extraction of lipase was explored.

Lipase is selected for this study due to high demand of lipase as a biocatalyst and due to their broad substrate specificity, thermostability and high tolerance towards organic solvents (Ooi, Tey, Hii, Kamal, et al., 2009). Alcohol/salt system has few advantages over polymer/polymer, polymer/salt and surfactant such as they are lower in cost, low viscosity, simple recovery of alcohol by evaporation and it is simple for scaling up in industrial production (Z. J. Tan, Li, & Xu, 2013). Despite these advantages, researchers are concern on the large scale application of LBF due to the high chemical demand and the environmental problems that may arise from the elevated salt concentration in waste disposal (H. Yang, Goja, Cui, & Li, 2013). The concentration of salt in two-phase system has a major effect on the partition coefficient (Iqbal et al., 2016). Most of the LBF system requires at least 200g/L of salt in their bottom phase for biomolecules separation (Md Sidek et al., 2016). Each time of separation process, large amount of salts is required. Thus, there is a need to establish an effective method of LBF application for sustainable recovery of biomolecules.

Phase forming polymer such as PEG which is not recyclable set a limitation to the LBF process, whereas the use of EOPO copolymer in LBF results in high cost

and slow separation of two-phase which subsequently limit the usage of LBF at large-scale. To the authors' knowledge there is no extensive research done concerning the recycling of the large scale phase components for LBF method. In a previous study (Show et al., 2013b), lipase was recovered using recycled hydrophilic organic solvent/inorganic salt LBF however, recycling of component was done separately. The top organic phase was recycled five successive cycles utilizing fresh aqueous phase each time. The fourth and fifth LBF cycle utilized aqueous phase that was recovered from the previous LBF (Show et al., 2013b). The waste water produced in each LBF cycle causes environmental concern that leads to the need for a sustainable LBF recycling technique to reduce the amount of waste water produced and the usage of salt. In the direction to improve the established LBF system, an extra economic and eco-friendly LBF with the ability to recover phase components for large scale has been proposed in this study.

The aim of this study was to investigate the effect of full and continuous recycling of both alcohol and salt phase components using LBF system. The objectives of this study are to reduce waste disposal and high salt consumption in the lipase separation. The bottom aqueous phase was reused eleven successive times to find the optimised condition for recycling, whereas the alcohol was recycled by evaporation. The salt in bottom phase was then crystallized through dilution crystallization by methanol and was tested for the possibility of recycling crystallised salt. The experiment is focused on the mono-dimensional approach, whereby each parameter was studied based on one factor-at a time. In this study, the proposed design of operating conditions was taken based on few literatures related to lipase separation method using LBF (Mathiazakan et al., 2016; Show et al., 2013a). These literatures focused on the operating conditions for lipase

separation process. Since this study emphasizes on the recycling, ranges of operating conditions from previous literatures were selected to perform recycling study.

This chapter highlights the results obtained from this study. This chapter begins with the results and discussion concerning the selection of crude feedstock concentration to choose the best concentration for maximum lipase yield and purification factor. Following that, the effect of different types of salt was experimented to obtain the suitable salt for effective lipase partitioning. Next section discusses the optimization of recycling process for following parameters: (i) volume of crude lipase added for recycling, (ii) types of alcohol and finally on concentration of 1-propanol. This optimization study was done to obtain the greatest conditions that give the highest lipase yield, separation efficiency, selectivity and purification factor upon recycling process. Succeeding that will be on the results and discussion from the experiment of recycling of phase components using recovered salts. Complete results and discussion from all the experiments will be explained comprehensively in the following sections. Final section from this chapter will be on the conclusion of this objective which covers the advantages, disadvantages and possibilities of future work that could be carried out.

4.2. Results and Discussion

4.2.1. Selection of crude feedstock concentration

This study begins with the selection of the appropriate lipase feedstock concentration and type of salt that able to give maximum lipase separation efficiency, yield and purification factor. This study is necessary to obtain the

rightest concentration and salt to be used for the subsequent recycling study. The concentration of crude feedstock plays a significant role in LBF. Increasing crude load benefits the recovery processes via LBF technique. Few findings have indicated that the amount of crude feedstock could significantly influence the partitioning behaviour of protein (Selvakumar, Ling, Walker, & Lyddiatt, 2010). Thus, it is vital to select the optimum crude load in the LBF system for effective lipase separation. In this study, 10-50 % w/w of crude feedstock was analysed on the 1500 ml aqueous phase. **Figure 19** explains the effect of crude feedstock concentration on the yield of lipase (Y), separation efficiency (E) and purification factor (P_{FT}). Based on the **Figure 19**, it can be seen that the separation efficiency and yield of lipase demonstrates the highest with 80.07 % and 64.64 % respectively at 20 %w/w of crude feedstock concentration. The observation of P_{FT} indicates that the crude load in the LBF system affects the P_{FT} results. The maximum P_{FT} 3.32 was achieved at 20 % (w/w) concentration of crude loading which shows that high crude loading results in decrease performance of LBF. This is because of the alteration in volume ratio and the partition behaviour of target protein (Ooi, Tey, Hii, Ariff, et al., 2009). Increase in crude concentration lead to reduction in volume ratio which then ultimately reduces the quantity of lipase partitioned to the top phase (Lin et al., 2015). In addition, increasing the concentration of crude feedstock will lead to increase in contaminants in the system and causes accumulation of precipitate at the interface that consequently reducing the performance of lipase separation (Amid et al., 2012). Therefore, 20 % w/w of sample loading was selected for further studies. After obtaining the optimized crude concentration, next parameter that needs to be investigated is on the types of

salt. The effect of different types of salt on the partitioning behaviour has been discussed in the following section.

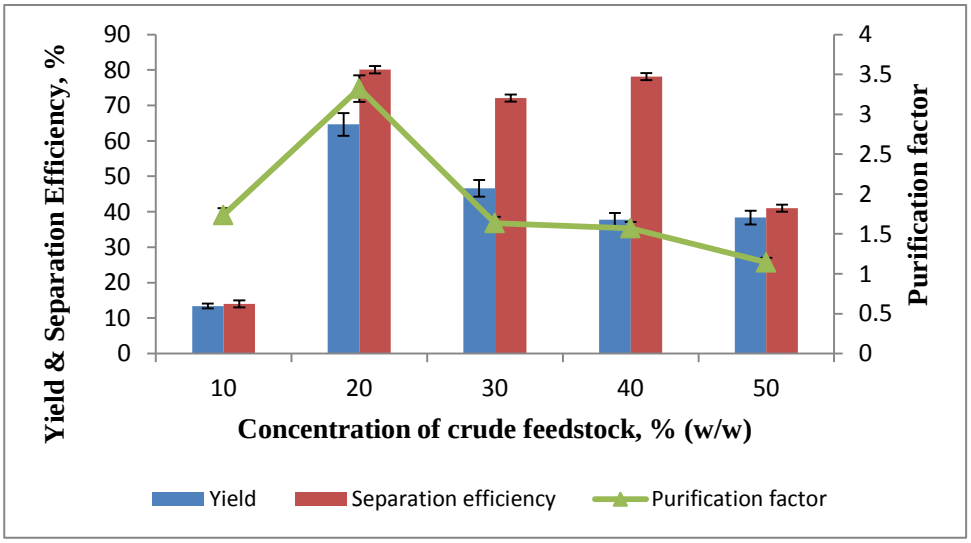


Figure 19: The effect of concentration of crude feedstock upon the yield, separation efficiency and purification factor of lipase in the LBF system.

4.2.2. Effect of salt types

This section is on the investigation to obtain the appropriate salt for high lipase yield, selectivity and efficiency. The performance of LBF varies with different types of salts and concentrations. The addition of salt into the solution will increase the surface tension of the water which results in increase of hydrophobic interaction between protein and water (Wingfield, 1998a). Due to the salting out effect, the target protein will then be accumulated in the hydrophilic solvent phase. The concentration of salt plays a major role in sustaining an immiscible two-phase system through salting-out effect. Generally, LBF requires high concentration of salt to maintain steady immiscible two-phase (Lee et al., 2016). Based on previous literature for the large scale LBF of lipase purification, it has been reported that the optimum volume of salt solution was at 1.5 L and optimised salt concentration was 250 g/L (Mathiazakan et al., 2016). Since this

study focuses on the recycling, the concentration of salt with 250 g/L from previous study was selected for further studies.

The choice of salts for the LBF differs based on their ability to support hydrophobic interactions between biomolecules (H. Yang et al., 2013). Five different types of salt were used to carry out LBF in order to select the best salt for superior LBF performance. According to **Table 4**, ammonium sulphate illustrated the highest selectivity (S) with 4.35, E 78.61 % and high Y of lipase with 76.73 %. Despite potassium phosphate has higher yield of lipase compared to ammonium sulphate, however, the S achieved was 1.14 and E of 60.48 % of lipase which is lower than ammonium sulphate. Besides that, ammonium sulphate is the traditional kosmotropic salt that encourages the stability of protein via favouring hydrophobic interactions (Pakhale & Bhagwat, 2016). Therefore, this explains for the high selectivity and separation efficiency of ammonium sulphate and hence, it is the best salt for lipase separation. From the optimization study, 20% of crude feedstock and ammonium sulphate have been optimized. These optimized conditions are used for the following recycling studies that are conducted to evaluate the following parameters: (i) crude load feedstock, (ii) types of alcohol and (iii) concentration of alcohol. The effects of these parameters are required to obtain the optimized conditions for the recycling studies.

Table 4: Effect of different types of salts on the selectivity, separation efficiency and yield of lipase in LBF system.

Types of salt	Selectivity	Efficiency (%)	Yield (%)
Potassium phosphate	1.14	60.48	78.76
Ammonium sulphate	4.35	78.61	76.73
Magnesium sulphate	0.26	26.62	24.43
Tri-sodium citrate	1.82	70.82	31.52
Sodium phosphate	3.43	57.11	50.05

4.2.3. Recycling of phase forming component: Effects of crude load feedstock

Based on the optimized result obtained from the previous sections, the influence of crude load of lipase feedstock in the recycling of the phase forming component is conducted. This section elaborates the results obtain from this particular study. Lipase partitioning in LBF containing alcohols and polymers has been reported in several studies (Mathiazakan et al., 2016; Show et al., 2011, 2013b). Utilizing salting-out effect in the recovery has resulted with high extraction efficiency. However, there are several drawbacks that have not been resolved. The main concern is regarding environmental issue which were elevated from high salt concentration in waste disposal (H. Yang et al., 2013). In addition, certain salt such as K_2HPO_4 able to alter the environment pH that is an important issue that need to be paid attention (Gu & Shih, 2004). The use of high concentration of salts for partitioning possibly will proceed to undesirable reactions (Dhamole, Mahajan, & Feng, 2010a). The application of LBF systems composed of alcohol and salts appears to be economically unjustified because of the high concentration of salts required in a single extraction step. Up to date, there is no study done on recycling for large scale of LBF due to the concern of large chemical consumption that will consequently lead to extra cost.

In this study, we have studied the possibility of recycling both top and bottom phases for large scale of LBF. For each set of experiment first extraction of lipase was begun with 20 % concentration of crude feedstock and subsequent recycling was done with four different volumes; 25, 50, 75 and 100 mL. The effect of different volume of crude feedstock added for the recycling of salt phase was

studied. This was done to identify the optimised amount of crude to be added for the recycling of bottom phase for better separation efficiency, selectivity and purification factor. From **Figure 20**, it is distinguished that 75 ml of crude volume for recycling showed better E with first extraction 78.41 %, first recycling 72.12 %, second recycling 73.23 %, while for S first extraction showed 4.36, first recycling 4.69 and second recycling 4.75 and as for P_{FT} first extraction was 2.8, first recycling 4.12 and second recycling 4.1. After the first extraction, most of the lipase in the bottom phase has been transferred to the top phase, low volume (25ml and 50ml) of crude feedstock that was added for first and second recycling demonstrates low E, P_{FT} and S. When high volume (100 mL) of crude lipase was added in the system, lower E of 32.35 % for first extraction, 28.48 % for first recycling, 13.15% for second recycling was obtained. The decrease in E was due to the alteration in the volume ratio of the developed two-phase system. 75 ml of added crude volume showed the best in terms of E of 73.23 %, P_{FT} of 4.1 and S of 4.75 after two times of recycling compared to other crude volume. Hence, it was selected for further studies. The following section will discuss on the recycling study using different types of alcohol.

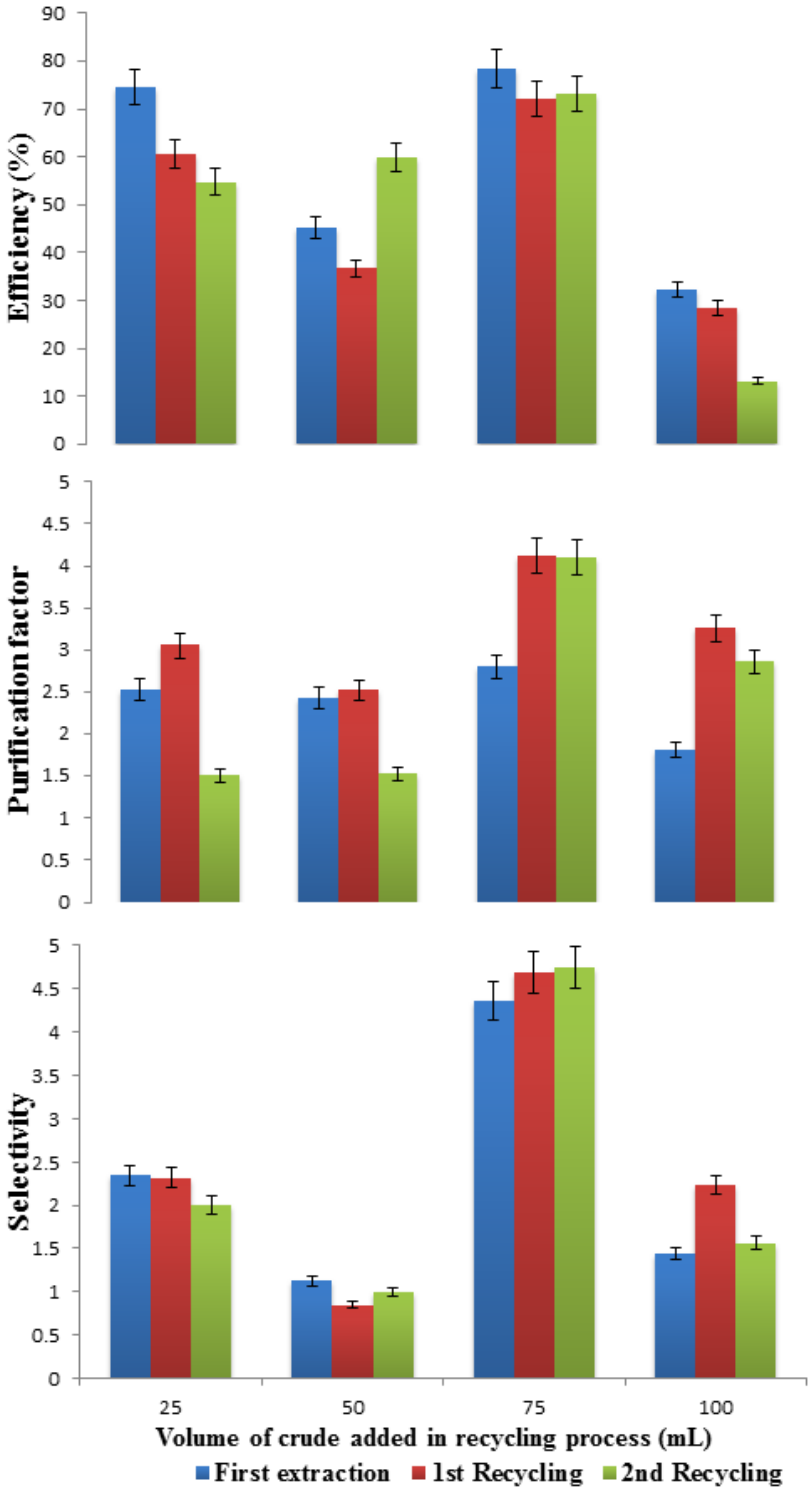


Figure 20: Effect of volume of crude feedstock added on the selectivity, purification factor and separation efficiency of recycling for large scale LBF.

4.2.4. Recycling of phase forming component: Effect of different types of alcohol

Following the study on the effect of crude load volume, the influence of different types of alcohol is investigated. This section focusses on the recycling effect of phase components using different types of alcohol. This study aims to find the most suitable type of alcohol for highest lipase purification factor using recycling phase components. The choice of suitable alcohol phase in LBF system is crucial because the interaction of alcohol with lipase varies according to the type of alcohol. For this experimentation, four different alcohols; 1-propanol, 2-propanol, ethanol and methanol were used and their recyclability for lipase separation was investigated. From the results obtained from **Figure 21**, 1-propanol exhibited as the best alcohol for lipase separation with the P_{FT} of 2.11 for first extraction, 1.85 for first recycling and 2.83 for second recycling. This can be explained due to the long hydrocarbon chain of propanol compared to ethanol and methanol that enhance the interaction with lipase which consequently boost the partitioning (Ooi, Tey, Hii, Kamal, et al., 2009). Additionally, alcohols with longer carbon chain is said to assist in the formation of biphasic system given that lower concentration of the less-polar alcohol is necessary for the formation of two-phases (Greve & Kula, 1991b). The study on the performance of 1-propanol during recycling displayed that the purification fold increases in the second recycling and this maybe because lipase was accumulated in the aqueous phase from previous cycles.

It is observed that, ethanol and methanol were incapable of the formation of two phases with inorganic salts higher concentration was needed to form two separate phases. The use of methanol leads to undesirable results whereby precipitation of salts occurs and this causes the hindrance of alcohol recycling

(Mathiazakan et al., 2016). As demonstrated in the **Figure 21**, after first recycling the purification factor decreases drastically to 0 which makes it unsuitable for recycling. **Figure 22**, demonstrates the effect of recycling alcohol and salt phases focusing on the Y and E of lipase. From **Figure 22**, it can be seen that the E decreases from 95.08% at first extraction to 65.38 % at second recycling. As the number of recycling increase, the efficiency decreases this is possible due to the increase of contaminants such as cell debris and unwanted proteins in the bottom phase from prior recycling process that may affect the salting out effect (Show et al., 2013b). Nonetheless, the Y of lipase showed slight increase from 75.11% in the first extraction to 86.96% in first recycling and slight decrease in the second recycling 63.32%. This may occur due to the residual lipase from the primary extraction that retained in the bottom phase. With the addition of crude for the secondary extraction, accumulated lipase from the primary bottom phase might have partitioned to the top phase that causes the increase in the yield. The decrease of Y in the second recycling may be due to low amount of lipase in the and possibly due to high contaminants of cell debris and unwanted proteins in the bottom phase (Show et al., 2013b). Since 1-Propanol gave the best result in the recycling study, the effect of different concentrations of this alcohol needs to be evaluated. The following recycling study investigates the effect of 1-propanol concentrations.

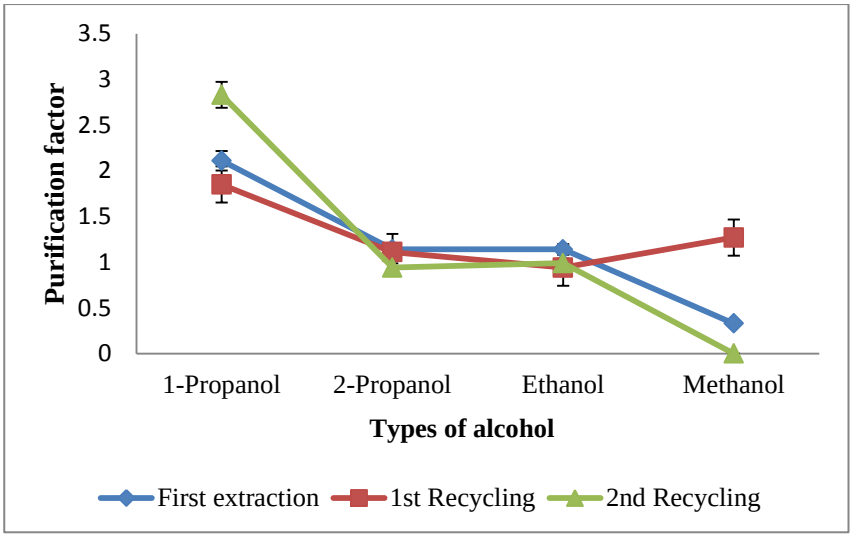


Figure 21:Comparison on the recyclability of different types of alcohol on the purification factor of lipase.

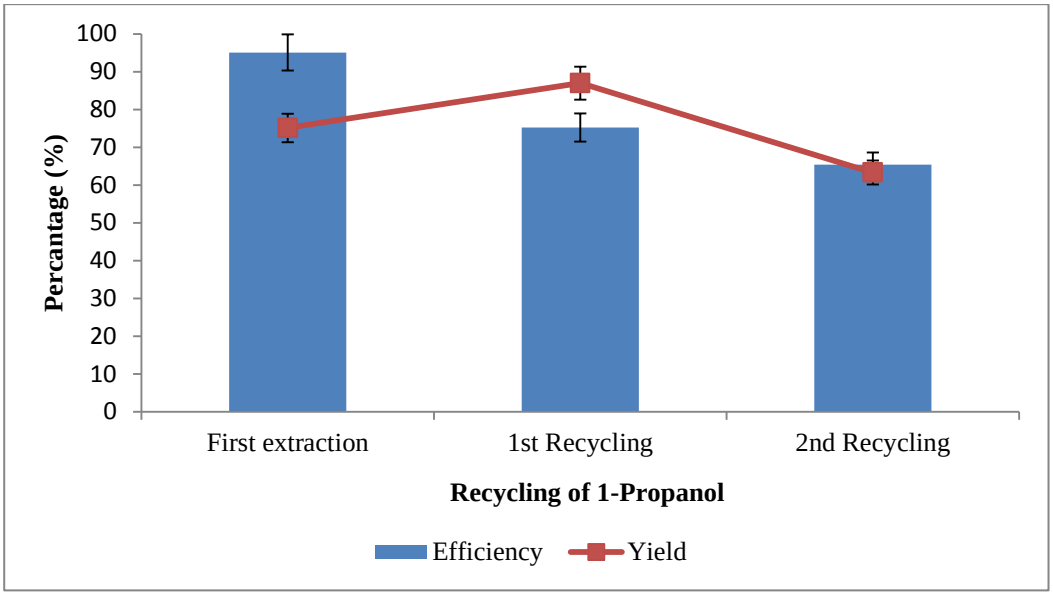


Figure 22: The effect of recycling of top and bottom phase on the yield and separation efficiency of lipase via LBF system

4.2.5. Recycling of phase forming component: Effect of different concentration of 1-propanol

1-propanol was found to be the best alcohol in the recycling study for maximum lipase purification factor. In this section, the effect of different concentration of 1-propanol will be investigated. The recycling effect in terms of

selectivity and efficiency by applying different concentrations of 1-Propanol were studied. From previous literatures it is clearly known that with a concentration of alcohol less than 50 %, the biphasic system could not retain in two phases and it exist in homogenous solution (Show et al., 2013a). Therefore, in this research the recyclability of 1-Propanol was studied using concentration of 1-propanol with 50 % and above. From **Figure 23,** the concentration of alcohol at 50 % w/w showed better E with 96.25 % after twice of recycling. As for the S at 50 %, the graph shows an increasing trend as the alcohol is recycled from 6.03 at first extraction, 32.97 at first recycling and slight decrease in second recycling with 23.38. The increase in selectivity indicates that lipase is preferably separated to the top phase leaving the unwanted protein in the bottom phase. This shows that recycling of both phase components able to separate lipase better in relation to the total protein in the salt-rich phase. The increase of lipase selectivity after recycling possibly due to the increase of total protein content in the subsequent cycle when more crude lipase is added. Meanwhile on the observation of E it can be seen that it almost remain stable after recycling twice from 88.29 % at first extraction to 97.45 % first recycling and 96.25 % at second recycling. The high S and E of recycling phase components shows that the higher protein accumulated in the bottom phase does not affect the separation of lipase to the top phase. From this study, 50% of 1-propanol was selected as the optimized condition and was used for the following study.

Table 5, illustrates the recovery effect of different concentration of 1-propanol for each recycling. According to the **Table 5**, the recovery of alcohol after 1st extraction increases from 76.67 %, 78.00 %, 83.33 % and 85.00 % as the concentration increases 50 %, 70 %, 90 % and 100 % respectively. This

circumstance possibly happens due to dilution of low concentration of alcohol in the bottom phase. Due to the low concentration of alcohol, they tend to mix and dilute into the bottom phase, thus reduces the recovery of alcohol. For higher concentration of 1-propanol, they tend to be more viscous and dense. This makes them difficult to mix in the bottom phase thus, it is easier to recover high amount of higher concentration of alcohol compared to lower concentration. Though the recovery of 100% of 1-propanol is high, the separation efficiency and selectivity for lipase separation is low. This indicates that 100 % of 1-propanol is not suitable for the lipase extraction and was not selected as the best concentration. This explained the trend of the recovered alcohol obtained in **Table 5**.

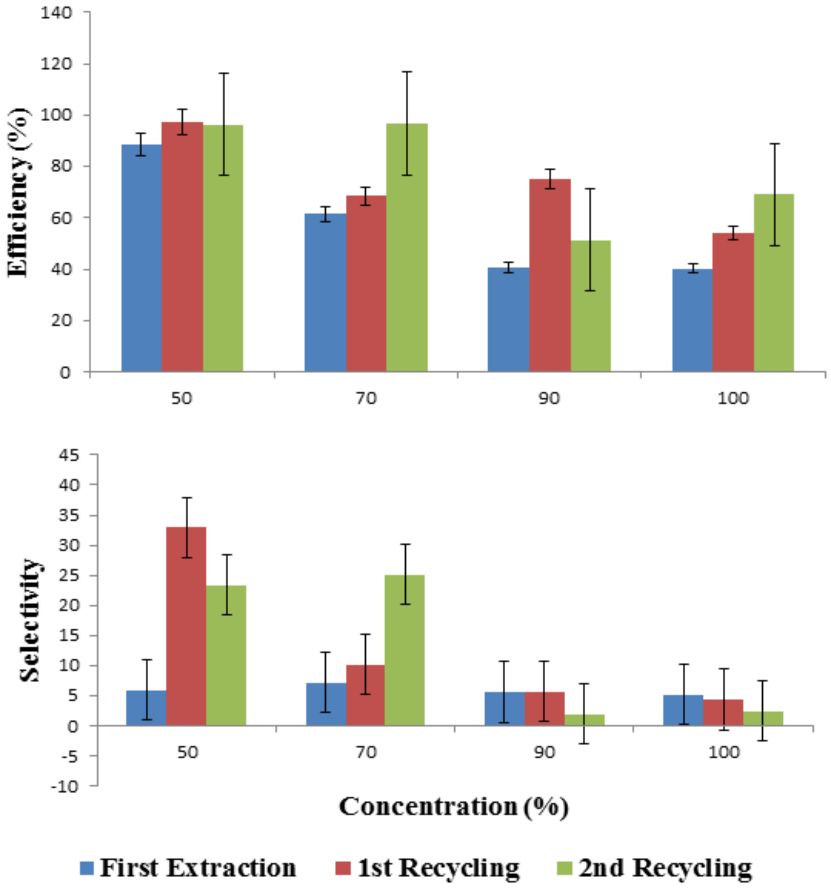


Figure 23: Effect of different concentration of 1-Propanol on the selectivity and efficiency of recycling phase components.

Table 5: Comparing the recovery of alcohol after each cycle of recycling

Concentration of alcohol (%) w/w	1st Recycling (%)	2nd Recycling (%)
50	76.67	77.33
70	78.00	79.33
90	83.33	82.96
100	85.00	83.33

Based on the recycling study, the optimized conditions for maximum lipase extraction are 75 mL of crude load feedstock and 50% of 1-propanol. Upon obtaining the optimized conditions for effective recycling, the effect of using recovered salts for recycling study is further investigated.

4.2.6. Recovery of lipase and recycling of the phase-forming component in LBF using recovered salts

This study focuses on the recycling ability of using recovered salt. The purpose of this study is to discover the potential use of the recovered salt and to study the recycling effect of the recovered salts. Given that ATPS requires high amount of salt consumption, a simple, rapid, and environmental friendly method to effectively utilize the salts is essential. Greve et al., studied three different methods for recycling salts in ATPS on small scale, and the best method was using aqueous ethanol solution to extract salt (Greve & Kula, 1991b). Recycling of salt is a vital issue that need to be solved at industrial scale because more than 30% (w/w) of the solvents and salts are found in the top and bottom phases, respectively (Z. Li, Teng, & Xiu, 2011).

In this study, the performance of LBF was investigated using ammonium sulphate that was recovered from previous extractions. Ammonium sulphate from previous sets of experiments was recovered by dilution crystallization of methanol.

The optimum volume ratio of methanol added to the salt-rich phase for maximum recovery of salt was found to be 2:1 (Z. Li et al., 2010) and by using this method salt was recovered. Recovering salts by this approach is achievable for industrial scale since the cost of methanol is cheap. In addition, methanol has low boiling point compared to other alcohols. Thus, the energy requirement and cost for methanol recycling were relatively low compare to other alcohols (Z. Li et al., 2011). Methanol that was used for crystallization could be recycled either by using rotary evaporator or by distillation. Even though many other solvents are utilised in ATPS that can be recovered effortlessly via distillation however, some of them for example isopropanol is unsuitable for industrial application. This is because isopropanol is an expensive solvent, the loss of isopropanol during extraction and distillation in large scale will increase the total cost of the process (Z. Li et al., 2010).

In a study by Greve et al., on the cost of salt recycling from the bottom phase in protein extraction process that was done, it was found that the amount spent on salt recycling are in the range of the purchase price of the salt (Greve & Kula, 1991a). The cost for extraction of salt from the system is found to be equivalent as for the total expenditure of the purchase price of salt together with the extra expenses for deposition of the primary bottom phase (Greve & Kula, 1991a). Hence, recovery of salt via dilution crystallization of methanol is a promising and economical approach for large scale.

From **Figure 24**, the Y of lipase decreases after first extraction from 71.69 % to 58.87 % and increases at second recycling to 79.39 %. As for the third and fourth recycling the Y decreases 58.09 % and 55.49 % respectively, this could happen since there are contaminants overloaded in aqueous phase and it causes

decrease in the Y. From this study, the result obtained explains that the ability of recovered salt for two-phase system decreases as the number of recycling increases. As for the P_{FT} of lipase, there was slight increase up to third recycling however, significant decrease occurred in the fourth recycling. In general, the recycling of LBF composing recycled salt showed that the Y of lipase after twice of recycling able to achieve approximately up to 80.00 %. Despite the decrease in yield after four times of recycling, the yield attained was reasonably high with more than 50 %.

In another study of salt recovery, phosphate was converted to KH_2PO_4 by reacting with phosphoric acid and some amount of the phosphate was crystallized via dilution crystallization of methanol (Z. Li et al., 2011). Besides that, it was found that in there is a decrease of 34.00 % of alcohol dosage and 60.00 % of the price for the recovery of phosphate via methanol ATPS compared to recovery of ammonium sulphate via ethanol ATPS (Z. Li et al., 2011). Furthermore, the energy consumption for methanol recovery is lower compared to ethanol due the azeotrope formation of ethanol with water that is absent in methanol. Consequently, the cost of methanol recovery is much lower than ethanol. By recycling phase components, the change of environmental pH and wastewater production of high salt concentration can be reduced. Thus, this proves that recycling of phase components is a cost-effective and promising approach for large-scale purification.

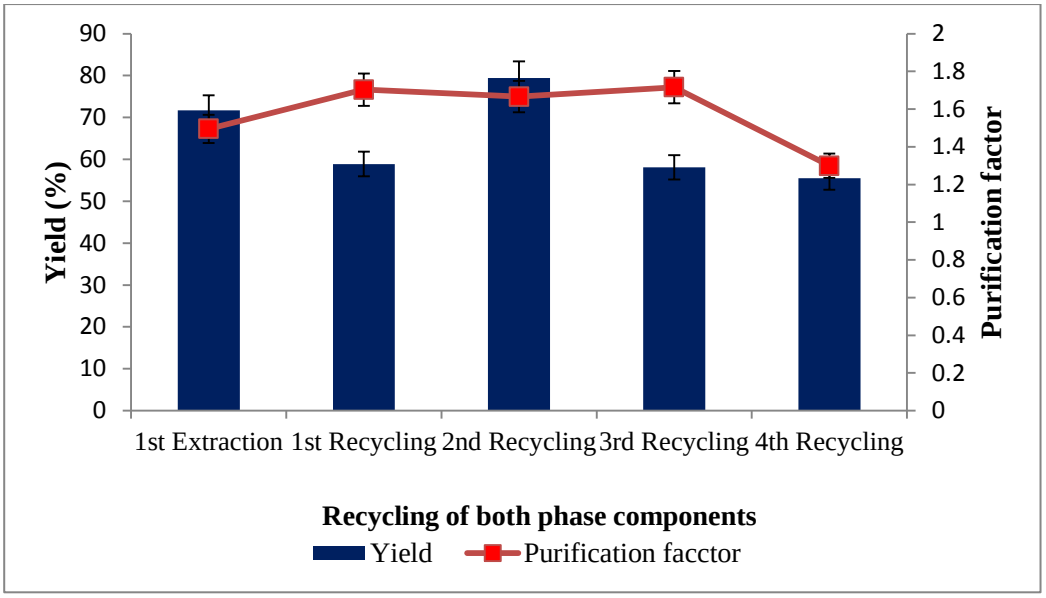


Figure 24: Effect of recycling upon lipase yield and purification factor utilizing recovered salts

The purity of lipase from *B. cepacia* using recycling phase components was assessed using SDS-PAGE. This assay is done to confirm that the lipase has been extracted to the top phase. **Figure 25**, described the bands obtained from the analysis. Lane 2 represents contaminant proteins in crude feedstock. Lane labelled as R1-R11 shows samples that were obtained from LBF using recycled 1-propanol/ ammonium sulphate phase components. Generally, lipase from *B. cepacia* strains have molecular weight of about 33 kDa (Show et al., 2011). This study has proven pure lipase extraction employing sustainable recycling LBF. As demonstrated from **Figure 25**, lipase was extracted by using recycling 1-propanol and ammonium sulphate. Most of contaminants and undesired proteins were eliminated from the crude feedstock.

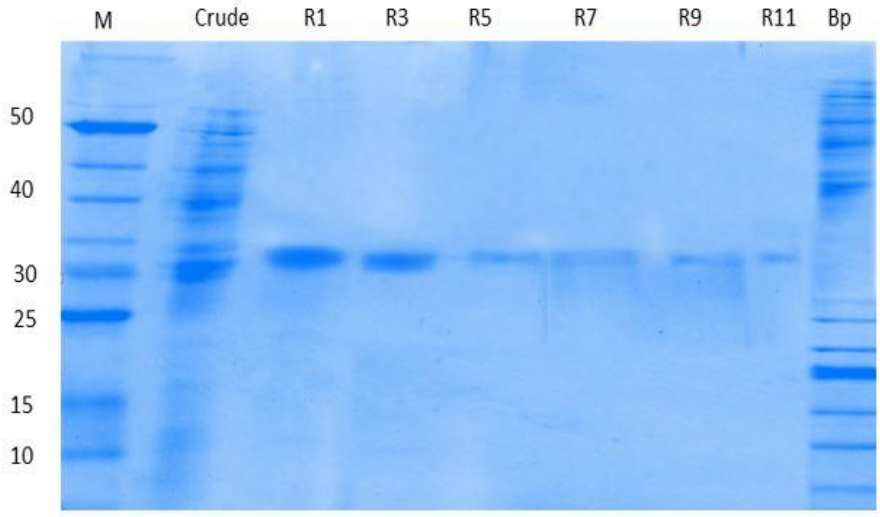


Figure 25: : SDS–PAGE assay on recycling 1-propanol/ammonium sulphate using LBF. The molecular weight of standard protein marker ranged from 10 to 50 kDa. In the SDS–PAGE, Lane 1: Marker; Lane 2: Crude sample; Lane R1: sample of recycling LBF conducted using recovered chemicals from first LBF; Lane R3: sample from third recycling of LBF; Lane R5: sample from fifth recycling of LBF; Lane R7: sample from seventh recycling of LBF; Lane R9: sample from ninth recycling of LBF; Lane R11: sample from eleventh recycling of LBF Lane BP: Bottom phase.

4.3.Conclusions

In this study, it is reported for the first time on the recycling of both phase components for large-scale lipase separation via LBF system. It has been demonstrated that the optimised operation condition for the recycling of both phase components of LBF was; (1) concentration of crude feedstock: 20 % w/w, (2) type of inorganic salt: ammonium sulphate, (3) crude volume added for subsequent recycling: 75 ml, (4) type of alcohol: 1-propanol, (5) concentration of alcohol: 50 % w/w. The significance of this study is to demonstrate the performance of fully phase component recycling and to establish this approach for sustainable recovery of lipase.

From this study, it evidently shown that by reusing the bottom phase twice after primary extraction it is able to sustain separation efficiency higher than 70 %. In addition, the maximum yield of lipase with 80 % was achieved by recycling the recovered salt in the LBF system. Besides, this study showed that the recovered phase components could be recycled effectively up to four cycles and be able to produce significantly high yield of lipase. The recycling of large scale LBF of both phase components comprising 1-propanol and ammonium sulphate is practically ideal in terms of efficiency, time, economic and sustainability. This study demonstrated that bottom aqueous phase could be recycled and reused for eleven times and still gives high lipase yield with more than 50%. Compared to previous study in which number of recycles that was done only five times and the phase components were not recycled entirely, fresh alcohol and aqueous phase were used. The method that is introduced in this study which uses full recycling of phase components appear to be advantageous in large scale industries as it is low-cost whereby phase components can be recycled more times whilst giving high lipase yield. This research has demonstrated the economical and sustainable competencies of a scaled-up downstream bioprocess using LBF with the ability for full recycling of phase components.

Possible drawback of this study is the number of experiments of large scale done for the sample collection and the outcome of combined effect of operating conditions. This current study can be basis for future computational approach and using different techniques of DoE in selecting the operating conditions. In addition, further study of DoE can also be applied to study the combined effect of operating conditions. This method is possibly applied for the sustainable recovery of other biomolecules.

In conclusion, undeniably, this is a logical approach towards reducing the total cost of lipase separation and it is an efficient technique. In spite of the advantages mentioned earlier, there are few disadvantages of using salt/alcohol phase components which includes corrosion of equipment due to high salt concentration and the use of alcohol that could cause environmental pollution. Thus, it is essential to find an alternative phase components that is environmentally friendly and could substitute the available phase components for lipase recovery.

5. Chapter 5: Lipase Extraction using Novel Phase Components Surfactant/Xylitol

5.1. Introduction

Previous chapter has discussed on the usage of alcohol/salt as the phase components in the recycling study for lipase extraction. In this study, a sustainable, eco-friendly and recyclable phase forming components consisting surfactant and sorbitol using LBF system is investigated to improve the conventional LBF method. This chapter focuses on the application of LBF composing novel phase components that are surfactant and xylitol for lipase extraction and on the recycling ability. In most of LBF study, the phase forming components used are either polymer/salt or alcohol/salt. The main shortcomings of using polymer/salt, polymer/polymer and alcohol/salt includes expensive polymer, complications in segregating desired biomolecules from polymer phase, difficulties in effective recycling of phase components, expensive production cost and environmental pollution. Thus, there is a need to search for novel phase components as a substitute. Up to current date, no study has been done on the recovery of lipase using LBF comprising surfactant/sorbitol.

Xylitol is sugar alcohol or also known as polyol that has a hydroxyl group attached to each carbon atom in its chain (Albuquerque, Da Silva, De MacEdo, & Rocha, 2014). Xylitol has beneficial health properties and represents a substitute to existing traditional sweeteners (Granström, Izumori, & Leisola, 2007). In a previous study, it has been reported the use of xylitol in the ATPS system able to reduce the overall cost and simplified the method of biomolecules separation from the two-phase solution (Amid, Manap, et al., 2015). In this present study, surfactant rich top phase and xylitol as bottom phase for the separation of lipase from fermentation broth via biphasic flotation was investigated. The objective of this

work is to develop a sustainable and environmentally friendly LBF lipase extraction method using recycling principles with the aim for high separation efficiency and economic viability in industrial scale. In this study, lipase from the fermentation broth of *Burkholderia cepacia* is extracted using LBF system composing recycling phase components of surfactant/xylitol. The effect of recycling with a goal to employ the recycled phase components for high lipase separation and high phase components recovery was explored for both small and large scale.

This chapter comprises the results and detailed discussion for this particular objective. Since this study utilized novel phase components, the stability of lipase in the presence of the phase components that are Triton X-100, Tween 20, Tween 80, SDS and xylitol was first studied. Additionally, different concentrations of these phase components on the lipase stability were also studied. The phase components and the concentration (top and bottom phases) that produces highest lipase activity will be selected for further optimization study for the separation process using LBF system. From the stability test, Triton X-100 and Xylitol with concentration of 20 % were selected for further optimization study. Parameters that were investigated for the optimization of lipase extraction using Triton X-100 and xylitol are as follows: (i) different xylitol concentration, (ii) types of surfactants, (iii) different concentrations of Triton X-100, (iv) crude lipase concentration, (v) volumes of top and bottom phases, (vi) pH and (vii) flotation time. Upon obtaining the optimized parameters for maximum lipase recovery, yield, purification factor and selectivity, recycling study was investigated. Recycling study was conducted in small and large scale. Results obtained will be discussed explicitly later in this

chapter. Finally, the last part of this chapter will conclude the overall research of this particular objective.

5.2.Results and Discussion

5.2.1. Study on lipase stability with different phase components

Before conducting the extraction process using the novel phase components, the stability of lipase in the presence of these phase components needs to be investigated. This study aims to obtain suitable phase components and concentration that are able to maintain high lipase stability. Surfactant such as Triton X-100, Tween 20, Tween 80 and SDS were used to test on the lipase stability. As for the bottom phase component xylitol was used and the effects of different concentration of various phase components were studied as well. This study has been done at room temperature because the LBF system that is used for the extraction process will be done at room temperature. **Table 6** shows the influence of individual phase forming component on lipase activity. Based on this preliminary study, lipase is tolerant and stable in the presence of Triton X-100, Tween 20, Tween 80, SDS and xylitol. This implies this new surfactant/xylitol grounded LBF may possibly be applicable for the separation of lipase.

Triton X-100, Tween 20 and Tween 80 are nonionic polyoxyethylene detergents whereas SDS is an anionic (Salameh & Wiegel, 2010). Different surfactants will influence lipase activity differently. From the four different surfactants, it can be seen that lipase was stable in Tween 20 and Tween 80 at low concentration which is 20%. As the concentration increases the lipase activity decreases. Despite the fact that Tween 20 and Tween 80 are non-ionic detergents as Triton X-100, lipase activity in Tween 20 and Tween 80 was much lower

compared to TritonX-100. This is possibly due to the long acyl ester chains of these detergents (Glogauer et al., 2011). Triton X-100 showed activating effect at low concentration, and as the concentration increases the lipase activity decreases. Surfactant is known to have the ability to intensify the accessibility of reaction sites and stimulate hydrolysis rate (Iyer & Ananthanarayan, 2008). Thus, this explains the enhanced activity of lipase in Triton X-100. Triton X-100 performed as a stimulator for enzyme activity in the enzyme-substrate interaction (Amid, Manap, et al., 2015). The decrease in activity at higher concentration of surfactants is due to lipase inhibition that resulted from high affinity of surfactants for the interface (Diaz, Cordova, Baratti, Carriere, & Abousalham, 2007).

For the anionic surfactant SDS, the lipase activity is highest at 20% concentration with 83.71%. As the concentration of SDS increases the activity of lipase decrease and to some extent deactivated the enzyme. In addition, the decrease of lipase activity was due to the conformation changes in the active site as the result of SDS binding to the proteins. This cause inhibition, partial reversible unfolding of the original structure of the lipase globular proteins' and subsequent inactivation of enzyme (Dos Prazeres, Cruz, & Pastore, 2006). As for the bottom phase component, xylitol showed the highest activating effect with 113.82% in the presence of lipase. It is reported that xylitol increases lipase activity by sustaining the enzyme's open conformation as a result of revealing the active crevice surface site (Amid, Manap, et al., 2015). In addition, polyols is known to increase the surface tension of water which is believed to be the main factor in protein stabilization by sugars (J. K. Kaushik & Bhat, 1998). As the concentration of xylitol increases from 20% to 100%, significant decrease was observed on the lipase activity and this happens because enzymes denatured at higher

concentration. The denatured enzyme becomes incompatible for substrate interaction. The same trend was obtained in another study of xylitol (Amid, Manap, et al., 2015).

Based on the observation made for all the phase components from 20% to 40 %, there is significant drop in the relative activity of lipase. This is possibly due to the disruption of the lipase active site at higher concentrations of phase components (Amid, Manap, et al., 2015). As the concentration rises lipase activity is inhibited which causes the low activity (Hemachander & Puvanakrishnan, 2000). From this preliminary study, it is evident that Triton X-100 and xylitol are suitable for the separation of lipase. Further studies using both of these as phase components for lipase separation via LBF system will be investigated.

Table 6: Effects of different phase components with different concentration on lipase activity

Phase components	Concentration % (w/w)	Relative activity (%)
Triton X-100	**20**	**103.49 ± 1.22**
	40	77.62 ± 1.41
	60	72.43 ± 0.51
	80	70.56 ± 2.14
	100	2.18 ± 3.04
Tween 20	20	82.49 ± 0.17
	40	58.45 ± 0.19
	60	34.47 ± 1.41
	80	23.91 ± 1.34
	100	12.99 ± 1.77
Tween 80	20	86.09± 0.09
	40	41.09 ± 1.65
	60	15.24 ± 0.26
	80	18.05 ± 0.29
	100	13.96 ± 0.56
SDS	20	83.71 ± 0.26
	40	29.65 ± 0.96
	60	27.33 ± 0.87
	80	25.90 ± 0.79
	100	24.46 ± 0.26

Xylitol	**20**	**113.82 ± 0.26**
	40	81.12 ± 0.66
	60	80.87 ± 0.39
	80	80.09 ± 2.18
	100	65.27 ± 3.26

Crude lipase was maintained at room temperature for 60 min in different phase forming concentration and components. The ratio of crude lipase to phase composition used was 1:1. Control was run under similar conditions without the presence of phase components. Each test was performed in three times. Highlighted value indicates the maximum lipase activity obtained from the study and it is selected for the following study.

5.2.2. Optimization of parameters for lipase separation using LBF system

Upon obtaining the suitable phase components and concentration which are 20 % of Triton X-100 and 20 % xylitol, the optimization study for lipase separation is investigated. Optimized conditions for maximum lipase separation efficiency, yield, purification factor and selectivity are obtained by considering the following parameters: xylitol concentrations, types of surfactant, and concentrations of Triton X-100, crude concentrations, volume of bottom and top phases, pH value and flotation time.

5.2.2.1. Effect of xylitol concentration

This study aims to select the right xylitol concentration for maximum lipase separation efficiency and yield. Generally in two-phase system consisting salt as phase component, salt concentration is a critical factor for immiscible two-phase system formation (Show et al., 2013b). Salt usually increases the surface tension of water which then results in the increase of hydrophobic interaction between protein and water that leads to the transfer of biomolecules to the top phase (Wingfield,

1998b). Similar to salts, sugars also have the ability to increase surface tension of water and in this case, xylitol will show the same effect (J. K. Kaushik & Bhat, 1998). From the preliminary study mentioned in **Table 6**, result showed that lipase stable at low concentration of xylitol and the highest lipase activity obtained was at 20% thus, for this study low concentration of xylitol varying from 5 % to 30 % (w/w) was studied.

Figure 26 shows the E and Y of lipase recovered from different concentrations of xylitol. Maximum E and Y of lipase from the LBF system was obtained at 25 % w/w of xylitol concentration with 55.70% and 68.83 % respectively. Each system has its specific minimum concentration of phase components for the formation of two-phase and for utmost recovery of biomolecules (Show et al., 2013b). In this study, 5 % of xylitol concentration was not able to form two-phase system which resulted in low E and Y of lipase. The concentration from 10 % to 20 % was able to form two-phase however, the performance of LBF is the best at 25 % based on the highest E and lipase Y obtained. At higher concentration 30% the E and Y of lipase reduced with 45.99% and 40.45% correspondingly. High concentration of phase component will inhibit lipase activity, this explains for the low E and Y of lipase in the system. Thus, the optimum concentration of xylitol selected for further study was 25% w/w.

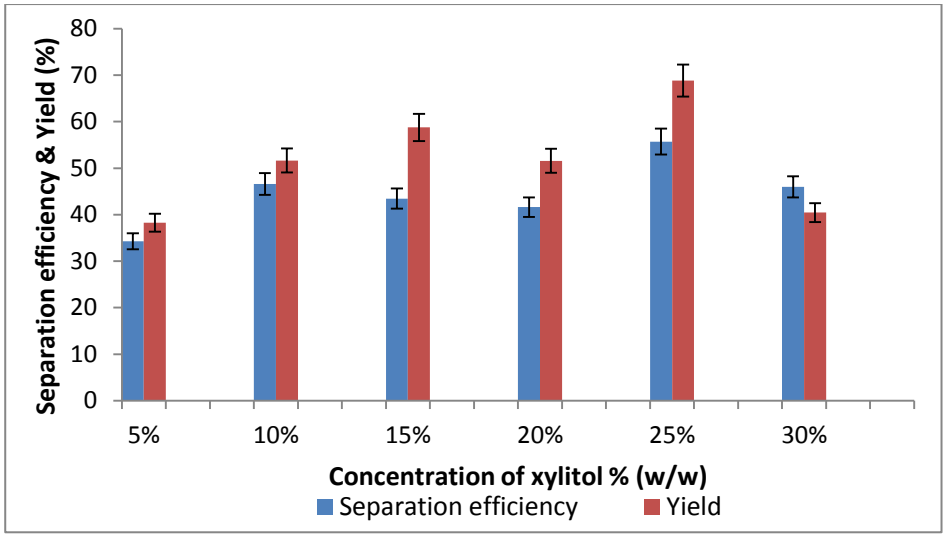


Figure 26: Effect of concentration of xylitol on lipase partitioning in LBF process. The concentrations of xylitol were ranged from 5 to 30% (w/w). Experiment was performed in triplicate.

5.2.2.2. Effect of different type of surfactants

The selection of suitable surfactant is important as dissimilar types of surfactant have diverse interaction with lipase. This study is done to obtain the appropriate surfactant for effective lipase partitioning. The influence of few types of surfactant on lipase Y and S via LBF system was investigated. Four different surfactants were used to study on the LBF performance based on their lipase Y and S. From the preliminary lipase stability test with different concentration of surfactants, it was revealed that lipase is stable at lower concentrations of surfactants. In another study, surfactant has proven to promote the accessibility of reaction sites and increase the hydrolysis rate (M. G. Antov, 2004). Thus, for this study 20 % of surfactant was used in the LBF system for lipase recovery. The effect of lipase partitioning in the presence of different surfactants is determined based on the selective interactions between chemicals that was induced by enzyme

structure and chemical properties of the surfactant (Amid, Murshid, Manap, & Hussin, 2015).

Based on the **Figure 27**, Triton X-100 exhibited highest lipase Y and S with 65.62 % and 1.53 respectively. Non-ionic surfactants demonstrated higher lipase yield and selectivity compared to anionic surfactant which is SDS. This is possibly due to amino acid shielding lids which protect the active site of the enzyme (M. G. Antov, 2004). In addition, the greater lipase yield in non-ionic surfactant is said to be due to the conformational changes of the active site of the enzyme in the presence of non-ionic surfactant (Amid, Murshid, et al., 2015). Partitioning of lipase is highest in Triton X-100 as surfactant, this is because Triton X-100 is believed to be mild detergent in which it provides gentle environment for lipase (A Salabat, Moghadam, & Far, 2010). Lipase enzyme remains active in this environment and hence, it is suitable for separation process. The binding of ionic surfactant to protein probably disrupts the tertiary protein structure. Studies have confirmed that proteins that are bounded to ionic surfactant are linked collectively via electrostatic and hydrophobic forces (V. Kaushik & Roos, 2007; Sunantha Ketnawa, Benjakul, Ling, Martínez-Alvarez, & Rawdkuen, 2013). The interaction between protein-surfactant plays an important role for strong ionic bonds formation which acts as key factor for enzyme partitioning (Amid, Manap, et al., 2015). In this section of experiment, Triton X-100/Xylitol LBF system presented maximum lipase Y and S therefore; it was selected for further optimization.

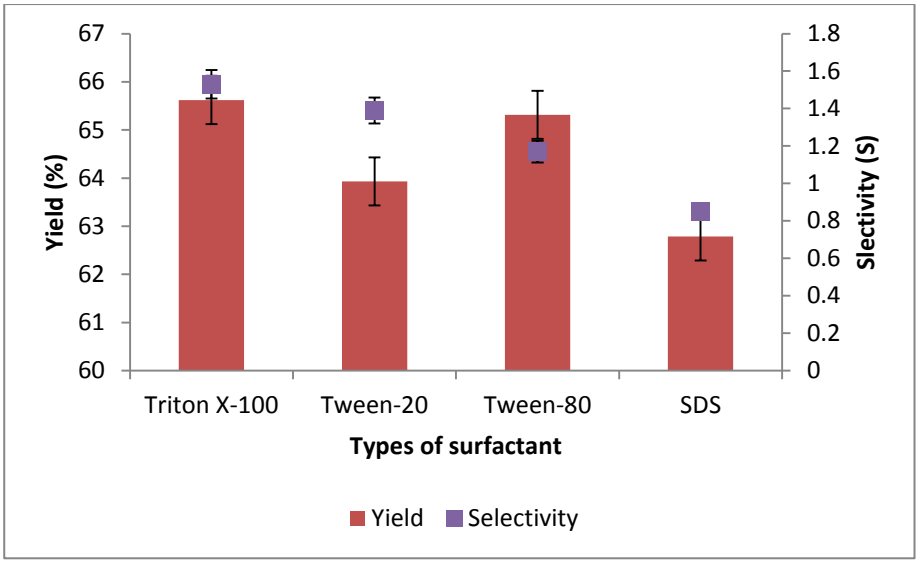


Figure 27: Effects of different type of surfactants on the LBF performance. Lipase yield and selectivity were obtained. Each experiment was performed in triplicate.

5.2.2.3. Effect of lipase partition in different concentrations of Triton X-100

In this particular study, the effect of different concentration of Triton X-100 for greatest lipase yield and purification factor is investigated. This study is necessary to select the optimal Triton X-100 concentration for effective lipase partitioning. **Figure 28**, revealed that the optimal requirement for lipase partitioning is 15 % (w/w) Triton X-100. From the result obtained, it can be inferred that lipase partitioning achieved high Y and P_{FT} at low concentrations of surfactants. At higher concentration, lipase yield and activity were found to be low. In a previous study on Triton X-100 and lipase, it was demonstrated that the activity of lipase was inhibited with the existence of high concentration of Triton X-100 in reaction mixture. The inhibition is due to high affinity of surfactant for the interface and the decrease in the lipase adsorption rates (Diaz et al., 2007). Whereas, at low concentration of Triton X-100, lipases able to escape interfacial denaturation which resulted in higher activity and yield. In addition, in a separate

study it has been described that at high concentrations of surfactants, stability of amino acids decreases significantly (Amid, Manap, et al., 2015). Based on this study maximum Y and P_{FT} was found to be at 15 % (w/w) with 70.70 % and 2.56 respectively and this was selected for further optimization study.

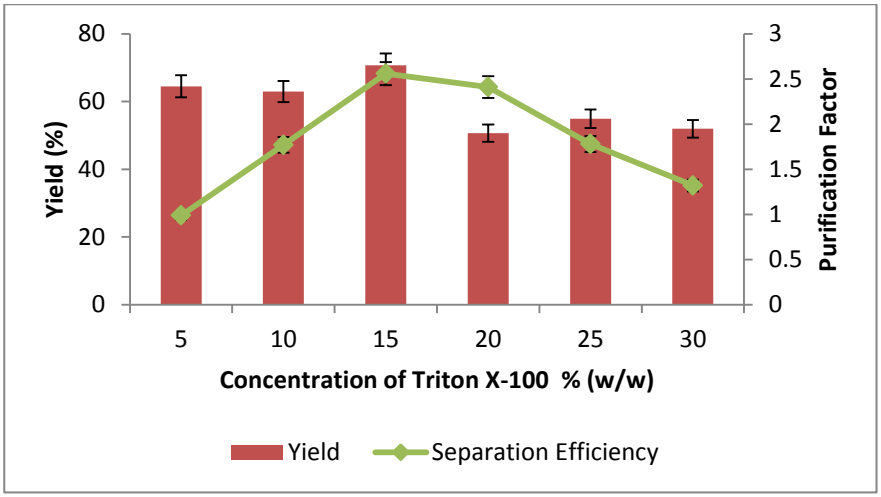


Figure 28: Effect of different concentrations of Triton X-100 on lipase separation efficiency and yield.

5.2.2.4. Effect of crude concentration on lipase partitioning

The purpose of this particular study is to obtain absolute crude lipase concentration for effective lipase partitioning. Generally concentration of crude feedstock plays a significant role in the performance of LBF system. The partitioning behaviour of target protein is affected by the concentration of crude feedstock. The change in crude feedstock concentration causes alteration in the volume ratio and composition of LBF which influences the partitioning performances (Mathiazakan et al., 2016). Therefore, in this experiment the influence of crude feedstock concentration varying from 20% w/w to 100% w/w was investigated. From the results obtained in **Figure 29**, lipase Y and P_{FT} increases with increasing crude extract concentration. Maximum Y and P_{FT} factor

of lipase was found to be 84.06% and 2.7 respectively at 80% of crude feedstock concentration.

Generally in ATPS system, higher load concentration will cause reduction in the partitioning performance due to alteration of volume ratio and composition of ATPS. This will consequently influence the biomolecules partitioning (Ooi, Tey, Hii, Ariff, et al., 2009). However, such behaviour was not identified in this LBF system. It is reported that higher concentration of crude load is beneficial in the recovery process of greater enzyme extract volumes (J. S. Tan et al., 2014). Similar phenomena is attained from another study on partitioning of BLIS from *Pediococcus acidilactici* Kp10 in ATPF system (Md Sidek et al., 2016). 100% crude load concentration showed reduction in the Y and P_{FT}. This is due to high amount of contaminants in the system that causes alteration in the composition and properties of LBF thus, resulting low LBF performance. From this study, 80% w/w of crude lipase was selected for further optimization process.

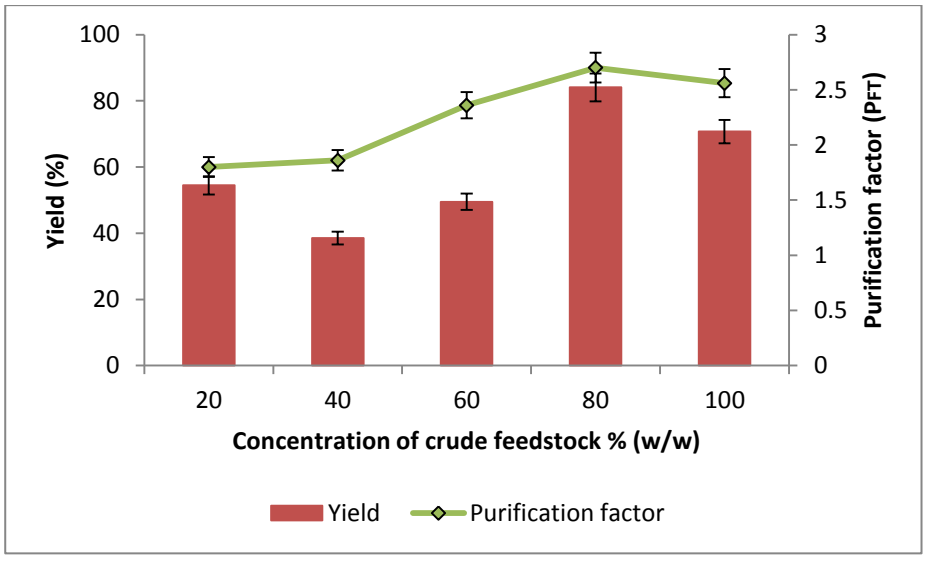


Figure 29: Influence of different crude feedstock lipase concentration on the lipase separating behaviour in the system composing xylitol and Triton X-100.

5.2.2.5. Effect of bottom and top phase volume

This study aims to choose suitable bottom and top phases volume for greatest lipase separation efficiency and selectivity. In LBF, the amount of aqueous bottom phase is larger compared to amount of organic top phase. The effect of bottom phase and top phase on the LBF performance was investigated in this section. As for the bottom phase the range tested was from 20 mL to 40 mL (volume ratio range from 0.15 to 0.075) while the top phase was fixed with 3 mL. This is to study on the lipase E and S, **Figure 30** shows the result obtained. The optimum volume was found to be at 35mL with a volume ratio of 0.089, where highest E and S were obtained with 70.59% and 2.57 S respectively. Based on the results, increase in volume of bottom phase leads to increase in lipase E and S until it reaches a volume of 35mL. Volume higher than that causes a decrease in E and S. Volume ratio (VR) is an important parameter that will affect the partitioning of enzyme and it is expressed as the fraction of quantity between top phases to bottom phase (S Ketnawa, Rungraeng, & Rawdkuen, 2017). As the volume increases the volume ratio decreases and lowest volume ratio which was at 40 mL gives the least E and S. This is because low VR causes low free volume of alcohol in the top phase for lipase partitioning. Optimum VR was found to be at 0.089 with 35 mL as bottom phase volume and was selected for further optimization study.

Figure 31 shows the effect of top phase volume on the LBF performance. Volume of top phase ranging from 1 to 5 mL was tested for maximum lipase E and S. Graph represents the top phase volume in terms of VR. From the graph it is evidently shown that 0.114 of VR which is 4 mL of Triton X-100 achieved the maximum E and S with 71.29 % and 2.62 S respectively. As the volume increases

from 1 to 4 mL there is an increase in E and S however, at 5 mL E and S decreases. This is due to the threshold level of the top phase that causes the accumulation of impurities in the Triton X-100 resulting lower value of S and E. The increase in E and S is due to the increase in free volume available as the top volume increases (Lin et al., 2015). In this study, the minimum volume of Triton X-100 applicable to the LBF system was found to be at 2 mL with VR of 0.057, because volume lowers than that affects the sampling of lipase due to thin layer of the top phase. From this investigation, 4 mL of Triton X-100 was found to be the optimum volume of top phase for the LBF system and was selected for further study.

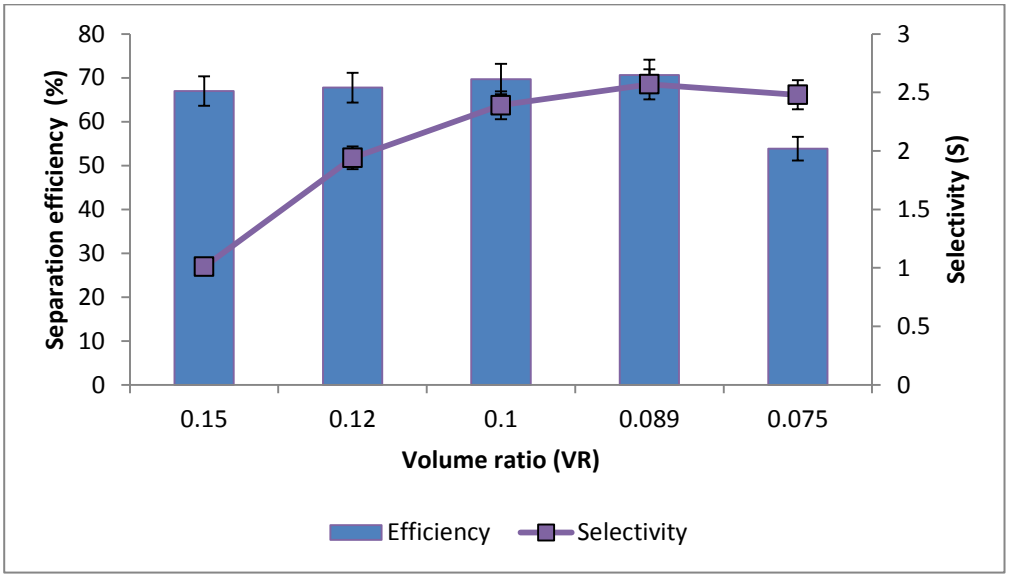


Figure 30: Influence of bottom phase volume in terms of volume ratio on the lipase selectivity and separation efficiency using LBF system

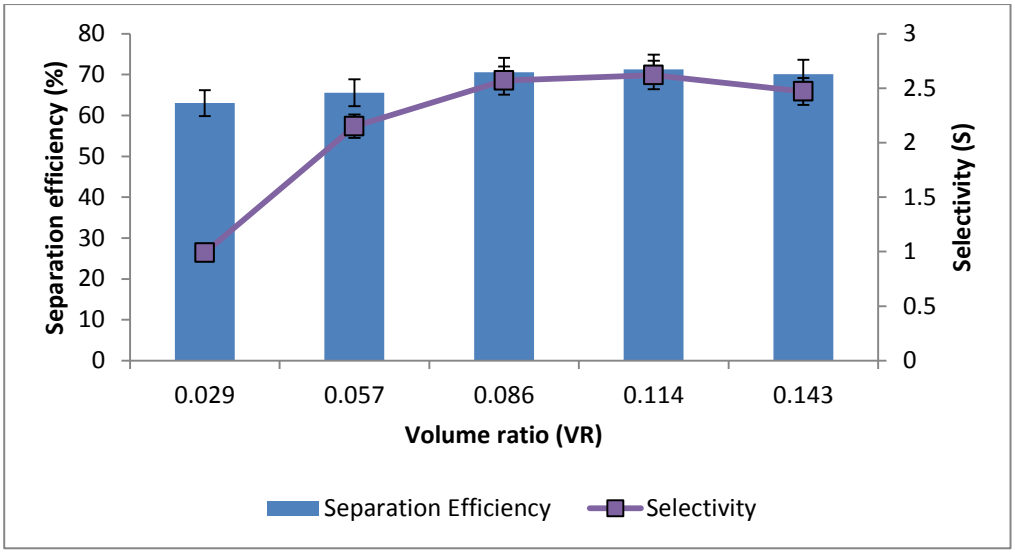


Figure 31: Influence of top phase volume in terms of volume ratio on lipase selectivity and separation efficiency

5.2.2.6. Effect of pH and flotation time on lipase partitioning

The objective of this section is to choose optimal pH and flotation time for effective lipase partitioning. Similarly to ATPS, pH value in LBF system plays a crucial role in biomolecules partitioning. Change in pH will influence the mass transfer of LBF system due to alteration in the adsorption of surface active compounds on the gas bubbles. The natural media containing lipase with xylitol without any alteration in pH is 4. When pH is altered, it causes properties transformation of biomolecules surface that involves surface net charge, molecular shape, surface hydrophobicity and the existence of specific binding site which eventually have an impact on the mass transfer (Lee et al., 2016). In general, lipase is an amphiphilic molecule which comprises of polar and non-polar group naturally. This specific characteristic of lipase initiates the attachment on the exterior layer of ascending gas bubbles in LBF system. Diverse pH values will

modify the net charge and surface properties in the system that ultimately alter the electrostatic relations involving the lipase and liquid aqueous which then disturbs the mass transfer proficiency (Ooi, Tey, Hii, Ariff, et al., 2009). Lipase has a pH approximately 6.3 (Ishimoto, Sugimoto, & Kawai, 2001), at pH 7 the lipase to some extent is negatively charged to neutral and the partitioning will predominantly be influenced by the surface properties as a substitute to net charge. At pH beyond 7~8, lipase is usually negatively charged and they are preferred to be separated into the hydrophobic region. The change in segregation behaviour of lipase is expected from its protein charge. Based on **Figure 32**, the S and Y of lipase is found to be maximum at pH 7 with 3.16 and 87.49% respectively. The partitioning of lipase is lower at pH lower than 7 which maybe due to the fact that lipase is usually active and stable in alkaline pH. Activity of lipase may decrease in acidic pH however, based on the result it can be seen at pH 4 the Y is comparatively high this could be because the neutral condition of the media which able to keep the lipase stable and active. From this study, pH 7 obtained the highest Y and S and it was selected for further optimization study.

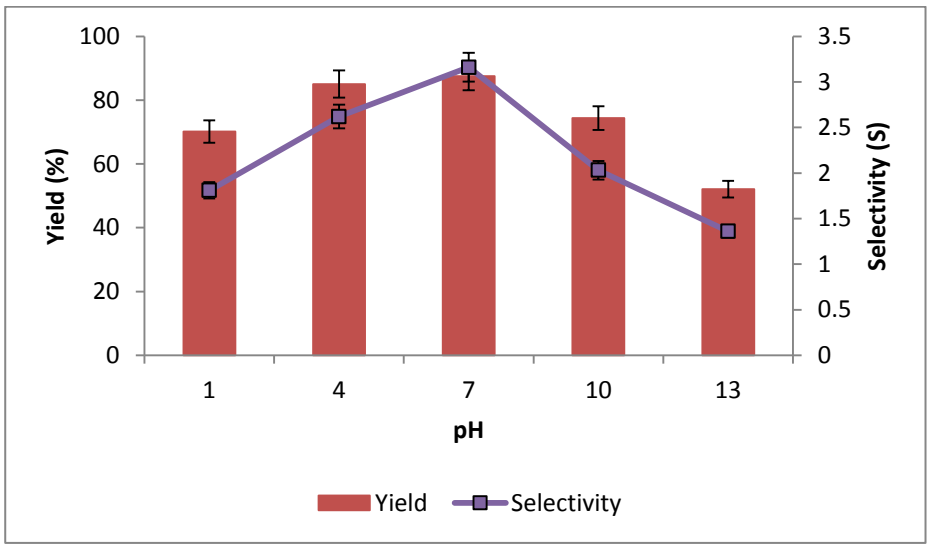


Figure 32: Effect of pH on the lipase yield and selectivity

Flotation time plays an important role in the partitioning of lipase in LBF system. In most cases of LBF system, percentage of separation efficiency of biomolecules will escalates as the flotation time increases till it reaches a stationary state (Show et al., 2013b). The capacity of separation has specific limits in which once the limit is reached to its maximum capacity, further increase in flotation time will not increase the separation efficiency. In this study, the effect of different flotation time from 5 to 30 minutes was studied. From **Figure 33**, it is clearly seen that the E value rose sharply in the first 15 min and beyond that slight decrease was observed. The maximum E and S obtained at 15 min were 86.46% and 3.63 respectively. In addition, long flotation time requires high energy consumption which is costly, non-environmentally friendly and it is not suitable for large scale process. Therefore, from this study 15 min of flotation time was selected for further study.

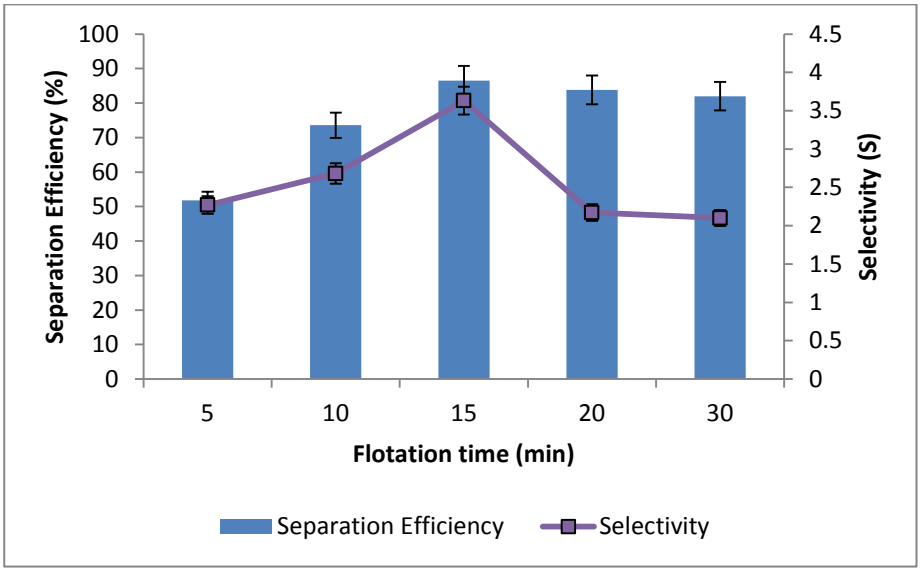


Figure 33: Effect of flotation time on lipase partitioning using LBF system composing surfactant and xylitol

5.2.3. Recycling study of the phase components

After obtaining the optimized conditions for lipase separation, the recycling effect of this novel phase component is investigated. The purpose of this study is to evaluate the efficiency and recycling ability of Triton X-100 and xylitol in the extraction of lipase. Advantage of these phase components in LBF system is the recycling capability of the components. Recycling of xylitol and Triton X-100 in LBF system is a new, inexpensive, environmentally friendly and effective method for biomolecules separation. In this study, recycling effect on the lipase separation and the recovery of phase components were investigated for both small and large scale with the ratio used was 1 to 5. Percentage of Triton X-100, xylitol recovered and lipase separation efficiency using recovered phase components was obtained from this study. **Table 7** demonstrates the performances of LBF in five sequential runs for lipase extraction in small and large scale of LBF system. In all the LBF systems, initial LBF system was set up using fresh phase components. The next four consecutive LBF systems was operated using recovered Triton X-100 and xylitol from previous LBF systems.

Table 7 shows that both phase components Triton X-100 and xylitol can be recovered with high percentage above 97% in both small and large scale LBF system. From both systems, initial value for lipase separation efficiency demonstrated the maximum since new chemical facilitates the LBF to accomplish better lipase separation (Show et al., 2013b). From the first to fourth recycling LBF, there was slight reduction in the efficiency of lipase retrieval. This happens because unwanted proteins and contaminants saturated in aqueous phase which eventually causes decrease in separation efficiency. However, the decrease in

separation efficiency is minor when compared to fifth recycling as there was a significant decrease in separation efficiency after the fourth cycle. Thus, it is recommended to recycle the phase components up to four times for better separation efficiency. Based on the table, it can be evidently seen that there are negligible losses of Triton X-100 and xylitol happened in the recycling steps. This gives an advantage of utilizing these phase components for extraction of biomolecules in industries. In addition, the performance of lipase separation is not affected by the recycled phase components.

On the whole, the sustainable LBF for lipase extraction composing recycled Triton X-100 and xylitol used for small and large scale was successfully achieved up to four times of recycling. This study has shown the reliability of recycling phase components in larger scale. From large scale LBF system, high recovery of surfactant and xylitol were obtained with 97.87%, 98.80% respectively and separation efficiency of 82.77 % after four times of recycling. Hence, this technique demonstrated to be an efficient, ideal, sustainability. Since it is possible to recycle top and bottom phase components, it is a green technology which is able to promote a cleaner bioprocessing technique. Based on the previous objective which uses alcohol/salt as phase components this novel phase components (Triton X-100 and Xylitol) gave higher E, Y, S and $P_{FT.}$ This study has demonstrated that Triton X-100 and Xylitol are better phase components for lipase extraction. In addition, this study also has shown that high recoveries of Triton X-100/xylitol were obtained than alcohol/salt after five times of recycling (Show et al., 2013b). Compared to previous recycling study of 2-propanol/potassium phosphate results obtained showed lower recovery of 2-propanol with only 69.8% whereas in this

study high recovery of Triton X-100 was obtained with 97.17% after five times of recycling.

The purity of lipase from *B. cepacia* using recycling phase components was assessed using SDS-PAGE. This assay is done to confirm that the lipase has been extracted to the top phase. **Figure 34**, describes the bands obtained from the analysis. Lane 2 represents contaminant proteins in crude feedstock. Lane labelled as R1-R5 shows samples that were obtained from LBF using recycled phase components. Generally, lipase from *B. cepacia* strains have molecular weight of about 33 kDa (Show et al., 2011). This study has proven pure lipase extraction employing sustainable recycling LBF. As demonstrated from **Figure 34**, lipase was extracted by using recycled TritonX-100/xylitol process. Most of contaminants and undesired proteins were eliminated from the crude feedstock

Table 7: Recyclability study on surfactant, xylitol recovered and separation efficiency of lipase using recovered phase components in small and large scale of LBF system

		Initial	1st Recycling	2nd Recycling	3rd Recycling	4th Recycling	5th Recycling
Small scale	Surfactant recovered (%)	99.67	99.00	98.33	98.00	97.33	97.17
	Xylitol recovered (%)	99.33	99.17	99.10	99.00	98.33	98.17
	Separation efficiency (E) (%)	86.26	85.09	84.63	83.54	81.73	77.75
Large scale	Surfactant recovered (%)	99.33	98.33	98.20	98.00	97.87	97.20
	Xylitol recovered (%)	99.67	99.47	99.13	99.00	98.80	98.67
	Separation efficiency (E) (%)	87.45	85.58	84.21	83.39	82.77	75.87

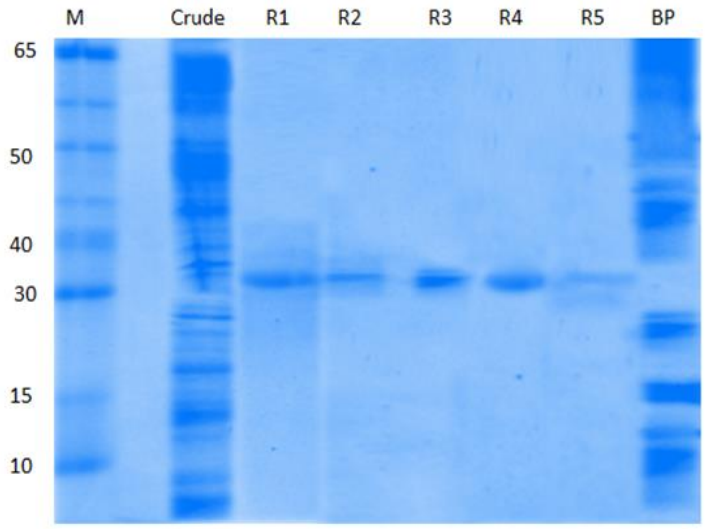


Figure 34: SDS–PAGE assay on recycling LBF. The molecular weight of standard protein marker ranged from 10 to 65 kDa. In the SDS–PAGE, Lane 1: Marker; Lane 2: Crude sample; Lane R1: sample of recycling LBF conducted using recovered chemicals from first LBF; Lane Lane R2: sample from second recycling of LBF; Lane R3: sample from third recycling of LBF; Lane R4: sample from fourth recycling of LBF; Lane R5: sample from fifth recycling of LBF; Lane BP: Bottom phase.

5.3.Conclusion

Recently, the major concern of researchers in biomolecules recovery is the large consumption of chemicals, expensive cost production and environmental issues due to huge chemical waste disposal. These limitations can be avoided or reduced by recycling the phase components. In this study, it is reported for the first time on the recycling of both phase components for small and large-scale lipase separation from *B. cepacia* via LBF system using surfactant and xylitol. It has been demonstrated that the optimised operation conditions for maximum lipase separation and efficiency using LBF system are 25 % w/w of xylitol concentration, 15 % (w/w) Triton X-100, 80% w/w of crude lipase, 4 mL of top phase, 35 mL of bottom phase, pH 7 and 15 minutes of flotation time. Recycling study for both small and large scale study have showed high recovery of Triton X-100 and xylitol could be achieved. In addition, high lipase separation efficiency was obtained using

recycled phase components. The recycling of phases Triton X-100 and xylitol LBF system is an excellent process due to the fact that it is sustainable, environmentally friendly, time saving and effective in terms of cost. Besides, this innovative method is practical and relevant to be employed for the sustainable extraction of other biomolecules. This technique opens up a new prospect for bio-based product extraction in biotechnology field.

The focus on the past two chapters have been on the application of LBF system on recyclable phase components and the discovery of novel phase components for lipase extraction. The remaining concern that has not been discussed is, the innovation of separation method that has minimum number of steps. The following chapter will discuss on the integration process of the upstream and downstream process using LBF system with the aim to minimize the number of step and to improve protein downstream processing.

6. Chapter 6: Integration Process of Fermentation and Liquid Biphasic Flotation for Lipase Separation from *Burkholderia cepacia*

6.1. Introduction

In recent era, developments in biotechnology have intensified the potential applications of biological products. The main challenge encountered by biotechnology industries for sustainable achievement and commercialization are the advancements of simplified and economical bio-separation method that are able to produce extensively purified products which are free with contamination. Biomolecules separation which indicates the extraction and purification especially of enzymes from several fermentation feed streams is deemed to be major unit operation in the bioprocess industry (Gaikaiwari et al., 2012a).

Up to date, all researches concerning LBF system primarily focuses on separation process. In this study, the idea of integrating fermentation and separation process in a single LBF system is proposed. To date, no research has been made on combining fermentation and separation in LBF system. Recently, the implementation of integration process in a single system appears to have immense potential in enhancing the efficiency of many bioprocesses for industrial scale bioproducts (Fresewinkel et al., 2014). In this thesis, the novel approach of integrating fermentation and lipase separation process as a single process was investigated.

In this chapter, the process integration methods for lipase production and separation were investigated with the goal to demonstrate that this novel method is able to increase the productivity significantly at a shorter period with limited step. A detailed study was made to obtain optimum operating conditions for the integration process of lipase production and extraction. The aim of this investigation is to exhibit the performance of flotation system in the fermentation

process and to demonstrate that this process integration for lipase production in a single unit flotation system is an efficient technology which has potential to be applied in industries.

This chapter covers the results obtained from this study and detailed discussion of the results has been provided. This chapter begins with the study on the fermentation using flotation system that is using LBF system. This initial study was conducted to investigate the cell growth and lipase production of bacteria in the presence of alcohol and salt. From the results obtained, it was shown that bacteria unable to grow in the presence of alcohol and salt. To overcome this, fermentation in the flotation system was initially done before adding in the phase components for the separation process. Following this study, the comparison of fermentation between the conventional method which is with the shaker system and aeration with the LBF system was conducted. Detailed results have been explained later in this chapter.

Next studies conducted were on the effect of glucose concentration and air flow rate on the cell growth and lipase production. After obtaining the best glucose concentration and air flow rate for maximum lipase production, optimization study for the integration process was carried out. Parameters that were optimized in this study were (i) crude feedstock concentration, (ii) type, concentration and volume of salt solution, (iii) type, concentration and volume of alcohol (iv) air flow rate and flotation time. This optimization study was performed to obtain the operating conditions that are able to give high lipase separation efficiency, yield and purification factor. Subsequent section was on the comparison study between conventional with optimized integrated flotation method. Final discussion is on the

large scale study of the integration process. This chapter ends with the conclusion obtained from this objective.

6.2. Results and Discussion

6.2.1. Fermentation via flotation system

Before conducting the integration study, it is essential to investigate the effect of fermentation using flotation system. Extractive fermentation and bioconversion in ATPS has been studied widely for over a decade (Sinha, Dey, & Panda, 2000). This process involves the integration of production and in situ product recovery. The advantages of this integration are that it ensures primary recovery and intensifies product formation rate by reducing inhibition by the end-product throughout fermentation (Show et al., 2012). In all the former extractive fermentation studies performed, shake flask was used as the mode of the process. Separation and purification of biomolecules via flotation system known as LBF has emerged as efficient method compared to conventional method ATPS as it provides higher separation efficiency, straightforward operation and milder conditions (Pakhale, Vetal, & Rathod, 2013).

Since separation via flotation has more benefits compared to conventional methods, the idea of integrating fermentation and separation of biomolecules using flotation system as a single system was proposed. This integration process deemed to produce higher lipase production in a shorter period of time. In this current research, flotation system as mode of the fermentation and separation of lipase from *B. cepacia* was studied. The problem with extractive fermentation of lipase from *B. cepacia* in the flotation system using salt and alcohol as phase components is the inhibition of the cell growth due to high concentration of salts and alcohol in

the system. In this section, experiments were conducted to investigate the cell growth and lipase production of bacteria in the presence of alcohol and salt. Fermentation was performed comparing blank (without alcohol/salt), with the presence of alcohol and with presence of salt in the medium. Cell growth is measured in terms of optical density (OD) while lipase production is measured in terms of lipase activity.

Table 8 shows the cell growth and lipase production with the presence of 10 % of alcohol in the fermentation media for 48 h. 48 h of fermentation was selected based on the preliminary study. Longer fermentation time above 48 h will not promote bacteria growth and lipase production and lesser fermentation time was not sufficient for the bacteria to grow. In this section, negative results indicate that there is no bacterial growth and no lipase obtained. **Table 8** clearly shows that even with low concentration of alcohol which is 10 %, the cell growth and lipase production is inhibited. Negative results were obtained for the growth and lipase production for 1-propanol and ethanol, whereas methanol gave 0.1052 OD for cell growth however, lipase production is negative. As for 2-propanol, positive results were obtained for both cell growth and lipase activity with 0.3853 OD and 0.0119 U/mg respectively yet, when compared with blank (without alcohol) the difference was significant. Better result with high cell growth and lipase production was obtained for the blank fermentation. Thus, it is not suitable to grow bacteria in media containing alcohol. **Table 9** shows the cell growth and lipase production in presence of 5 % (w/w) of salt in fermentation media for 48 h. Similarly, all the salts except for tri-sodium citrate gave negative result for lipase production. Tri-sodium citrate gave 0.0621 U/mg however, when compare to blank the reading was much lower. High salt concentration in the fermentation medium inhibits the cell growth

and lipase production, this is supported with another study (Ooi et al., 2011). To overcome this, fermentation in the flotation system was initially done before adding in the phase components for the separation process. The first part of the study, demonstrate the ability of fermentation in the flotation system. Optimizations of glucose concentration and flow rate for the fermentation in the flotation system were carried out to obtain the best parameters for high lipase production.

Table 8: Effect of 10 % of alcohol in the fermentation media on the cell growth and lipase production for 48 h of fermentation process.

Alcohol (10 %)	Cell growth (OD 600)	Lipase Activity (U/mg)
2-Propanol	0.3853	0.0019
1-Propanol	Negative	Negative
Ethanol	Negative	Negative
Methanol	0.1052	Negative
Blank	4.248	0.348

Note: Negative result indicates that there are no cell growth and lipase production in the medium after fermentation.

Table 9: Effect of 5 % (w/w) of salt in fermentation media on the cell growth and lipase production for 48 h of fermentation process.

Types of salt	Cell growth (OD 600)	Lipase Activity (U/mg)
Ammonium sulphate	Negative	Negative
Magnesium sulphate	0.0424	Negative
Tri-sodium citrate	0.136	0.0621
Pottasium phosphate	Negative	Negative
Sodium phosphate	0.2589	0.0041
Blank	4.819	0.3164

6.2.2. Comparison of fermentation between aeration and agitation system

Previous study has demonstrated that fermentation could be done in the flotation system. In this particular study, the comparison between shaker and flotation system for fermentation was studied. This preliminary study is substantial to demonstrate that fermentation process can perform better using the flotation system. Oxygen transfer is an important factor in the fermentation process as oxygen is vital for the microbial growth, which consequently affects the protein and enzyme productions (Najafpour, 2015). Traditional method of fermentation is usually done in a shake flask or a bioreactor whereby the transfer of oxygen is transmitted by means of agitation. Conventional fermentation technique has few limitations such as low product yield and product inhibition (Sinha et al., 2000). To overcome these limitations and to improve the efficiency of upstream and downstream processes, the use of aeration via air bubbles flotation system for fermentation process was proposed in this study. Flotation unit operation has wide unexplored potential in biotechnology field. Preliminary study of fermentation in the flotation system was studied before further investigations on the integration of fermentation and extraction of lipase using LBF system. This study compares the cell growth and lipase production of *B. cepacia* during fermentation by means of flask shaker and by flotation gas bubbles.

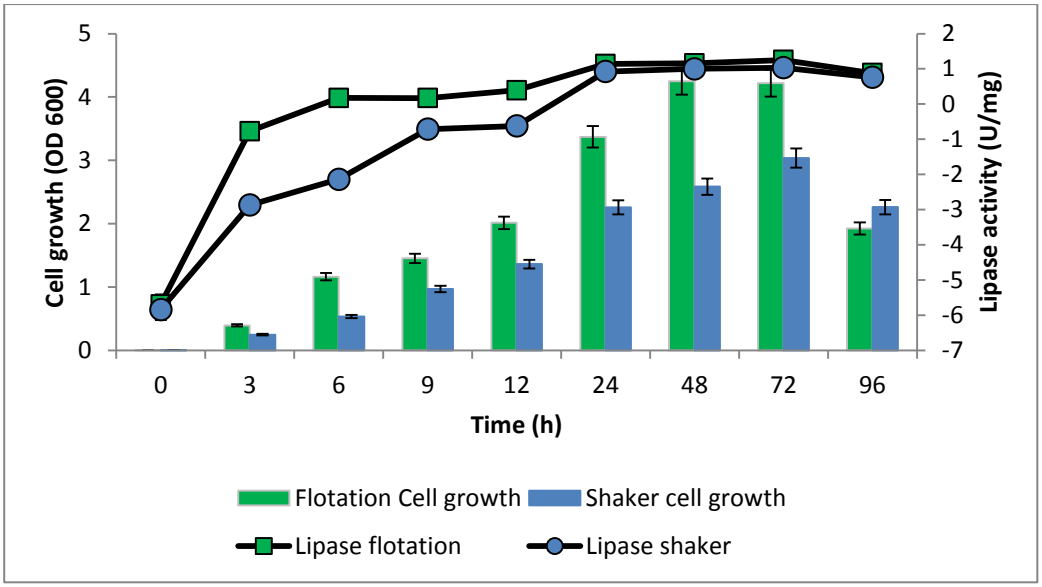


Figure 35: Comparison of cell growth and lipase production during fermentation between shaker and flotation.

Figure 35 shows the difference in the cell growth of *B. cepacia* during fermentation via two different systems. Both fermentation processes were done at room temperature as the flotation system's design does not have temperature controlling facility. **Figure 35** displays the capability of bacterium to grow in a flotation system. There is difference in the bacterial growth between shaker and flotation. As illustrated in the **Figure 35**, the concentration of bacteria and lipase production is high in the flotation system compared to shaker. Bacteria that grow in the flotation system achieved its maximum growth at 48 h with 4.25 OD and slight decrease at 72 h with 4.22 OD and the growth declined greatly at 96 h with 1.93 OD. As for the shaker, the maximum OD was achieved at 72 h with 3.04 OD and decreased to 2.26 at 96 h. Production of lipase was found to be higher in the flotation following the trend of cell growth. Higher cell concentration leads to higher lipase production. For both system, maximum lipase activity was found at 72 h with 1.25 U/mg for flotation whereas the shaker system it is 1.03 U/mg. Based on the results obtained from both system, it can be comprehended that bacteria able

to grow faster and produce higher lipase via flotation system. Generally, in fermentation process, microorganism consumes dissolved oxygen in the liquid media for its growth and that is why aeration and agitation is the utmost important parameter in a fermentation process (Najafpour, 2015). The difference in the growth and lipase production from both systems might be due to the mode of oxygen transfer in the media. In shaker, fermentation was performed based on the agitation mode and the oxygen transfer from the medium whereas in the flotation system, aeration of the gas bubbles helps in the oxygen transfer to the bacteria. The oxygen passes across the gas liquid interface, then the bulk of liquid and in the end into the microbial cell (Bailey & Ollis, 1986). This causes the higher cell growth in the flotation system and subsequently higher lipase production compared to shaker.

Due to the efficient oxygen transfer in the flotation system, the bacterial growth and the lipase production is higher and faster compared to shaker system. The shaker system took 72 h to reach the maximum growth of 3.03 OD and lipase production with 1.03 U/mg, whereas the flotation system took only 24 h to reach almost the same amount of cell growth with 3.37 OD and lipase production with 1.14 U/mg. Thus, this shows flotation system took shorter period to produce the higher amount of cell growth and lipase production from the shaker. From this study, it has been demonstrated 24h of fermentation could give high cell growth and lipase production and 24h as fermentation time is selected for further study. The aim of this preliminary study is to demonstrate that fermentation can be done using flotation system and based on the result obtained, it is clearly shown that flotation system is able to produce higher cell growth and lipase production at shorter period and thus proving that it is relatively better system for fermentation.

The above primary study has proven the efficiency of flotation system for the fermentation. It has been proven that fermentation can be done in flotation with shorter period for lipase production, for the following experiment, fermentation was carried out up to 24 h. Fermentation of *B. cepacia* for 24 h is sufficient to produce adequate amount of lipase for the separation process. The advantages of this system includes higher productivity with less time, energy saving as the fermentation time is shorter and cost effective.

6.2.3. Effect of glucose concentration on cell growth and lipase production

Since the previous study ensured that flotation system gave better cell growth and lipase production, optimization study is conducted to investigate the optimal glucose concentration for maximum lipase production. In this study, the parameter for the fermentation via flotation system was investigated. It was found that glucose utilised as sugar additives in the fermentation process has a profound influence on the production of lipase (Rathi, Saxena, & Gupta, 2001). Studies have shown that, addition of glucose in the fermentation medium stimulates the lipase production which is found in many fungi and additionally in *Mucor hiemalis* (Akhtar, Mirza, & Chughtai, 1980) and *Aspergillus wentii* (Chander, Batish, Sannabhadti, & Srinivasan, 1980). The effect of glucose concentration on the cell growth and lipase production in the flotation unit was studied using five different glucose concentrations from 1 to 9 %. **Figure 36** illustrates the effect of glucose concentration on the cell growth and lipase production. From **Figure 36,** there was an increase in lipase production with an increase in glucose concentration until 5 % and thereafter it decreases. Maximum cell growth was achieved at 5 % glucose with 3.49 OD which is in accordance with lipase production with 7.13 U/mg. High

cell growth contributed to high lipase production. Glucose is used as the carbon source and it was found that glucose concentration is important for fast lipase production (M. D. F. Lopes, Cunha, Clemente, Carrondo, & Crespo, 1999). With high glucose concentration, cells will be able to grow faster and at the same time produce more lipase. As the glucose concentration gets higher, the cell growth becomes slower and thus lower lipase production. Lipase production is hence interrelated to the glucose concentration in the medium and to the growth rate of the cells (M. D. F. Lopes et al., 1999). From the figure, higher glucose concentration above 5 % hindered the cell growth and lipase production.

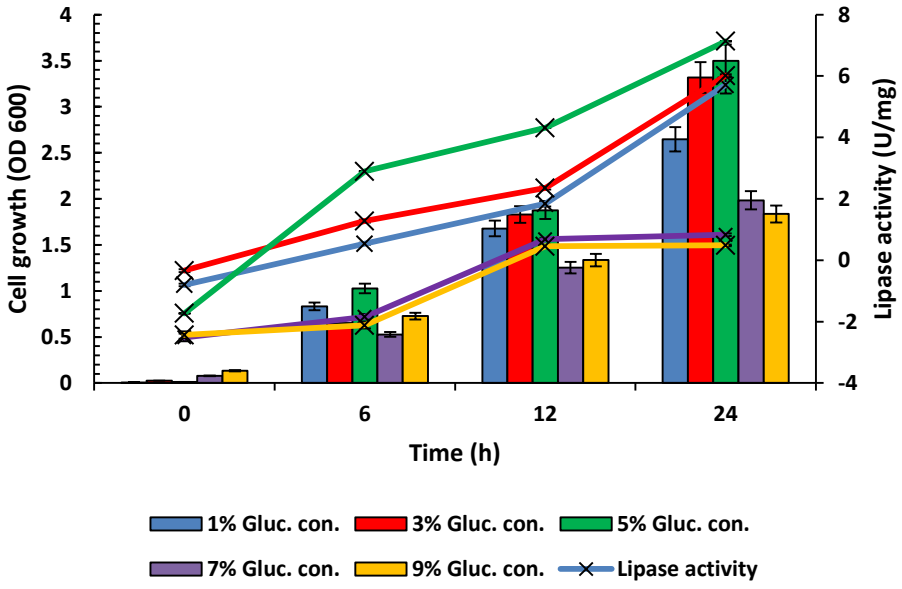


Figure 36: Comparing cell growth and lipase production during fermentation via flotation system at different glucose concentration.

6.2.4. Effect of air flow rate on the cell growth and lipase production

This particular study aims to obtain the best flow rate to be used in the flotation system for maximum lipase production and cell growth. The effect of aeration on the lipase fermentation is shown in **Figure 37**. Aeration is essential in a

fermentation process as it supplies oxygen to the microorganism (Bernardo & Filho, 2005). Four different air flow rates ranging from 50 to 200 cc/min were tested to study the optimum air flow rate for the fermentation via the flotation system. The range of air flow rate was selected based on the preliminary study that was done. From the figure, it is evident that at different air flow rate has resulted in different levels of cell growth and lipase production. Increasing trend of both cell growth and lipase production were observed as the flow rate increases. Based on the maximum cell growth and lipase production at 24 h with OD 4.03 and lipase 7.73 U/mg, 200 cc/min was found to be the optimum flow rate. Higher flow rate above 200 cc/min causes high foam formation. It is not recommended to have the formation of foam during fermentation as it induces contamination and overflows which then consequently leads to loss of broth and products (Al-Masry, 1999). In addition, foam formation causes reduction of mass transfer in the media that subsequently results in lower cell growth and lipase production (Prins & van't Riet, 1987). The structure of the bubbles in the foam is polyhedral, like a honey-comb. Foams are usually formed due to the dispersion of gas bubbles in the liquid phase as the proportion of gas bubbles volume is higher compared to the liquid volume (Al-Masry, 1999). Considering that the flow rate increases, more gas bubbles is form in the vessel. The increasing of bubbles across the surface of the liquid within the vessel coupled with the presence of surface-active molecules stimulates the formation of foam (Delvigne & Lecomte, 2010). Besides, higher flow rate can cause shear effects on microbial cells, which then lead to reduce biomass concentration and lipase production. Thus, 200 cc/min flow rate was chosen for further optimization.

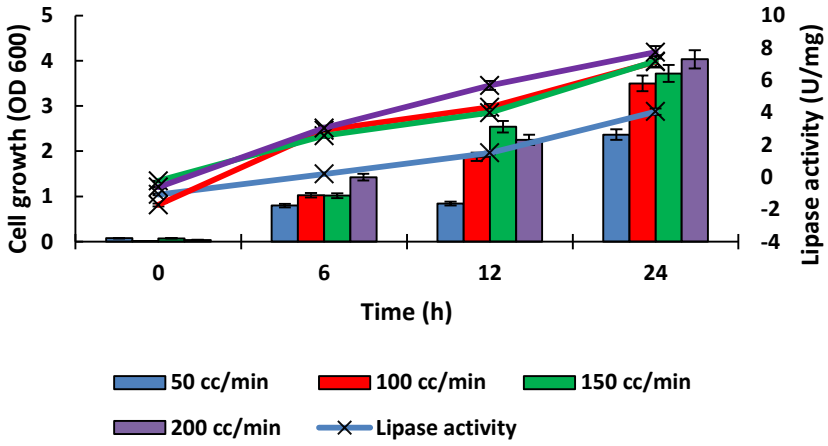


Figure 37: Comparing cell growth and lipase production during fermentation via flotation system at different flow rate.

6.2.5. Optimization of parameters for the separation of lipase after fermentation

This part of study concerns on the separation of lipase from the fermentation media. From the previous section, the optimized conditions obtained for the fermentation in the flotation was 5 % of glucose concentration and the flow rate was 200 cc/min. The fermentation was carried out for 24 h before proceeding for the separation of lipase. The subsequent studies describe the optimization of process parameters for the lipase separation continuing from 24 h fermentation using the optimized condition obtained.

6.2.5.1. Effect of crude feedstock concentration

This section aims to explore the optimal crude feedstock concentration for the integration of fermentation and lipase extraction. The effect of different crude concentration from 20 to 100 % (w/w) was examined (Show et al., 2013b). Preceding studies have been reported that the loading of crude feedstock will alter

the partition behaviour of target protein and separation efficiency (Selvakumar et al., 2010). **Figure 38** illustrates the effect of different concentration of lipase on the lipase Y and E of the flotation system. Highest Y and E were obtained at 40 % of crude feedstock concentration with 80.67% and 83.79% respectively. Higher crude concentration reduces the separation performance, this happens due to high contaminants and impurities in the system (Mathiazakan et al., 2016). The high concentration of unwanted components in the feedstock causes the change in the LBF properties which lead to the decrease in yield and efficiency (Lin et al., 2015). This occurrence can be elucidated with the increase of precipitate at the interface of alcohol and salt phases that leads to the loss of target biomolecules in sync with other impurities (Show et al., 2011). In addition, fermentation of lipase was done in the flotation system before the addition of phase components for the lipase separation process, with these circumstances, higher possibility for the contaminants to be present in the system with higher crude concentration. Thus, 40% (w/w) of crude feedstock was selected as the optimum concentration for further investigation.

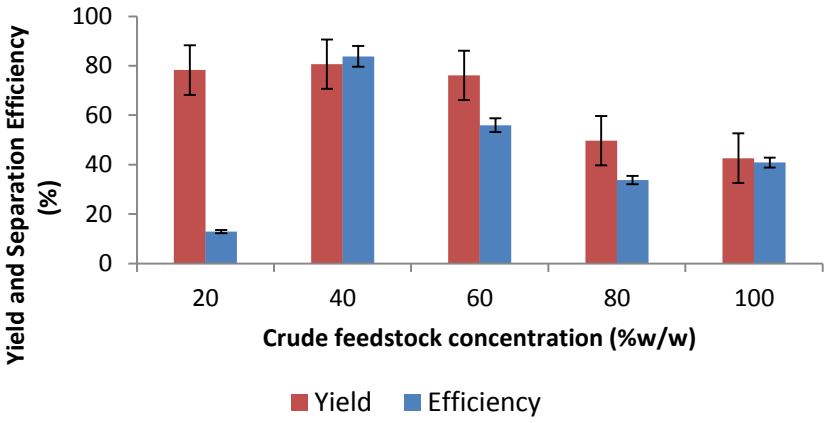


Figure 38: Effect of crude lipase feedstock concentration on the partitioning of lipase in LBF process. The concentrations of crude feedstock were ranged from 20 to100 % (w/w).

6.2.5.2. Effect of the type, concentration and volume of salt solution

This optimization study is done to select the effective type, concentration and volume of salt for maximum lipase separation efficiency, purification factor, yield and selectivity. Salt as a phase-forming component of LBF plays vital influence on liquid−liquid phase equilibria. Surface tension of water increases when salt is added into the solution which then leads to the increase of hydrophobic interaction between protein and water (Wingfield, 1998a). The target biomolecule will shift and accumulate from the aqueous phase to the hydrophilic solvent phase due to salting-out effect. It is reported that the ability of salt for formation of two-phase with the hydrophilic alcohol in LBF is dependent on the Gibbs free energy of hydration of salts (Lu, Yu, Tan, & Yan, 2016).

In this section, the effect of LBF performance from five different types of salt namely ammonium sulphate, tri-sodium citrate, potassium phosphate, sodium phosphate and magnesium sulphate were investigated. The utilization of different salts causes alteration in the environmental phase system which consequently modifies the behaviour of protein partitioning (L. Yang et al., 2010). The selection of salt used for the LBF varies based on their ability to support hydrophobic interactions between biomolecules (H. Yang et al., 2013). Based on **Figure 39**, ammonium sulphate displayed highest S and E with 10.54 and 83.79% respectively. Potassium phosphate showed comparatively high S with 9.94 and E with 62.07%. However, the E of ammonium sulphate is much higher. Ammonium sulphate is better in the separation because generally anions (SO_4^{-2} > HPO_4^{-2} > acetate) are the best for proficient partitioning compared to cations (NH_4^+ > K > Na^+ > Mg^{2+} > Ca^{2+}) (H. Yang et al., 2013). In addition, ammonium sulphate is

known as traditional kosmotropic salt which promotes the stability of protein by favouring hydrophobic interactions (Pakhale & Bhagwat, 2016). Hence, this explains for the high S and E of ammonium sulphate and it is selected for further study.

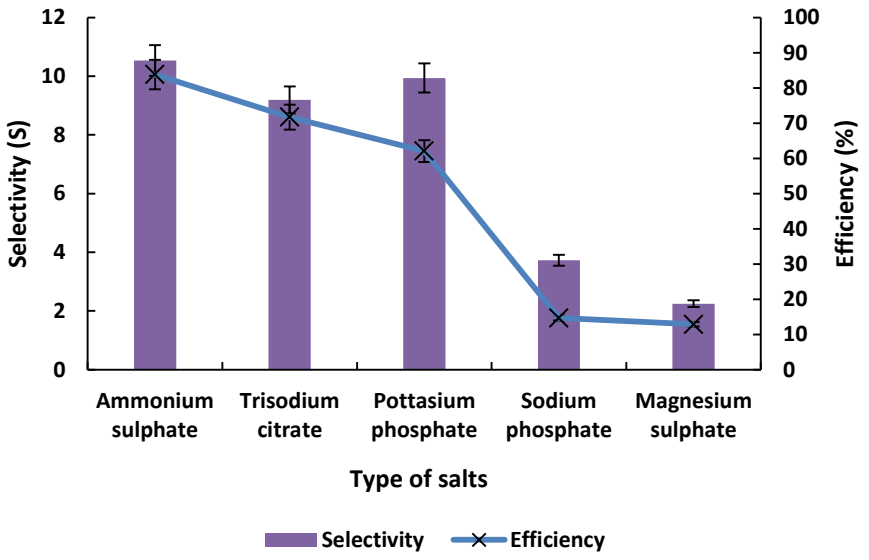


Figure 39: Results showing effects of the type, concentration on the integration performance of LBF. Effect of different types of salt on selectivity and efficiency of lipase extraction.

The concentration and volume of inorganic salt are the two critical factors that need to be considered for the formation of an immiscible two-phase system via salting-out effect. Compared to traditional batch extraction via ATPS, usually LBF method requires higher concentration of salt to maintain a steady immiscible two-phase via salting- out effect. This is because, LBF process applies high volume ratio of aqueous salt to hydrophilic alcohol (Lee et al., 2016). In this section, the effect of different concentration of ammonium sulphate ranging from 200 to 400 g/L on the performance of LBF was examined. This range was selected from previous literature study (Show et al., 2013b). The partitioning of lipase in both hydrophilic alcohol phase and inorganic salt phase of LBF was studied and the

results are shown in **Figure 40**. As the concentration of salt increase to 300 g/L, both S and P_{FT} of lipase increased to 14.44 and 5.81 respectively. Further increase on concentration beyond 300 g/L lead to decrease in the selectivity and purification factor of lipase. Similarly, the study on the separation of puerarin using flotation system showed that the separation efficiency decreases with the increase of ammonium sulphate concentration (P. Y. Bi et al., 2010). The decrease in the lipase S and P_{FT} is possibly due to the strong ionic strengths that are present at high salt concentration solution which influence the reactivity and active sites of enzymes (Ng et al., 2013). Besides, the survivability of lipase in the existence of hydrophilic alcohol phase and inorganic salt phase is valid till a specific extent. This is to ensure ideal phase-forming components environment for the lipase recovery via hydrophilic organic solvent and inorganic salt LBF (Show et al., 2013b).

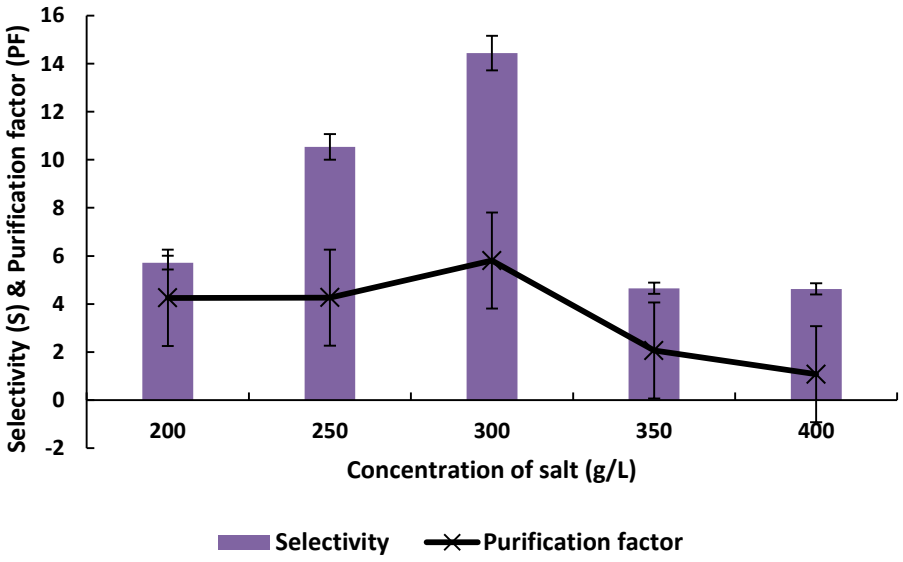


Figure 40: Results showing effects of the type, concentration on the integration performance of LBF. Effect of different concentration of ammonium sulphate on the selectivity and purification factor of lipase.

In LBF the volume of aqueous phase (salt solution) is always higher than the volume of alcoholic phase (alcohol top phase) (Show et al., 2013b). The effect

of ammonium sulphate solution volume on the LBF performance was explored. The range tested was from 150 to 350 mL of ammonium sulphate solution as the top alcohol phase was fixed at 20 mL. This volume range was selected based on the preliminary study. **Figure 41** shows the result obtained for the lipase Y and P_{FT} from the investigation. Based on the results, as the volume increases the lipase yield and purification factor increases until it reaches a volume of 300 mL. As the aqueous volume increases, the volume ratio decreases. It is proposed that higher volume ratio leads to higher free volume of alcohol in the top phase that attracts more lipase for higher P_{FT}. However, in this case at a volume of 300 mL of aqueous phase which is low in volume ratio gives the highest P_{FT} of 10.43 and Y of 90.74 %. Despite the lipase yield for the volume from 150mL to 300 mL were almost similar but the purification factor varies. The low purification factor in the lower volume of aqueous phase might be due to the excessive free volume in the top phase which causes additional impurity protein to be segregated to the alcohol top phase in LBF (Lin et al., 2015). The volume of aqueous solution with 300 mL corresponding to the highest lipase purity and yield was therefore selected for the following studies.

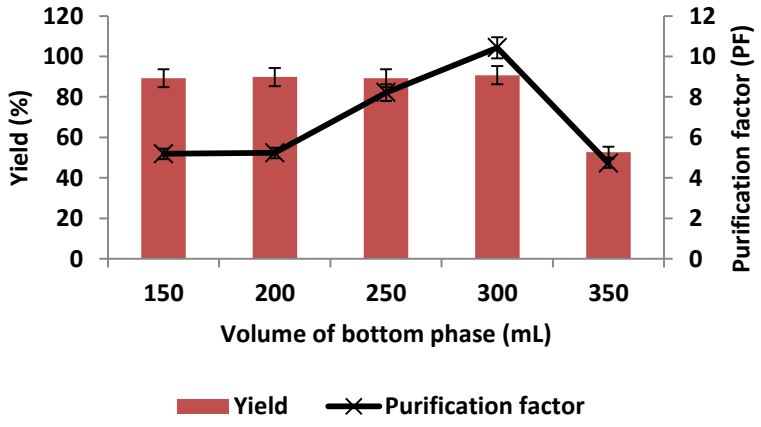


Figure 41: Effect of ammonium sulphate volume on the purification factor and yield of lipase. The results are expressed as the mean of triplicate readings with an estimated error of ±5%.

6.2.5.3. Effect of the type, concentration and volume of alcohol

This experiment is conducted to obtain appropriate type, concentration and volume of alcohol that give the effective lipase partitioning in the integration process. The selection of suitable hydrophilic organic solvent (alcohol) in LBF is important as the interaction of alcohol with lipase differs according to the type of alcohol. For this research, 1-propanol, 2-propanol, methanol, ethanol and acetonitrile were selected to study their effects on the performance of LBF. **Figure 42** illustrates the result of E and Y of lipase. From the result obtained, 1-propanol exhibited the maximum E and lipase Y with 83.36 % and 83.54 % respectively. Propanol was found to be the best alcohol compared to the other solvents, this is due to long hydrocarbon chain of propanol which enhance the interaction of lipase and subsequently increase the partitioning (Ooi, Tey, Hii, Kamal, et al., 2009). As for the methanol, high concentration of methanol was required for the formation of two-phase and they performed the least E and lipase Y with 30.50 % and 24.23 % respectively. Methanol was found to be unsuitable for lipase separation due to excessive rate of evaporation which resulting in great loss of solvent from top phase consequently limited the accumulation of lipase in the alcohol phase (Mathiazakan et al., 2016).

Acetonitrile which has properties of aprotic and strongly polar is an interesting solvent that can be used for the salting-out effect (Cardoso et al., 2014). However, it was found that the efficiency of acetonitrile is lower compared to propanol and this may be due to the low eluotropic strength of acetonitrile compared to propanol (Gilar, Jaworski, & McDonald, 2014). The comparison of elution strength of methanol, propanol and acetonitrile can be arranged following

the order of increasing elution power: methanol < acetonitrile < isopropanol (Gilar et al., 2014). When comparing the performance of LBF between 1-propanol and 2-propanol, excellent lipase separation efficiency and yield was obtained via 1-propanol. This occurrence is possibly due to high hydrophobicity of 1-propanol which makes them form two-phase system with ease (Ooi, Tey, Hii, Kamal, et al., 2009) consequently leading to greatest performance and was chosen for further experimentation.

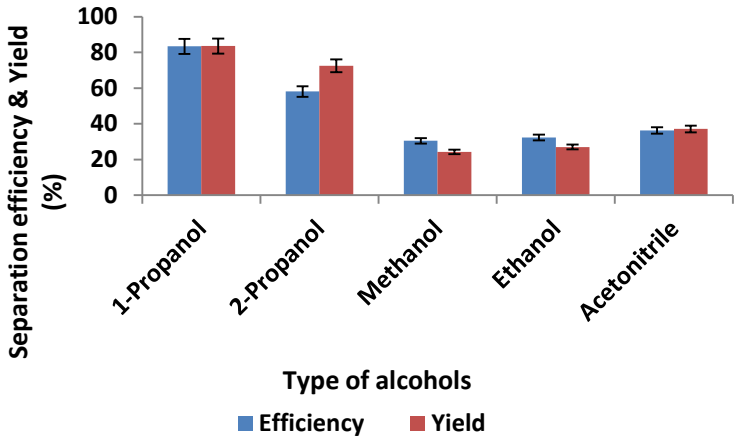


Figure 42: Effect of type of alcohol on the lipase partitioning performance via integration method using LBF system. Influence of alcohol types on lipase separation efficiency and yield. 50% of alcohol with a volume of 20 mL of the top phase and 300 mL of bottom phase was used.

The effect of 1-propanol concentration on the performance of LBF was studied from the range of 35 to 95 (% w/w) (Mathiazakan et al., 2016). From **Figure 43**, the optimal concentration of alcohol was found to be at 80% (w/w) with highest lipase Y and P_{FT} of 89.25 % and 8.22 respectively. Concentration below 50 % (w/w) of alcohol, the ability to form two-phase system is low. System existed as homogenous solution at concentration of alcohol below 50 % this explains the low lipase Y and P_{FT} with 37.15 % and 0.57 accordingly. This result was coherent with the result achieved from a past study (Mathiazakan et al., 2016). As the

concentration of alcohol increases, the Y of lipase increases up to 80 % (w/w) and decreases at 95 %(w/w). Despite there is a slight reduction in the Y of lipase at 65 % (w/w), however the difference is minute which can be ignored. The optimum concentration of 1-propanol of 80 % (w/w) was selected for the next study.

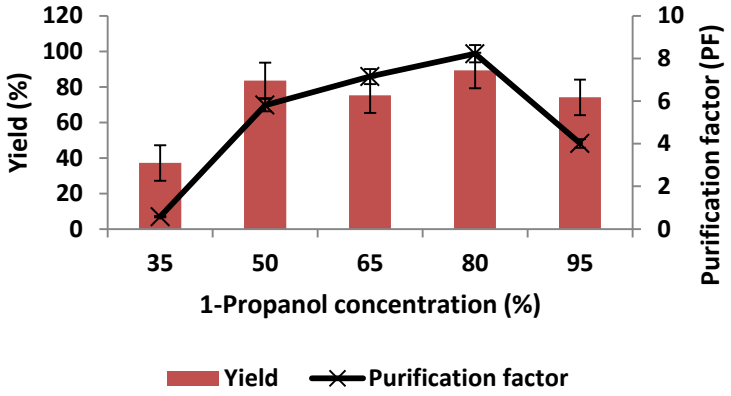


Figure 43: Results of different concentrations % (w/w) of 1-propanol on the performance of lipase yield and purification factor. 20 mL of the top phase and 300 mL of bottom phase was used. All results are expressed as the mean of triplicate readings with an estimated error of ±5%.

As for the volume of top phase, volume of alcohol was examined by using 20 to 60 mL of 1-propanol. **Figure 44** illustrates the separation efficiency and selectivity of lipase at different volume of 1-propanol. The optimum volume was achieved at 30 mL of 1-propanol with E of 84.91 % and lipase S of 28.99 As the volume of alcohol increases, the free volume available would be congruently increased which results in high lipase separation efficiency and selectivity from 20 to 30 mL. However, beyond 30 mL of top phase, there were a decrease of both efficiency and selectivity due to the deposit of impurities in the alcohol top phase. This implies that, there is a threshold of the alcohol volume present in the LBF system for efficient lipase E. In addition, since fermentation was performed in the flotation system prior to separation process more contaminants and impurities are

present in the system. This may cause high possibilities for the impurities to be accumulated at the high free volume of alcohol rich top phase, and thus resulting in low lipase separation efficiency and selectivity. Thus, the optimum volume for excellent lipase separation of LBF system is 30 mL and it was selected for further studies.

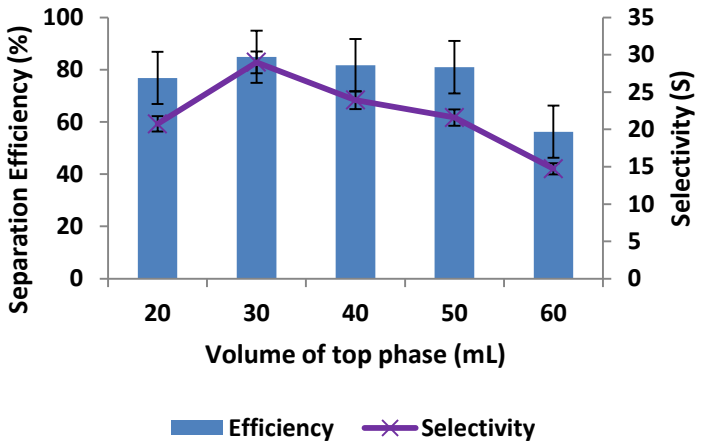


Figure 44: Influence of different volumes of 1-propanol (top phase) on the performance of lipase separation efficiency and selectivity. 80 % (w/w) of 1-propanol and 300 mL of bottom phase was used. All results are expressed as the mean of triplicate readings with an estimated error of ±5%.

6.2.5.4. Effect of air flow rate and flotation time on the performance of LBF for lipase separation

This section examines on the effect of air flow rate and flotation time in the integration process. This study aims to select the optimal flow rate and flotation time for maximum lipase yield and selectivity. Air flow rate and flotation time play major roles in the LBF system as they are able to influence the area of air-water interface per unit volume of aqueous solution in time (Naimi-Jamal, Hamzeali, Mokhtari, Boy, & Kaupp, 2009). **Figure 45** demonstrates the performance of lipase yield and selectivity based on the different air flow rate of the LBF system. It was anticipated that as the air flow rate increases, lipase Y and S increases equally

since the interfacial area and turbulence increases (Pakhale et al., 2013). Based on the **Figure 45** it can be observed that the lipase Y and S increases when the air flow rate increases up to 100 cc/min and beyond that the lipase yield and selectivity declined. The highest Y and S obtained were 93.24 % and 33.03 respectively. Air flow rate at above 100 cc/min causes decrease in the yield because, at high flow rate the interface of the system is disturbed by high turbulence mixing that results in re-dissolution of lipase in bottom phase (P. Y. Bi et al., 2010). Besides, in a study it has been reported that greater number of gas bubbles that are produced from high flow rates could not rupture at the similar rate which then results in accumulation of bubbles on the top phase of flotation system (M. Li & Dong, 2010). Hence, the optimum flow rate selected for further study was 100 cc/min.

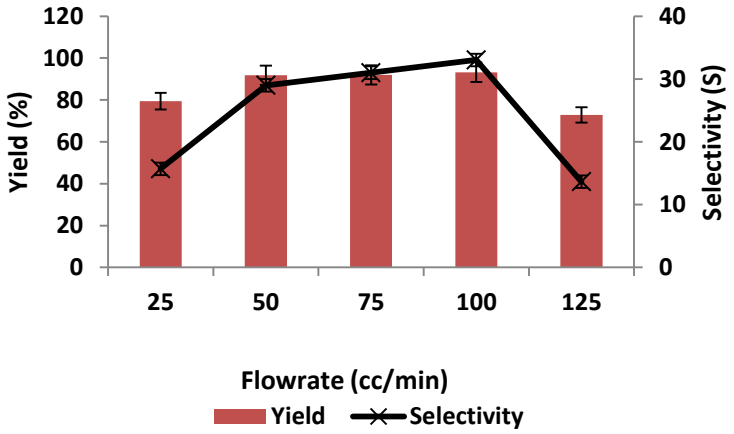


Figure 45: Effect of flow rate on the lipase yield and selectivity. Experiment carried out at 30 mL volume of 80% (w/w) 1-propanol and 300 g/L of ammonium sulphate.

As for the flotation time, the effect of different flotation time ranging from 15 to 60 min was studied. **Figure 46** shows the results obtained for the lipase Y and S at different time. From the figure, it can be observed that the lipase Y and S increased from the first 15 min and reached its maximum at 30 min with 95.73 %

and 35.88 accordingly. After 30 min there was slight decrease in the selectivity however, the Y is above is 80% yet the maximum Y was achieved at 30 min. Thus, it was selected as optimum flotation time for the large scale study.

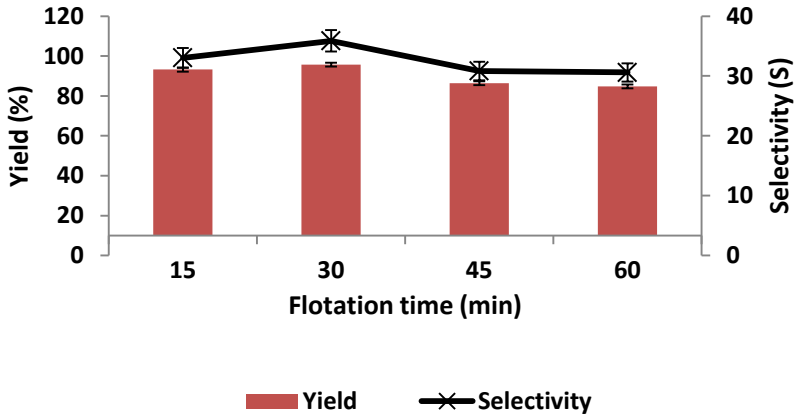


Figure 46: Effect of flotation time upon the performance of LBF. At 30 mL volume of 80% (w/w) 1-propanol and 300 g/L of ammonium sulphate at 100 cc/min of gas flow rate on the lipase yield and selectivity in LBF.

6.2.6. Comparison between conventional and optimized integrated flotation method

After obtaining the optimized conditions for greatest lipase partitioning effect, the comparison between conventional and integration process was conducted. A comparison study of lipase separation methods has been made between the conventional flotation method and integrated (fermentation and separation) flotation method. This study is essential to evaluate the differences between these two methods. Conventional method involves separate fermentation in the shake flask at 24 h and introducing them into flotation system with phase-components for two-phase partitioning process. As for integrated flotation method, both fermentation and separation process were performed continuously in the single flotation column. Since, integration method was performed as a single process, centrifugation process for cell removal for both systems were not

performed for fair comparison. The optimum conditions obtained for the fermentation process via flotation system are 5 % of glucose concentration of fermentation medium and flow rate of 200 cc/min for the fermentation process. This was followed with separation process using the optimized conditions obtained. Both systems were compared using optimized conditions which were 40 % (w/w) crude lipase concentration, 300 mL of 300g/L of ammonium sulphate, 30 mL of 80 % 1-propanol and 100 cc/min of flow rate with 30 min of flotation. **Table 10** shows the comparison study based on the conventional and integrated flotation method for lipase separation. From **Table 10**, it can be evidently seen that novel integrated flotation method performed the best for lipase extraction as it achieved highest E, Y, S and P_{FT} of lipase with 92.29 %, 95.73 %, 35.88 and 11.48 respectively. From the initial study, flotation unit has demonstrated high lipase yield with shorter time from fermentation process compared to shaker. This gives benefit for the integration process for better performance of lipase separation. Continue process in a single system enable the reduction of operation steps, reduces energy consumption and subsequently decreases the overall cost. It is reported that integration method poses significant potential advantage for the recovery of intracellular proteins (Phong et al., 2016). In conclusion, integrated flotation method was proven to perform better than conventional method and hence, was selected for large scale investigation.

Table 10:Comparison study of conventional and integrated flotation system.

Method	Total Lipase Activity (mg/mL)	Efficiency (%)	Yield (%)	Selectivity (S)	Purification factor (P_{FT})
Conventional	5.17	80.11	80.65	25.69	8.11

Integrated	7.74	92.29	95.73	35.88	11.48

Note: Conventional method comprises two separate methods where fermentation done in the shake flask (24 h) and then introducing them into flotation system for separation process. Integration method involves in a single flotation unit whereby fermentation done in the flotation unit and subsequent separation with phase-components for two-phase partitioning process.

6.2.7. Large scale integration of fermentation and separation of lipase

In this section, the scale up of integrated fermentation and separation of lipase in LBF system was conducted. All the parameters that were optimized earlier are kept constant for the large-scale study. This investigation is carried out to demonstrate the ease, reliability and efficiency of this novel method in scale up quantity. For large scale purpose the volume of both phases were increased seven times from the optimized volume obtained. The ratio of small scale : large scale which is 1:7 was selected based on the maximum working volume that can be used for the large scale LBF system. A total of 2.5 L working volume with 2.1 L of bottom phase and 0.21 L of alcohol phase was used. After analysing the results obtained from **Table 11**, relatively high S, P_{FT} and E with 33.95 S, 11.67 P_{FT} and 89.53 % respectively were obtained from this method despite using larger volume. This integrated method has been proven for its consistency for large-scale which is beneficial for direct recovery of other bio-products at industrial scale.

Table 11: Results obtained from small and large scale integration process.

LBF system	Selectivity (S)	Purification Factor (P_{FT})	Efficiency (%)
Small scale	35.88	11.48	92.30
Large scale	33.95	11.67	89.53

Note: Small scale consists of 290 mL of working volume, whereas large scale consists of 2.5L working volume. The results on S, P_{FT} and E were compared between these two systems.

6.3. Conclusion

This chapter introduced a novel integration method of fermentation and separation of lipase using flotation system. The study primarily emphasised on the effects of process parameters on the lipase separation performance. The comparison study between conventional and integrated method showed that integrated method is superlative over traditional method. This study has demonstrated integration method able to extract lipase with high yield in shorter period of time. This could improve the production time of biomolecules and reduce the cost for various biomolecules production.

The usage of LBF system to integrate two processes in a single method is found to be an effective technique in the downstream processing. This study has been a stepping stone for other innovative findings and prospective applications for example protein separation. There are many other potential processes that could be integrated into this LBF system for efficient extraction process. Next chapter will be the study on the potential integration of sonication process with the LBF system.

7. Chapter 7: Integrating ultrasonication and LBF system utilising sugaring-out effect for the extraction of protein from microalgae biomass.

7.1. Introduction

Previous chapters have focused on the different application of LBF in the lipase extraction. From the previous studies, it have been demonstrated that LBF is an efficient technique for lipase separation. In order to explore the potential use of this technique for other biomolecules, this chapter will study on the LBF application for protein separation. This chapter will also aim to study on the potential integration of ultrasonication process with the LBF system.

It has been found that the microalgae proteins contain various bio-functionalities that provide positive impacts not only to health but offer various functional applications in pharmaceuticals, cosmetic industry and nutraceuticals (Samarakoon & Jeon, 2012). It has been reported that protein is one of the main components in the biomass of microalgae other than carbohydrate and lipid (Blackburn & Volkman, 2012). Currently, ultrasonication has been acknowledged extensively by the researchers as an effective technique for cell disruption and many studies have been performed using this technique (Gerde et al., 2012; M. Wang et al., 2014; Wu et al., 2011). High shear forces that are produced from cavitation bubbles of ultrasound help in the cell wall disruption for protein extraction (Dey & Rathod, 2013). In this context, in this study, integration of cell disruption with protein extraction has been proposed for maximum recovery of protein from wet microalgae.

In general, LBF system utilizes the concept of salting-out effect for the separation. In this work, the concept of sugaring-out has been introduced for the extraction of protein from microalgae using LBF system. Therefore, this study emphasises utilising an LBF system to evaluate the effect of sugaring-out for

maximising the protein recovery. Thus, a novel approach of integrating ultrasonication and LBF system utilising sugaring-out effect for the extraction of protein from microalgae biomass of *Chlorella vulgaris* FSP-E was investigated. A thorough investigation was made with the goal to attain optimal operating conditions for protein recovery from the cultivated microalgae biomass using acetonitrile (ACN)/sugar LBF system. Parameters that were investigated include the effect of sonication, biomass concentration, the position/location of sonication probe, ultrasonic pulse mode that includes ON and OFF time, concentration of glucose, types of sugar, acetonitrile concentration, flow rate and scale-up.

This chapter discusses the results obtained for this particular integration process. Initial study is on the effect of ultrasonication and LBF on the protein separation and cell morphology. Following that is the optimization study for maximum protein separation efficiency and yield which includes following parameters (i) microalgae biomass concentration, (ii) location of sonication probe, (iii) sonication pulse mode, continuous sonication, resting period, (iv) glucose concentration and types of sugar and (v) acetonitrile concentration and air flowrate. Next study is on the scale up whereby after obtaining the optimized conditions, large scale of the integration study was conducted.

7.2. Results and Discussion

7.2.1. Effect of ultrasonication and LBF on protein separation and cell morphology

Initial study was conducted to observe the effect of sonication and the combined technique on the algae cell for the extraction of protein in terms of the morphology. Cell disruption is an important technique required in order to increase

the efficiency of protein extraction. There are few methods of algal cell disruption that include enzymatic approach, chemical, osmotic shock, mechanical grinding and others. These conventional methods are generally time consuming and laborious (Bleakley & Hayes, 2017). Recently, ultrasonication assisted protein extraction has attracted the attention of many researchers, as it results in higher extraction yield of biomolecules (Goula, Ververi, Adamopoulou, & Kaderides, 2017; H. L. Jiang, Yang, & Shi, 2017). Besides, ultrasound is easily scalable to large-scale and can be operated continuously (Mittal, Tavanandi, Mantri, & Raghavarao, 2017). Previous study exhibited the effort to extract protein by integrating the cell disruption and ATPS into one single step (Phong et al., 2016). Research investigations also demonstrated that the incorporation of cell disruption with primary extraction process facilitates the processing time with a minimum loss of product quality. This technique enables the enhancement of protein yield and quality (Buyel, Twyman, & Fischer, 2015; Rito-Palomares & Lyddiatt, 2002). LBF system has demonstrated to be an effective technique for the separation of many biomolecules with numerous advantages over ATPS. In this study, a promising approach of incorporating sonication and LBF as a single system has been introduced with the aim to simplify the unit operations in the downstream processing. This approach enables to minimize the overall production cost of protein.

In order to investigate the feasibility of integrating ultrasonication and LBF system to improve the protein extraction, initial study was conducted to observe the effect of sonication and the combined technique on the algae cell for the extraction of protein. **Figure 47** shows the effect of sonication on the cell morphology. This study is essential to evaluate the importance of sonication in the protein separation

process. **Figure 47A** shows the image of algae cell before subjected to sonication. The cells have spherical shape with the intracellular compartments intact within the cell. **Figure 47B** shows the cell morphology after sonication, whereby the cells are introduced to sonication for cell disruption. The cells here are disrupted as the shape is distorted substantially due to sonication. This proves that sonication is important for breaking the cell wall to facilitate the release of protein for the extraction. **Figure 47C** depicts the cell morphology after treating with the integrated method i.e. sonication and LBF system. The cells from this treatment are similar to the cells as observed from only sonication whereby the cell walls are damaged and small cell debris are surrounding the ruptured cells. This study demonstrates that the integrated approach has no significant effect on the cell morphology which allows for the combined process to be carried out for the separation of protein.

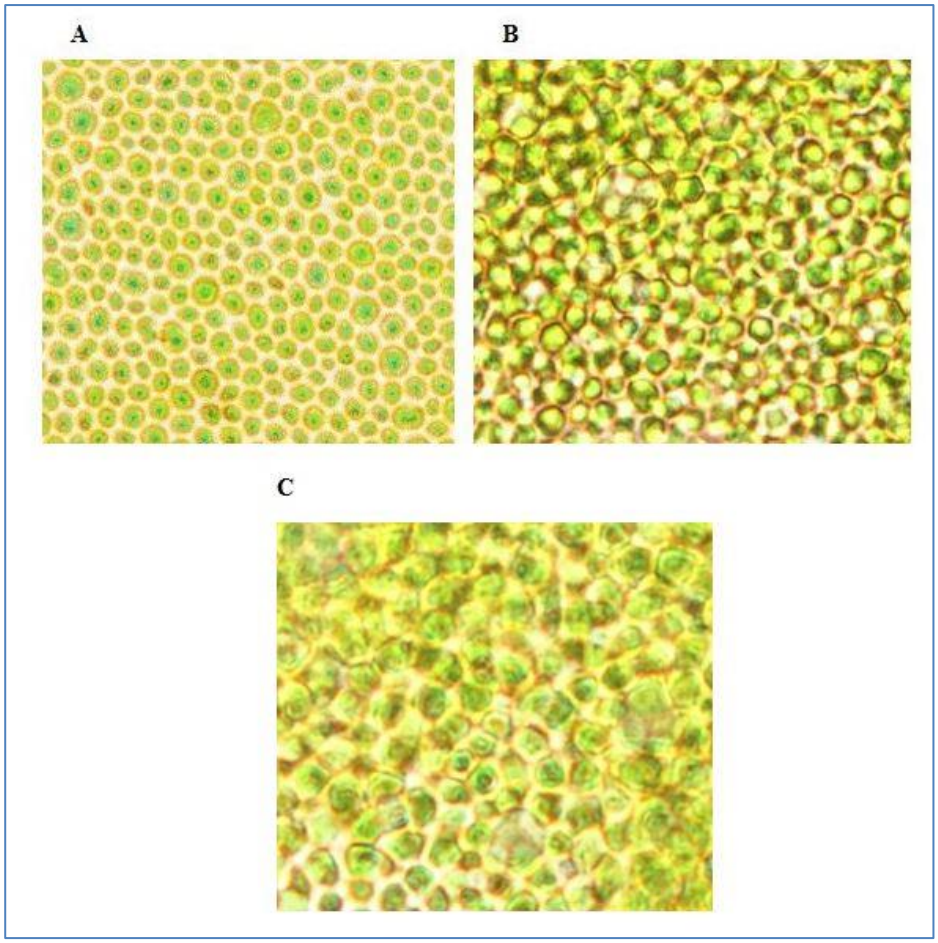


Figure 47: Microscope images of cell morphology of C. vulgaris cells under 1000x of magnification: (A) Before sonication (B) Ultrasonication (200W, 10min), (C) Integrated technique (Ultrasonication: 200W, 10min and LBF).

Next study is focused on the effect of protein separation efficiency and recovery using integration and without integration process. **Figure 48** demonstrates the effect of protein separation efficiency and recovery yield using three different systems. First system involves the protein separation via LBF whereby air bubbles are introduced into the system to aid sugaring out effect without any sonication. While, the second system comprises only sonication to facilitate the protein separation via sugaring out effect. Third system engages the integration of sonication and LBF (air bubbles) for protein extraction. Based on the figure, it could be seen that the integration technique gives the highest amount of protein E

and Y_P with 80.02% and 76.70% respectively, which indicates that the integrated process enables showing a better performance. Sonication is required to disrupt the cells in order to release the intracellular proteins into the solution for the extraction. Notably, without sonication less protein is available and thus the protein E and Y_P are lower. Generally, with the introduction of ultrasound, cavitation is induced in the system which helps in breaking the cells. Cavitation describes the formation and collapse of bubbles which occurs during the rarefaction cycle of sound waves (Rooze, 2012). Thus, when the bubbles collapse close to algae cell walls, the impact of shockwaves easily damages the cell walls thereby inducing the release of substances that are present within the cells (Mercer & Armenta, 2011). Comparing the effect of sonication with and without flotation system demonstrates that the presence of air bubbles facilitates the separation of protein and its yield. Air bubbles produced via flotation provide an additional assistance to cavitation. Without flotation, the system solely depends on the two-phase partitioning which is known as ATPS. It is reported that LBF has advantages as compared to ATPS system that include a higher concentration coefficient and a better separation efficiency (Pakhale et al., 2013). This may be due to the surface of air bubbles which enables the adsorption of surface active proteins which float to the top phase. This aids in the transfer and accumulation of proteins from the bottom phase to the top phase and resulting in higher separation efficiency as well as in their yield. Thus, from this investigation, the process strategy of integration demonstrated that the simultaneous disruption and LBF extraction able to attain a maximum protein separation efficiency and yield.

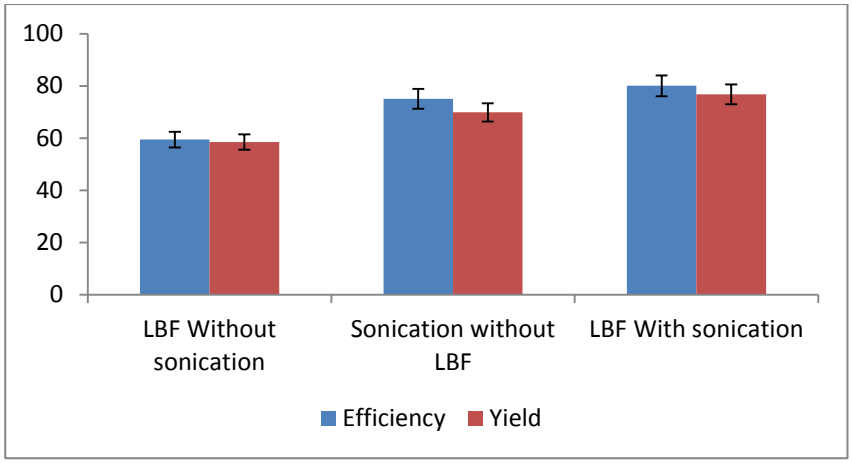


Figure 48: Effect of LBF, sonication and the combined LBF and sonication on the efficiency of protein separation and protein yield

7.2.2. Effect of concentration of microalgae biomass on the protein separation efficiency and yield

From the previous study, it has been demonstrated that integration method gave better results. Thus, further optimization study of the integration process is conducted to obtain optimal conditions for maximum protein separation efficiency and yield. This section focuses to obtain the optimal microalgae biomass concentration. It is known that by increasing the concentration of biomass in a batch process assists in increasing the protein extraction efficiency. In a previous study utilising a LBF system reported that the biomass concentration plays a major role in partitioning effect as it could change the partition behaviour of protein and hence the separation efficiency (Sankaran et al., 2018). For two-phase partitioning system, when the biomass feedstock is loaded above the critical quantity of 5% (w/w) of the system it could affect the protein separation (Selvakumar et al., 2010). It is also reported that as the biomass concentration reaches 5% (w/w) of the system the aqueous solution becomes viscous which then causes difficulties in controlling the air bubbles for the flotation (Phong et al., 2017). In this experiment,

by using the biomass concentration in the range from 0.2 to 1.0 % (w/w) which is below the threshold quantity of aqueous two-phase, the performance of protein partitioning was investigated.

Based on **Figure 49**, a biomass concentration of 0.6 % (w/w) gives a maximum protein E and Y_P of 80.35 % and 76.39 % respectively. With an increase in the loading of biomass concentration into the system it causes decreasing the protein partitioning effect. This occurs due to the accumulation of contaminants and impurities in the aqueous solution affecting the composition of LBF system and resulting in lower E (Show et al., 2011). Besides, a higher biomass results into an increase in the precipitation at the interface of acetonitrile/sugar phases that causes interference with sonication and thus leads to a lower protein separation and efficiency. From this study, 0.6% (w/w) of biomass concentration was selected for further optimization of parameters in the integration of processes.

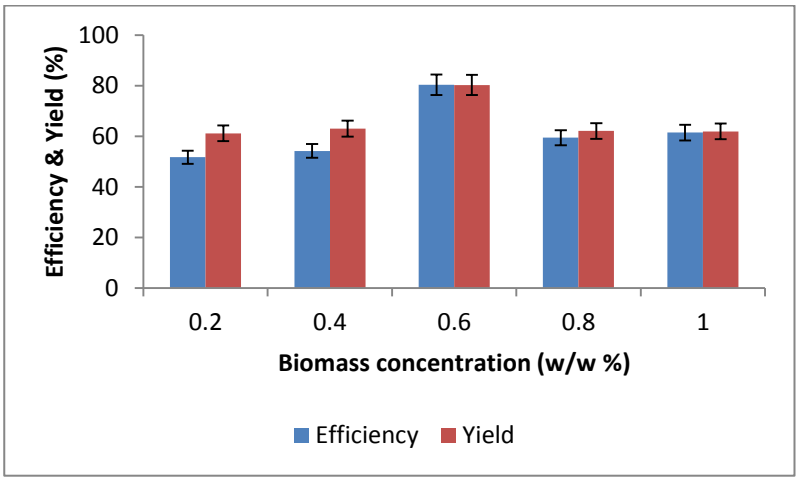


Figure 49: Effect of concentration of biomass feedstock loaded on the protein separation efficiency and yield

7.2.3. Effect of location of sonication probe on the protein separation and yield

Further optimization study is conducted to select the appropriate sonication probe location in the LBF system for highest protein yield and separation. In this study, ultrasonic probe (horn) was used as it provides higher intensity and hence, efficiency since the probe is directly immersed into the solution which delivers ultrasound directly into the liquid and hence it is more efficient as compared to using an ultrasonic bath (Miljevic et al., 2014). The placement or the location of a sonicating probe in the system plays a critical role in the sonication as it possibly causes proteins to degrade and denature (Pchelintsev, Adams, & Nelson, 2016). Suitable positioning of the probe in the system is very important for higher efficiency and to avoid the degradation of protein due to its high power. This study focuses on the effect of location of the probe in the system for the protein separation efficiency and recovery yield. Since two phases are formed in the system due to sugaring out effect, the probe was placed in three different positions i.e. top phase, interface and the bottom phase.

From **Figure 50**, it can be seen that the maximum protein E (82.11%) and Y_P (83.54%) were attained when the probe was placed in the interface of the system. Lowest E and Y_P were achieved when the probe was placed in the top phase which may be due to the absence of microalgae biomass in the top phase. In the liquid biphasic system, the two phases are formed whereby the bottom phase aqueous solution consists of sugar with microalgae biomass solution whereas acetonitrile is in the top phase. Most of the microalgae biomass is present in the bottom phase and there is a minute or no biomass in the top phase (acetonitrile) for the sonicator probe to induce the breaking of the cell walls. Due to this phenomenon, sonication

is affected due to the low biomass concentration and thus less protein is being released for the separation process resulting in lower protein E and Y_P.

When the probe is placed in the bottom phase, higher efficiency was attained as compared to the top phase. However, it is lower as compared to when the probe is in the interface. This is possible due to the direct immersion of the probe in the bottom phase where the presence of a maximum amount of biomass cells could be disrupted by the high intense probe sonication. It is found that higher sonication power could generate high temperature and pressure which could disrupt cells as well as the proteins (Pchelintsev et al., 2016). When the probe is placed directly to the microalgae biomass, the high pressure and temperature could damage the algae cell and proteins which results in lower E. This is absent at the interface which may be due to the even distribution of pressure and temperature in the top and bottom phases of the solution. Since the probe is in the interface there is no direct pressure on the cell which reduces the damage to the cells and protein. Therefore, from this study, the suitable position for the sonication probe to be placed is found to be in the interface (between the top and bottom phases) of the two-phase system.

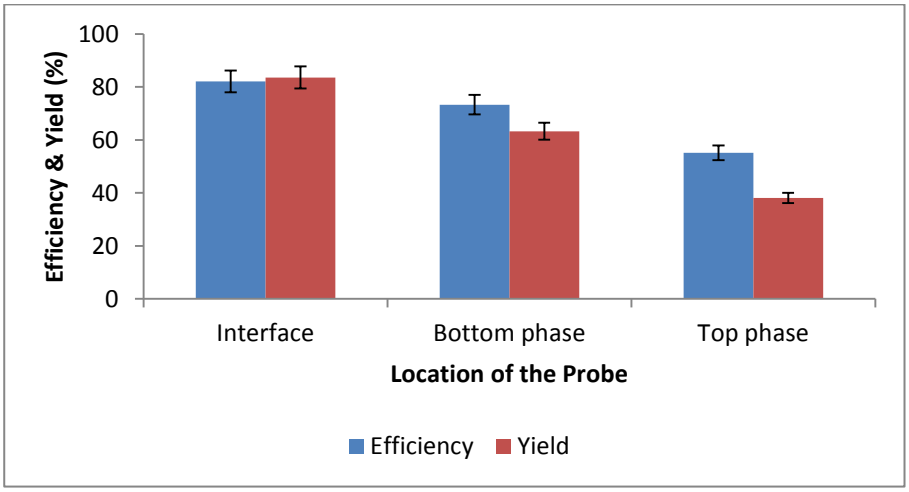


Figure 50: Effect of location of the sonicator probe in the integration system on the protein separation efficiency and yield

7.2.4. Effect of pulse mode, continuous sonication and resting period on the protein separation and yield

This section further explores on the optimization study with the goal to obtain insights on the influence of sonication time, pulse mode and continuous sonication on the protein E and recovery Y_P. This study is important to attain the optimized conditions of sonication for the maximum efficiency of protein separation in the integration process. First part of this section focuses on the effect of pulse mode of sonication. A total of 15 minutes (900 s) of processing time was fixed for this study and experiments were conducted by varying the pulse mode i.e. 5 s, 10 s, 15 s and 20 s ON and 20 s OFF (resting period). Based on the results obtained in Figure 5, it could be observed that shorter pulse mode gives higher E and Y_P with 82.65 % and 90.81% respectively. Comparing with the results obtained from continuous sonication, it clearly demonstrates that the pulse mode operation of ultrasound was found to increase the protein extraction efficiency and yield. This is possible due to the non-steady state of mass transfer operation contrasting to steady state mass transfer in continuous sonication mode (Sivakumar, Anna, Vijayeeswarri, & Swaminathan, 2009). Shorter pulse mode increases the regularity of sonication at a given time that may result in higher separation efficiency.

Figure 51 also demonstrates the effect of continuous sonication on the protein separation efficiency and yield. From this figure, it can be evidently seen that shorter sonication time provides maximum E and Y_P. Continuous sonication for a period of 5 minutes (300 s) achieved a higher protein E and Y_P with 82.19 % and 82.57% respectively. Though the result is lower as compared to the pulse

mode, it can be concluded that a shorter period of time enhanced the productivity and yield. As the sonication time increases, the yield of protein decreases which could be due to the damaging/degrading effect of ultrasonic wave on the proteins (Sengupta et al., 2007). At a higher power of ultrasound, the sound energy could be transformed to heat energy (Ravanfar et al., 2015) and as the sonication time prolonged this could damage and degrade the proteins which could result in lower E and Y_P of protein with longer sonication time.

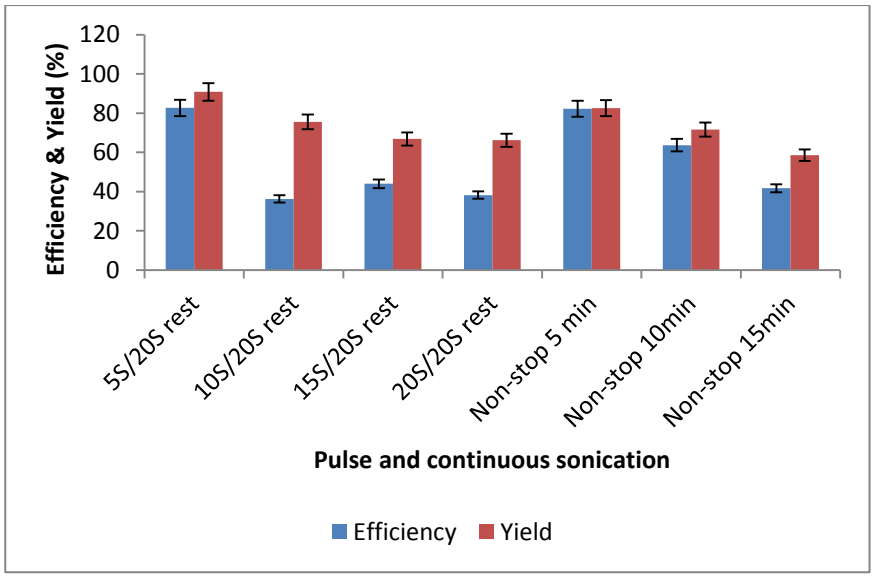


Figure 51: Effect of pulse mode and continuous sonication on the protein separation efficiency and yield

Following the observation of shorter sonication time with short pulse which could increase the separation efficiency, the effect of resting period during sonication was then investigated. Resting period during sonication could influence the protein separation efficiency and yield. In this study, a sonication time of 5 min (300s) and a pulse of 5 s were used to understand the effect of varying the resting period. **Figure 52** shows the results of different resting period ranging from 10s to 40s. It could be observed that shorter resting period contributes to higher E and Y_P with 83.22 % and 91.02 % respectively. With shorter resting time, the frequency of

sonication in 5 minutes is higher (the total effective sonication time) which could increase the cell disruption and increase the protein release for the separation process. This study indicates that at shorter sonication time with short pulse mode and short resting period gives the maximum protein E and Y_P. Through this study, 5 min of sonication with a pulse mode of 5 s "ON" and 10 s off "OFF" was selected for further studies.

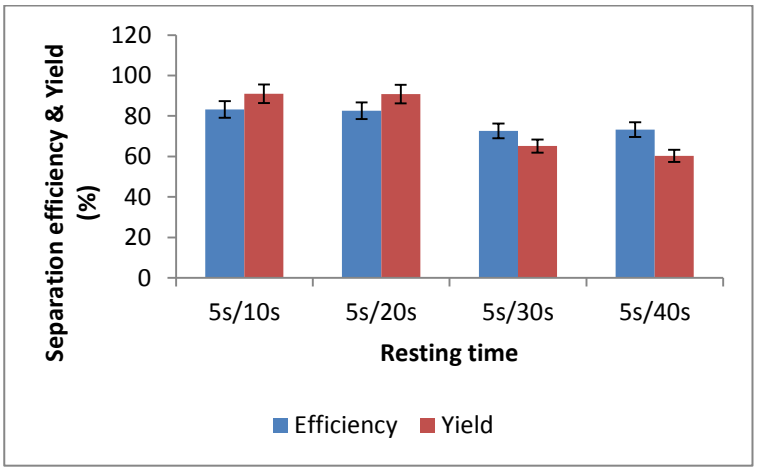


Figure 52: Effect of resting period of ultrasound pulse mode on the protein separation efficiency and yield

7.2.5. Effect of concentration of glucose and types of sugar on the protein separation and yield

This section investigates the influence of glucose concentration and types of sugar for effective protein extraction. Generally for the salting out effect, types of salts as phase forming component and the concentration of salt are important for the formation of two-phase system. Similarly, sugars including glucose, fructose and sucrose play a critical role in inducing the phase formation with acetonitrile for sugaring out effect. The formation of two-phase by acetonitrile–water-glucose is due to their interactions. It is reported that a three dimensional cluster is formed by acetonitrile molecules in which these cluster (acetonitrile–water molecules) are

enclosed together via hydrogen bonding and dipole-dipole interaction by the water molecules. These strong intermolecular interactions of acetonitrile are likely to be substituted by acetonitrile-water complexes (N–H–O) that cause the dimers to be damaged (Szydłowski & Szykuła, 1999). The hydrogen bonds between acetonitrile and water molecules are possibly substituted by sugar molecules (Dhamole et al., 2010b). This causes acetonitrile molecules to be forced out from the solution and subsequently resulting in the formation of a two-phase system. When a higher concentration of glucose is added into the solution, the volume of acetonitrile increased and a better formation of two-phase is observed (B. Wang et al., 2008).

As in the salting out effect, the concentration of salt in the partitioning of biomolecules is important for the formation of a biphasic system. In this study, the effect of glucose concentration ranging from 100 g/L to 300 g/L on the protein partitioning efficiency and yield was studied. This study is vital to obtain the best concentration for the maximum protein partitioning efficiency. **Figure 53** illustrates the results obtained, whereby at a glucose concentration of 200 g/L the highest E and Y_P were achieved by about 83.65 % and 92.26 % respectively. Given that a high concentration of glucose is added into the aqueous solution, less hydrogen bonding is available for the protein to be attached and therefore the proteins are removed to the top phase. As the glucose concentration increases, E and Y_P increased up to a certain limit and decreased beyond that, which is similar in the salting out effect whereby after reaching a limit the partitioning performance decreases (Sankaran et al., 2018). Higher glucose concentration increases the acetonitrile volume and since acetonitrile is non-polar (hydrophobic) in contrast to water which helps in the extraction of non-polar proteins (Dhamole et al., 2016).

From this study, 200 g/L of glucose is found to be the optimized concentration and was selected for further studies.

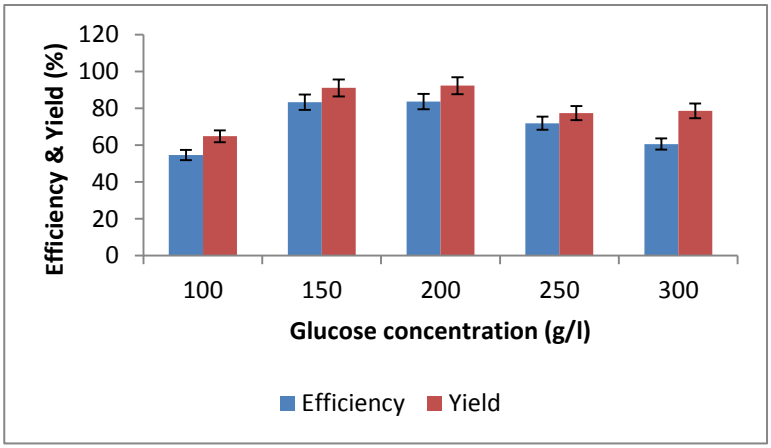


Figure 53: Effect of glucose concentration on the protein separation efficiency and yield

The effect of different types of sugar on the performance of protein partitioning was then investigated. Four types of sugars namely glucose, fructose, sucrose and maltose were used in and their influence on the separation efficiency and yield was studied in **Figure 54**. Based on the results, it could be found that E and Y_P were higher in glucose with 83.75% and 92.78% respectively as compared to other sugars. It is reported that the placement of hydroxyl group on carbon 4 influences the ATPS formation (De Brito Cardoso et al., 2013). By comparing the monosaccharides sugars i.e. glucose and fructose, it shows that glucose is much better in the separation which may be due to the presence of ketoses in the fructose (5 sided rings) which are not efficient in the formation of two-phase system (De Brito Cardoso et al., 2013). In case of sucrose, it is able to form eight hydrogen bonds with water, whereas glucose can form only five hydrogen bonds for a similar mass of sugar which in this study is 200g/L. Therefore, the number of hydrogen bonds formed by glucose is higher than sucrose which results in a higher volume of

acetonitrile to be pushed out to the top phase that causes a higher separation efficiency (Dhamole et al., 2016). This observation matches with the earlier study that demonstrated a higher separation efficiency of glucose as compared to sucrose (Bachchhav et al., 2017).

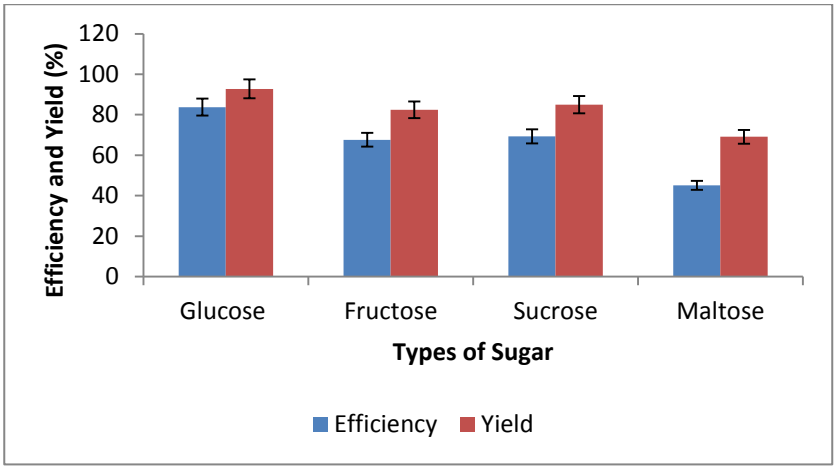


Figure 54: Effect of types of sugar on the protein partitioning performance

7.2.6. Effect of concentration of acetonitrile and the air flowrate in the flotation system

Next optimization study is on the acetonitrile concentration. The influence of acetonitrile concentration on the protein partitioning was investigated. **Figure 55** shows the results obtained on the performance of protein separation by varying the concentrations of acetonitrile i.e. 70%, 85% and 100%. Based on this figure, it can be seen that the best acetonitrile concentration was found to be at 100% which resulted in 84.02% and 92.25% of E and Y_P respectively. Lower concentration of acetonitrile could not sustain the two phase formation and consequently led to lower E and Y_P. It is predicted that at a lower concentration of acetonitrile there will be more hydrogen bonds between acetonitrile and water. Since more number of water molecules are available, there will be a higher chance for more glucose

and water hydrogen bonds resulting in a weak formation of two phase system. Owing to this, for the following study, 100% acetonitrile was selected as the optimized concentration.

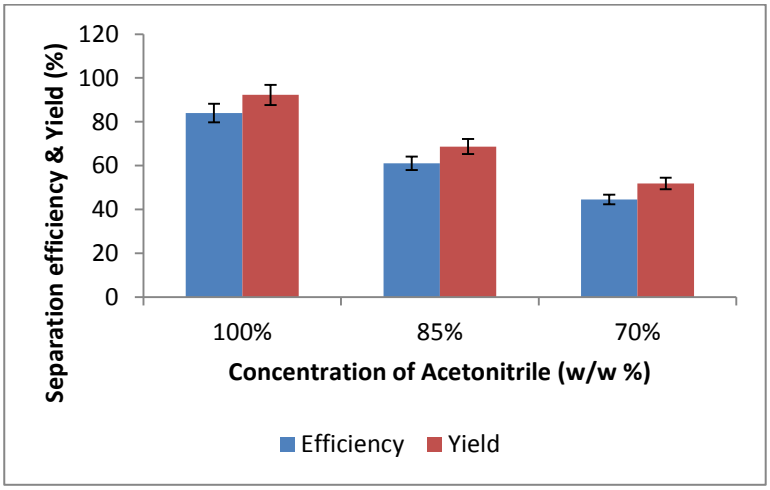


Figure 55: Effect of concentration of acetonitrile on the protein partitioning performance

Final optimization study is on the air flow rate of the flotation system. Air flow rate has a key role in the LBF system as it can influence the area of air-water interface per unit volume of aqueous solution in a specific time (Naimi-Jamal et al., 2009). **Figure 56** exhibits the performance of protein separation efficiency, E and Y_P, yield by varying the air flow rates of LBF system from 25 cc/min to 150 cc/min. According to Pakhale's prediction, E and Y_P increase as the air flow rate increases due to the increase in the interfacial area and turbulence (Pakhale et al., 2013). This can be clearly seen from the current study which shows that as the flow rate increases from 25cc/min to 100cc/min both E and Y_P increased and the highest E and Y_P recorded were 86.38% and 93.33% respectively. Beyond 100 cc/min, the E and Y_P showed a decrement which may be due to the intense turbulence resulting from high flow rate that causes re-dissolution of protein into the bottom phase (P.-

Y. Bi, Dong, & Dong, 2010). In addition, a higher flow rate could cause an influence on the cavitation phenomenon resulting from ultrasound.

As described earlier, cavitation is the process of formation and collapse of bubbles which occurs during the rarefaction phase of sound waves (Rooze, 2012). It is reported that the existence of dissolved gas could decrease the cavitation threshold. Also, it has been demonstrated that the flow of air bubbles influences the oxidative process (Gondrexon et al., 1997). In this case, instead of a dissolved gas, introducing air in the flotation system could affect the cavitation resulting from sonication which in turn might influence the protein separation. Higher air flow rates in the system could possibly disturb the cavitation process due to higher air pressure that subsequently affects the cell disruption and protein partitioning. From this study, 100 cc/min was selected as the optimized flow rate.

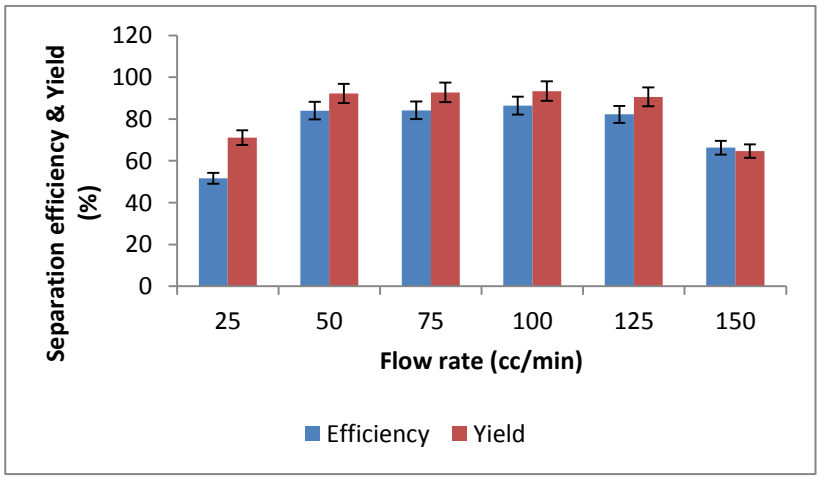


Figure 56: Influence of the flow rate in the flotation process on protein partitioning

From the optimization study, it was found that the optimal conditions for maximum protein separation efficiency and yield were as follows: 0.6% w/w of microalgae biomass concentration, sonication probe location at interface, 5 minutes of sonication with a pulse mode of 5 s "ON" and 10 s off "OFF", 200 g/L of

glucose, 100% acetonitrile and 100 cc/min. Based on this optimized conditions, further scale up study is conducted.

7.2.7. Scale-up of the integration system

Integration of sonication and separation of protein in LBF system on a large-scale was also investigated. Parameters that were optimized previously have been kept constant for the large-scale study. This exploration study was conducted to validate the ease, reliability and effectiveness of this new method in the scale-up. As for the large-scale study, the volumes of both upper and lower phases were increased to five times as compared to the small scale study. A total of 1.5 L of working volume with 0.75 L of bottom phase and 0.75 L of acetonitrile were used. After evaluating the results obtained from **Table 12**, a relatively higher E and Y_P with 85.25 % and 92.24 % respectively were attained from the integration method despite using a higher volume. Based on the results obtained, this integration study has been verified for its reliability in scale-up which is valuable for the extraction of other biomolecules on an industrial scale.

Table 12: Comparison between small and large-scale of the integration method for the protein separation

LBF system	Separation efficiency (E %)	Recovery Yield (%)
Small-scale	86.38	93.33
Large-scale	85.25	92.94

7.3. Conclusion

This investigation reports on a novel integration method of ultrasound and LBF system for the extraction of protein. This study provides comprehensive information on the effective extraction of protein from *Chlorella vulgaris* FSP-E and more importantly several parameters such as biomass concentration, position of sonication probe, sonication time, pulse mode, glucose concentration, types of sugar, acetonitrile concentration and flow rate were optimized for improving the separation efficiency and yield of protein by employing ultrasonication and sugaring-out effects. In addition, large-scale study was also conducted to verify the reliability of the developed integration system for larger volumes and for its reproducibility. Higher separation efficiency and yield of protein with 86.38% and 93.33% respectively were obtained at a biomass concentration of 0.6%, glucose concentration of 200 g/L, acetonitrile concentration of 100% with 5 min of 5s ON/10s OFF pulse mode and at a flow rate of 100 cc/min. The advantages of this process include simple process with fewer steps and having a less processing time which could provide in enhancing the yield of protein as compared to the conventional techniques. This integration method could be an alternative method for cell disruption and extraction of various bioactive compounds from plants.

Chapter 8: Conclusion and Future Studies

With the rapid development of biotechnology in the last two decades, many studies have been done to upgrade the downstream processing. Major challenge encountered by biotechnology industries today is economical bio-separation method for high purity products. In addition, high value bioproducts such as proteins are generally labile substances that require mild processing technique in order to maintain its biological activity and functionality. In most of the conventional biomolecules recovery techniques, comprises several steps of unit operations, costly, environmentally non-friendly and difficult in scaling up.

LBF technique is a relatively new method that is composed from the combination of the principles of aqueous two-phase system (ATPS) and solvent sublation (SS). This technique able to overcome the drawbacks related to previously-developed technologies. This includes simple operation, high separation efficiency, environmentally friendly, high concentration coefficient and economical approach. Additionally, this system is easy to adopt as it has simple operation conditions. Even though there are many advantages, the full potential of LBF system in large scale and prospective integration with other methods as simultaneous and continuous process is yet to be discovered.

In this dissertation, the diverse applications of LBF system for biomolecules separation were investigated. The use of LBF with green solvents and recyclable phase components can increase the potential applications of this system for numerous biomolecules. This study has open up a new research area of LBF by incorporating different processes with LBF system as one continuous process for effective downstream process. The combination of fermentation and ultrasound processes with LBF system has shown the diversification of LBF applications as an efficient tool for the upstream and downstream process.

From the researches done in this thesis, the results attained are very promising indicating that LBF has an amazing potential to become a powerful technique for the extraction of various biomolecules. LBF system has demonstrated efficient biomolecules recovery, simple operation, low-cost and environmental friendly approach. Since this is an emerging technique in the biotechnology field, there is a vast uncovered area of research in the field. To begin with, large scale study using working volume that is similar to industrial scale will give better impact of this LBF system in the industrial application. Up to date, large scale study has been made up to 2.5L, larger volume will improve the bioprocessing field.

Further research work can be done which includes the study on kinetics, thermodynamics and also on the development of theoretical models for LBF. In addition, complete modelling of LBF process is essential for rapid assessment of the outcome of diverse process parameters for potential scale-up of this system for extraction and purification of huge amount of biomolecules. Besides, there are many processes that could be integrated with LBF system for superior downstream process. In this regard, future studies can be done to investigate additional integration processes with LBF system.

Additionally, future computational approach and using different techniques of DoE in selecting the operating conditions could be an option for future works. This simulation work could study on the combined effects of the parameters and also on the inter reaction between different parameters that could improve the bioprocessing field. Besides that, improving the design of the LBF system could be done by understanding and modifying the mechanism of this LBF system able to enhance the downstream processing.

References

Aguilar, O., Albiter, V., Serrano-Carreón, L., & Rito-Palomares, M. (2006). Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by E. coli. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *835*(1–2), 77–83. https://doi.org/10.1016/j.jchromb.2006.03.016

Akhtar, M. W., Mirza, A. Q., & Chughtai, M. I. D. (1980). Lipase induction in Mucor hiemalis. *Applied and Environmental Microbiology*, *40*(2), 257–263.

Al-Masry, W. A. (1999). Effects of antifoam and scale-up on operation of bioreactors. *Chemical Engineering and Processing: Process Intensification*, *38*(3), 197–201. https://doi.org/10.1016/S0255-2701(99)00014-8

Al-Zuhair, S. (2007). Production of biodiesel: possibilities and challenges. *Biofuels, Bioproducts and Biorefining*, *1*(1), 57–66. https://doi.org/10.1002/bbb.2

Albuquerque, T. L. De, Da Silva, I. J., De MacEdo, G. R., & Rocha, M. V. P. (2014). Biotechnological production of xylitol from lignocellulosic wastes: A review. *Process Biochemistry*, *49*(11), 1779–1789. https://doi.org/10.1016/j.procbio.2014.07.010

Almeida, A. F. de, Terrasan, C. R. F., Terrone, C. C., Tauk-Tornisielo, S. M., & Carmona, E. C. (2017). Biochemical properties of free and immobilized Candida viswanathii lipase on octyl-agarose support: Hydrolysis of triacylglycerol and soy lecithin. *Process Biochemistry*, (September), 0–1. https://doi.org/10.1016/j.procbio.2017.10.019

Alves, R. W., Ulson De Souza, A. A., Ulson De Souza, S. M. D. A. G., & Jauregi, P. (2006). Recovery of norbixin from a raw extraction solution of annatto pigments using colloidal gas aphrons (CGAs). *Separation and Purification Technology*, *48*(2), 208–213. https://doi.org/10.1016/j.seppur.2005.07.014

Amid, M., Manap, M. Y., Hussin, M., & Mustafa, S. (2015). A novel aqueous two phase system composed of surfactant and xylitol for the purification of lipase from pumpkin (Cucurbita moschata) seeds and recycling of phase components. *Molecules*, *20*(6), 11184–11201. https://doi.org/10.3390/molecules200611184

Amid, M., Murshid, F. S., Manap, M. Y., & Hussin, M. (2015). A novel aqueous micellar two-phase system composed of surfactant and sorbitol for purification of pectinase enzyme from psidium guajava and recycling phase components. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/815413

Amid, M., Shuhaimi, M., Islam Sarker, M. Z., & Abdul Manap, M. Y. (2012). Purification of serine protease from mango (Mangifera Indica Cv. Chokanan) peel using an alcohol/salt aqueous two phase system. *Food Chemistry*, *132*(3), 1382–1386. https://doi.org/10.1016/j.foodchem.2011.11.125

Andrews, B. A., Head, D. M., Dunthorne, P., & Asenjo, J. A. (1990). PEG activation and ligand binding for the affinity partitioning of proteins in aqueous two-phase systems. *Biotechnology Techniques*, *4*(1), 49–54. https://doi.org/10.1007/BF00156610

Andualema, B., & Gessesse, A. (2012). Microbial Lipase and Their Industrial Applications: Review. *Biotechnology*. https://doi.org/10.3923/biotech.2012.100.118

Angajala, G., Pavan, P., & Subashini, R. (2016). Lipases: An overview of its current challenges and prospectives in the revolution of biocatalysis. *Biocatalysis and Agricultural Biotechnology*. https://doi.org/10.1016/j.bcab.2016.07.001

Antov, M. G. (2004). Partitioning of pectinase produced by Polyporus squamosus in aqueous two-phase system polyethylene glycol 4000/crude dextran at different initial pH values. *Carbohydrate Polymers*, *56*(3), 295–300. https://doi.org/10.1016/j.carbpol.2003.12.008

Antov, M., & Omorjan, R. (2009). Pectinase partitioning in polyethylene glycol 1000/Na2SO4 aqueous two-phase system: statistical modeling of the experimental results. *Bioprocess and Biosystems Engineering*, *32*(2), 235–240. https://doi.org/10.1007/s00449-008-0243-3

Asenjo, J. A., & Andrews, B. A. (2011). Aqueous two-phase systems for protein separation: A perspective. *Journal of Chromatography A*, *1218*(49), 8826–8835. https://doi.org/10.1016/j.chroma.2011.06.051

Asenjo, J. A., & Andrews, B. A. (2012). Aqueous two-phase systems for protein separation: Phase separation and applications. *Journal of Chromatography A*, *1238*(September 2011), 1–10. https://doi.org/10.1016/j.chroma.2012.03.049

Ashokkumar, M. (2015). Applications of ultrasound in food and bioprocessing. *Ultrasonics Sonochemistry*, *25*(1), 17–23. https://doi.org/10.1016/j.ultsonch.2014.08.012

Ayaz, B., Ugur, A., & Boran, R. (2015). Purification and characterization of organic solvent-tolerant lipase from Streptomyces sp. OC119-7 for biodiesel production. *Biocatalysis and Agricultural Biotechnology*, *4*(1), 103–108. https://doi.org/10.1016/j.bcab.2014.11.007

Aysun, Y., Oznur, S., Ceren, D.-D., Fatih, B., & Beraat, O. (2018). Optimisation of ultrasound-assisted extraction of protein from Spirulina platensis using RSM. *Czech Journal of Food Sciences*, *36*(No. 1), 98–108. https://doi.org/10.17221/64/2017-CJFS

Azevedo, A. M., Rosa, P. A. J., Ferreira, I. F., & Aires-Barros, M. R. (2007). Optimisation of aqueous two-phase extraction of human antibodies. *Journal of Biotechnology*, *132*(2), 209–217. https://doi.org/10.1016/j.jbiotec.2007.04.002

Azhdarpoor, A., Mortazavi, B., & Moussavi, G. (2014). Oily wastewaters treatment using Pseudomonas sp. isolated from the compost fertilizer. *Journal of Environmental Health Science & Engineering*, *12*(1), 77. https://doi.org/10.1186/2052-336X-12-77

Bachchhav, M. B., Kulkarni, M. V, & Ingale, A. G. (2017). An efficient extraction of phycocyanin by ultrasonication and separation using " sugaring out ." *Journal of Phycological Society, India*, *47*(2), 19–24.

Bailey, J., & Ollis, D. (1986). *Biochemical engineering fundamentals-2nd Edition*. McGraw-Hill Science/Engineering/Math.

Bajpai, D., & Tyagi, V. K. (2007). Laundry detergents: an overview. *Journal of Oleo Science*, *56*(7), 327–340. https://doi.org/10.5650/jos.56.327

Balachandran, S., Kentish, S. E., Mawson, R., & Ashokkumar, M. (2006). Ultrasonic enhancement of the supercritical extraction from ginger. *Ultrasonics Sonochemistry*, *13*(6), 471–479. https://doi.org/10.1016/j.ultsonch.2005.11.006

Baranyai, K. J., Deacon, G. B., MacFarlane, D. R., Pringle, J. M., & Scott, J. L. (2004). Thermal Degradation of Ionic Liquids at Elevated Temperatures. *Australian Journal of Chemistry*, *57*(2), 145. https://doi.org/10.1071/CH03221

Basheer, S. a, & Thenmozhi, M. (2010). Reverse Micellar Separation of Lipases : a Critical Review. *Int. J. Chem. Sci.*, *8*(5), 57–67.

Baskir, J. N., Hatton, T. a, & Suter, U. W. (1989). Protein partitioning in two-phase aqueous polymer systems. *Biotechnology and Bioengineering*, *34*(4), 541–558. https://doi.org/10.1002/bit.260340414

Ben Bacha, A., Al-Assaf, A., Moubayed, N. M. S., & Abid, I. (2016). Evaluation of a novel thermo-alkaline Staphylococcus aureus lipase for application in detergent formulations. *Saudi Journal of Biological Sciences*. https://doi.org/10.1016/j.sjbs.2016.10.006

Bernardo, O. Y., & Filho, F. M. (2005). Agitation , aeration and shear stress as key factors in inulinase production by Kluyveromyces marxianus. *Enzyme and Microbial Technology*, *36*, 717–724. https://doi.org/10.1016/j.enzmictec.2004.12.008

Beutel, S., Villain, L., & Scheper, T. (2016). Industrial Application of Membrane Chromatography for the Purification of Enzymes. In *Applied Biocatalysis: From Fundamental Science to Industrial Applications* (pp. 297–316). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527677122.ch13

Bhavya, S. G., Priyanka, B. S., & Rastogi, N. K. (2012). Reverse micelles-mediated transport of lipase in liquid emulsion membrane for downstream processing. *Biotechnology Progress*, *28*(6), 1542–1550. https://doi.org/10.1002/btpr.1637

Bhowal, S., Priyanka, B. S., & Rastogi, N. K. (2014). Mixed reverse micelles facilitated downstream processing of lipase involving water-oil-water liquid emulsion membrane. *Biotechnology Progress*, *30*(5), 1084–1092. https://doi.org/10.1002/btpr.1941

Bi, P.-Y., Dong, H.-R., & Dong, J. (2010). The recent progress of solvent sublation. *Journal of Chromatography. A*, *1217*(16), 2716–25. https://doi.org/10.1016/j.chroma.2009.11.020

Bi, P. Y., Chang, L., Mu, Y. L., Liu, J. Y., Wu, Y., Geng, X., & Wei, Y. (2013). Separation and concentration of baicalin from Scutellaria Baicalensis Georgi extract by aqueous two-phase flotation. *Separation and Purification Technology*, *116*, 454–457. https://doi.org/10.1016/j.seppur.2013.06.024

Bi, P. Y., Dong, H. R., & Wang, N. N. (2008). Effect of water droplet in solvent sublation. *Chinese Chemical Letters*, *19*(5), 623–626. https://doi.org/10.1016/j.cclet.2008.03.010

Bi, P. Y., Dong, H. R., & Yuan, Y. C. (2010). Application of aqueous two-phase flotation in the separation and concentration of puerarin from Puerariae extract. *Separation and Purification Technology*, *75*(3), 402–406. https://doi.org/10.1016/j.seppur.2010.09.010

Bi, P. Y., Li, D. Q., & Dong, H. R. (2009). A novel technique for the separation and concentration of penicillin G from fermentation broth: Aqueous two-phase flotation. *Separation and Purification Technology*, *69*(2), 205–209.

https://doi.org/10.1016/j.seppur.2009.07.019

Bi, P. yu, Dong, H. ru, & Dong, J. (2010). The recent progress of solvent sublation. *Journal of Chromatography A*, *1217*(16), 2716–2725. https://doi.org/10.1016/j.chroma.2009.11.020

Blackburn, S. I., & Volkman, J. K. (2012). Microalgae: A Renewable Source of Bioproducts. In *Food and Industrial Bioproducts and Bioprocessing* (pp. 221–241). Oxford, UK: Wiley-Blackwell. https://doi.org/10.1002/9781119946083.ch9

Blank, K., Morfill, J., & Gaub, H. E. (2006). Site-specific immobilization of genetically engineered variants of Candida antarctica lipase B. *ChemBioChem*, *7*(9), 1349–1351. https://doi.org/10.1002/cbic.200600198

Bleakley, S., & Hayes, M. (2017). Algal Proteins: Extraction, Application, and Challenges Concerning Production. *Foods*, *6*(5), 33. https://doi.org/10.3390/foods6050033

Borowitzka, M. A. (2013). High-value products from microalgae-their development and commercialisation. *Journal of Applied Phycology*, *25*(3), 743–756. https://doi.org/10.1007/s10811-013-9983-9

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Buyel, J. F., Twyman, R. M., & Fischer, R. (2015). Extraction and downstream processing of plant-derived recombinant proteins. *Biotechnology Advances*, *33*(6), 902–913. https://doi.org/10.1016/j.biotechadv.2015.04.010

Cardoso, G. de B., Souza, I. N., Pereira, M. M., Freire, M. G., Soares, C. M. F., & Lima, Á. S. (2014). Aqueous two-phase systems formed by biocompatible and biodegradable polysaccharides and acetonitrile. *Separation and Purification Technology*, *136*, 74–80. https://doi.org/10.1016/j.seppur.2014.08.020

Carta, G., & Jungbauer, A. (2010). Downstream Processing of Biotechnology Products. In *Protein Chromatography* (pp. 1–55). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527630158.ch1

Chander, H., Batish, V. K., Sannabhadti, S. S., & Srinivasan, R. A. (1980). FACTORS AFFECTING LIPASE PRODUCTION IN Aspergillus wentii. *Journal of Food Science*, *45*(3), 598–600. https://doi.org/10.1111/j.1365-2621.1980.tb04109.x

Chang, L., Wei, Y., Bi, P. Y., & Shao, Q. (2014). Recovery of liquiritin and glycyrrhizic acid from Glycyrrhiza uralensis Fisch by aqueous two-phase flotation and multi-stage preparative high performance liquid chromatography. *Separation and Purification Technology*, *134*(2014), 204–209. https://doi.org/10.1016/j.seppur.2014.07.045

Chauhan, M., Chauhan, R. S., & Garlapati, V. K. (2013). Evaluation of a new lipase from staphylococcus sp. for detergent additive capability. *BioMed Research International*. https://doi.org/10.1155/2013/374967

Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry*, *34*, 540–560.

https://doi.org/10.1016/j.ultsonch.2016.06.035

Chen, C.-Y., Lee, P.-J., Tan, C. H., Lo, Y.-C., Huang, C.-C., Show, P. L., … Chang, J.-S. (2015). Improving protein production of indigenous microalga Chlorella vulgaris FSP-E by photobioreactor design and cultivation strategies. *Biotechnology Journal*, *10*(6), 905–914. https://doi.org/10.1002/biot.201400594

Cherif, S., Fendri, A., Miled, N., Trabelsi, H., Mejdoub, H., & Gargouri, Y. (2007). Crab digestive lipase acting at high temperature: Purification and biochemical characterization. *Biochimie*, *89*(8), 1012–1018. https://doi.org/10.1016/j.biochi.2007.02.005

Cherif, S., Mnif, S., Hadrich, F., Abdelkafi, S., & Sayadi, S. (2011). A newly high alkaline lipase: an ideal choice for application in detergent formulations. *Lipids in Health and Disease*, *10*(1), 221. https://doi.org/10.1186/1476-511X-10-221

Choudhury, P., & Bhunia, B. (2015). Industrial Application of Lipase: a Review. *Biopharm Journal*, *1*(2), 41–47.

Chow, Y. H., Yap, Y. J., Anuar, M. S., Tejo, B. A., Ariff, A., Show, P. L., … Ling, T. C. (2013). Interfacial partitioning behaviour of bovine serum albumin in polymer-salt aqueous two-phase system. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *934*, 71–78. https://doi.org/10.1016/j.jchromb.2013.06.034

Chow, Y. H., Yap, Y. J., Tan, C. P., Anuar, M. S., Tejo, B. A., Show, P. L., … Ling, T. C. (2014). Characterization of bovine serum albumin partitioning behaviors in polymer-salt aqueous two-phase systems. *Journal of Bioscience and Bioengineering*, *120*(1), 85–90. https://doi.org/10.1016/j.jbiosc.2014.11.021

Coskun, O. (2016). Separation Tecniques: CHROMATOGRAPHY. *Northern Clinics of Istanbul*, *3*(2), 156–160. https://doi.org/10.14744/nci.2016.32757

Cravotto, G., & Binello, A. (2010). Innovative techniques and equipments for flavours extraction. *Household Pers. Care Today*, *5*(1), 30–32.

Crini, G., & Badot, P. M. (2008). Application of chitosan, a natural aminopolysaccharide, for dye removal from aqueous solutions by adsorption processes using batch studies: A review of recent literature. *Progress in Polymer Science (Oxford)*, *33*(4), 399–447. https://doi.org/10.1016/j.progpolymsci.2007.11.001

Dai, J.-Y., Ma, L.-H., Wang, Z.-F., Guan, W.-T., & Xiu, Z.-L. (2016). Sugaring-out extraction of acetoin from fermentation broth by coupling with fermentation. *Bioprocess and Biosystems Engineering*. https://doi.org/10.1007/s00449-016-1710-x

De Brito Cardoso, G., Mourão, T., Pereira, F. M., Freire, M. G., Fricks, A. T., Soares, C. M. F., & Lima, Á. S. (2013). Aqueous two-phase systems based on acetonitrile and carbohydrates and their application to the extraction of vanillin. *Separation and Purification Technology*, *104*(2013), 106–113. https://doi.org/10.1016/j.seppur.2012.11.001

de Medeiros e Silva, G. M., Viana Marques, D. de A., Porto, T. S., Filho, J. L. L., Teixeira, J. A. C., Pessoa-J??nior, A., & Porto, A. L. F. (2013). Extraction of fibrinolytic proteases from Streptomyces sp. DPUA1576 using PEG-phosphate aqueous two-phase systems. *Fluid Phase Equilibria*, *339*, 52–57.

https://doi.org/10.1016/j.fluid.2012.11.033

Deive, F. J., Rodríguez, A., Rebelo, L. P. N., & Marrucho, I. M. (2012). Extraction of Candida antarctica lipase A from aqueous solutions using imidazolium-based ionic liquids. *Separation and Purification Technology*, *97*, 205–210. https://doi.org/10.1016/j.seppur.2011.12.013

Delvigne, F., & Lecomte, J. (2010). Foam Formation and Control in Bioreactors. In *Encyclopedia of Industrial Biotechnology* (pp. 1–13). Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/9780470054581.eib326

Deng, H. T., Xu, Z. K., Wu, J., Ye, P., Liu, Z. M., & Seta, P. (2004). A comparative study on lipase immobilized polypropylene microfiltration membranes modified by sugar-containing polymer and polypeptide. *Journal of Molecular Catalysis B: Enzymatic*, *28*(2–3), 95–100. https://doi.org/10.1016/j.molcatb.2004.01.004

Dey, S., & Rathod, V. K. (2013). Ultrasound assisted extraction of β-carotene from Spirulina platensis. *Ultrasonics Sonochemistry*, *20*(1), 271–276. https://doi.org/10.1016/j.ultsonch.2012.05.010

Dhamole, P. B., Chavan, S., Patil, R. G., Feng, H., Bule, M., & Kinninge, P. (2016). Extraction of p-coumaric acid from agricultural residues and separation using “sugaring out.” *Korean Journal of Chemical Engineering*, *33*(6), 1860–1864. https://doi.org/10.1007/s11814-016-0020-y

Dhamole, P. B., Mahajan, P., & Feng, H. (2010a). Phase separation conditions for sugaring-out in acetonitrile-water systems. *Journal of Chemical and Engineering Data*, *55*(9), 3803–3806. https://doi.org/10.1021/je1003115

Dhamole, P. B., Mahajan, P., & Feng, H. (2010b). Sugaring out: A new method for removal of acetonitrile from preparative RP-HPLC eluent for protein purification. *Process Biochemistry*, *45*(10), 1672–1676. https://doi.org/10.1016/j.procbio.2010.06.020

Dhamole, P. B., Mahajan, P., & Feng, H. (2011). Sugaring out for separation of acetonitrile and extraction of proteins and antibiotics. *The 11th International Congress on Engineering and Food. Athens, Greece.*, 4–8.

Diaz, J. C. M., Cordova, J., Baratti, J., Carriere, F., & Abousalham, A. (2007). Effect of nonionic surfactants on Rhizopus homothallicus lipase activity: A comparative kinetic study. *Molecular Biotechnology*, *35*(3), 205–214. https://doi.org/10.1007/s12033-007-9000-0

Ding, X., Cai, J., & Guo, X. (2016). Extraction of ovalbumin with gemini surfactant reverse micelles – Effect of gemini surfactant structure. *SEPARATION AND PURIFICATION TECHNOLOGY*, *158*, 367–373. https://doi.org/10.1016/j.seppur.2015.12.042

Ding, Z., Zheng, K., & Cao, X. (2014). Lipase purification by affinity precipitation with a thermo-responsive polymer immobilized Cibacron Blue F3GA ligand. *Biotechnology and Bioprocess Engineering*, *19*(5), 892–899. https://doi.org/10.1007/s12257-014-0015-7

Dos Prazeres, J. N., Cruz, J. A. B., & Pastore, G. M. (2006). Characterization of alkaline lipase from Fusarium oxysporum and the effect of different surfactants and detergents on the enzyme activity. *Brazilian Journal of Microbiology*, *37*(4), 505–509. https://doi.org/10.1590/S1517-83822006000400019

Dos Santos, R. R., Moreira, D. M., Kunigami, C. N., Aranda, D. A. G., & Teixeira, C. M. L. L. (2015). Comparison between several methods of total lipid extraction from Chlorella vulgaris biomass. *Ultrasonics Sonochemistry*, *22*, 95–99. https://doi.org/10.1016/j.ultsonch.2014.05.015

Du, W., Li, W., Sun, T., Chen, X., & Liu, D. (2008). Perspectives for biotechnological production of biodiesel and impacts. *Applied Microbiology and Biotechnology*, *79*(3), 331–337. https://doi.org/10.1007/s00253-008-1448-8

Ekinci, A. P., D'Inçer, B., Baltaş, N., & Adigüzel, A. (2016). Partial purification and characterization of lipase from Geobacillus stearothermophilus AH22. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *31*(2), 325–331. https://doi.org/10.3109/14756366.2015.1024677

El-gawad, H. S. A. (2014). Oil and Grease Removal from Industrial Wastewater Using New Utility Approach. *Advances in Environmental Chemicstry*, *2014*(Article ID 916878), 6 pages. https://doi.org/10.1155/2014/916878

Escamilla-mejía, J. C., Rodríguez, J. A., Álvarez-romero, G. A., & Galán-vidal, A. (2015). Monoenzymatic Lipase Potentiometric Biosensor for the Food Analysis Based on a pH Sensitive Graphite-epoxy Composite as Transducer. *Journal of the Mexican Chemical Society*, *59*(1), 19–23.

Fernandes, M. L. M., Krieger, N., Baron, A. M., Zamora, P. P., Ramos, L. P., & Mitchell, D. A. (2004). Hydrolysis and synthesis reactions catalysed by Thermomyces lanuginosa lipase in the AOT/Isooctane reversed micellar system. *Journal of Molecular Catalysis B: Enzymatic*, *30*(1), 43–49. https://doi.org/10.1016/j.molcatb.2004.03.004

Fields, C., Li, P., O'Mahony, J. J., & Lee, G. U. (2016). Advances in affinity ligand-functionalized nanomaterials for biomagnetic separation. *Biotechnology and Bioengineering*, *113*(1), 11–25. https://doi.org/10.1002/bit.25665

Fresewinkel, M., Rosello, R., Wilhelm, C., Kruse, O., Hankamer, B., & Posten, C. (2014). Integration in microalgal bioprocess development: Design of efficient, sustainable, and economic processes. *Engineering in Life Sciences*, *14*(6), 560–573. https://doi.org/10.1002/elsc.201300153

Fuda, E., Bhatia, D., Pyle, D. L., & Jauregi, P. (2005). Selective separation of β-lactoglobulin from sweet whey using CGAs generated from the cationic surfactant CTAB. *Biotechnology and Bioengineering*, *90*(5), 532–542. https://doi.org/10.1002/bit.20412

Gaikaiwari, R. P., Wagh, S. A., & Kulkarni, B. D. (2012a). Efficient lipase purification using reverse micellar extraction. *Bioresource Technology*, *108*, 224–230. https://doi.org/10.1016/j.biortech.2011.11.126

Gaikaiwari, R. P., Wagh, S. A., & Kulkarni, B. D. (2012b). Extraction and purification of tannase by reverse micelle system. *Separation and Purification Technology*, *89*, 288–296. https://doi.org/10.1016/j.seppur.2012.01.043

Gerde, J. A., Montalbo-Lomboy, M., Yao, L., Grewell, D., & Wang, T. (2012). Evaluation of microalgae cell disruption by ultrasonic treatment. *Bioresource Technology*, *125*, 175–181. https://doi.org/10.1016/j.biortech.2012.08.110

Ghosh, R. (2003). Protein Bioseparation: An Overview. In *Protein Bioseparation Using*

Ultrafiltration (pp. 1–11). PUBLISHED BY IMPERIAL COLLEGE PRESS AND DISTRIBUTED BY WORLD SCIENTIFIC PUBLISHING CO. https://doi.org/10.1142/9781860949388_0001

Gilar, M., Jaworski, A., & McDonald, T. S. (2014). Solvent selectivity and strength in reversed-phase liquid chromatography separation of peptides. *Journal of Chromatography A*, *1337*(2014), 140–146. https://doi.org/10.1016/j.chroma.2014.02.048

Glogauer, A., Martini, V. P., Faoro, H., Couto, G. H., Müller-Santos, M., Monteiro, R. A., … Krieger, N. (2011). Identification and characterization of a new true lipase isolated through metagenomic approach. *Microbial Cell Factories*, *10*, 54. https://doi.org/10.1186/1475-2859-10-54

Glyk, A., Scheper, T., & Beutel, S. (2015). PEG–salt aqueous two-phase systems: an attractive and versatile liquid–liquid extraction technology for the downstream processing of proteins and enzymes. *Applied Microbiology and Biotechnology*, *99*(16), 6599–6616. https://doi.org/10.1007/s00253-015-6779-7

Golaki, B. P., Aminzadeh, S., Karkhane, A. A., Yakhchali, B., Farrokh, P., Khaleghinejad, S. H., … Mehrpooyan, S. (2015). Cloning, expression, purification, and characterization of lipase 3646 from thermophilic indigenous Cohnella sp. A01. *Protein Expression and Purification*, *109*, 120–126. https://doi.org/10.1016/j.pep.2014.10.002

Gondrexon, N., Renaudin, V., Boldo, P., Gonthier, Y., Bernis, A., & Petrier, C. (1997). Degassing effect and gas-liquid transfer in a high frequency sonochemical reactor. *Chemical Engineering Journal*. https://doi.org/10.1016/S1385-8947(96)03124-5

Goula, A. M., Ververi, M., Adamopoulou, A., & Kaderides, K. (2017). Green ultrasound-assisted extraction of carotenoids from pomegranate wastes using vegetable oils. *Ultrasonics Sonochemistry*, *34*, 821–830. https://doi.org/10.1016/j.ultsonch.2016.07.022

Granström, T. B., Izumori, K., & Leisola, M. (2007). A rare sugar xylitol. Part II: Biotechnological production and future applications of xylitol. *Applied Microbiology and Biotechnology*, *74*(2), 273–276. https://doi.org/10.1007/s00253-006-0760-4

Greve, A., & Kula, M.-R. (1991a). Cost structure and estimation for the recycling of salt in a protein extraction process. *Bioprocess Engineering*, *6*(4), 173–177. https://doi.org/10.1007/BF00369255

Greve, A., & Kula, M. R. (1991b). Recycling of salts in partition protein extraction processes. *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire : 1986)*, *50*(1), 27–42. https://doi.org/10.1002/jctb.280500105

Gu, Y., & Shih, P.-H. (2004). Salt-induced phase separation can effectively remove the acetonitrile from the protein sample after the preparative RP-HPLC. *Enzyme and Microbial Technology*, *35*(6–7), 592–597. https://doi.org/10.1016/j.enzmictec.2004.08.011

Guan, Y., Lilley, T. H., Treffry, T. E., Zhou, C., & Wilkinson, P. B. (1996). Use of aqueous two-phase systems in the purification of human interferon-ad from recombinant Escherichia coli. *Enzyme and Microbial Technology*, *19*(96), 446–455. https://doi.org/10.1016/S0141-0229(96)00051-8

Guldhe, A., Singh, B., Mutanda, T., Permaul, K., & Bux, F. (2015). Advances in synthesis of biodiesel via enzyme catalysis: Novel and sustainable approaches. *Renewable and Sustainable Energy Reviews*, *41*(2015), 1447–1464. https://doi.org/10.1016/j.rser.2014.09.035

Hadiyanto, H., & Suttrisnorhadi, S. (2016). Response Surface Optimization of Ultrasound Assisted Extraction (UAE) of Phycocyanin from Microalgae Spirulina platensis. *Emirates Journal of Food and Agriculture*, *28*(4), 227. https://doi.org/10.9755/ejfa.2015-05-193

Han, J., Wang, Y., Yu, C., Li, C., Yan, Y., Liu, Y., & Wang, L. (2011). Separation, concentration and determination of chloramphenicol in environment and food using an ionic liquid/salt aqueous two-phase flotation system coupled with high-performance liquid chromatography. *Analytica Chimica Acta*, *685*(2), 138–145. https://doi.org/10.1016/j.aca.2010.11.033

Harris, D. P., Andrews, A. T., Wright, G., Pyle, D. L., & Asenjo, J. A. (1998). The application of aqueous two-phase systems to the purification of pharmaceutical proteins from transgenic sheep milk. *Bioseparation*, *7*(1), 31–37. https://doi.org/10.1023/A:1007908703773

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*, *39*(2), 235–251. https://doi.org/10.1016/j.enzmictec.2005.10.016

Helena, L., Rodrigues, R., Arenzon, A., Raya-rodriguez, M. T., & Fontoura, N. F. (2011). Algal density assessed by spectrophotometry : A calibration curve for the unicellular algae Pseudokirchneriella subcapitata. *Journal of Environmental Chemistry and Ecotoxicology*, *3*(August), 225–228.

Hemachander, C., & Puvanakrishnan, R. (2000). Lipase from Ralstonia pickettii as an additive in laundry detergent formulations. *Process Biochemistry*, *35*(8), 809–814. https://doi.org/10.1016/S0032-9592(99)00140-5

Hemlata, B., Uzma, Z., & Tukaram, K. (2016). Substrate kinetics of thiol activated hyperthermostable alkaline lipase of Bacillus sonorensis 4R and its application in bio-detergent formulation. *Biocatalysis and Agricultural Biotechnology*. https://doi.org/10.1016/j.bcab.2016.08.008

Herrero, M., Sánchez-Camargo, A. del P., Cifuentes, A., & Ibáñez, E. (2015). Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. *TrAC - Trends in Analytical Chemistry*, *71*, 26–38. https://doi.org/10.1016/j.trac.2015.01.018

Ho, S. H., Chen, C. Y., & Chang, J. S. (2012). Effect of light intensity and nitrogen starvation on CO2fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. *Bioresource Technology*, *113*, 244–252. https://doi.org/10.1016/j.biortech.2011.11.133

Iftikhar, T., Niaz, M., Jabeen, R., & ul Haq, I. (2011). Purification and Characterization of Extracellular Lipases. *Pakistan Journal of Botany*, *43*(3), 1541–1545. https://doi.org/10.1007/s10295-008-0473-z

Iqbal, M., Tao, Y., Xie, S., Zhu, Y., Chen, D., Wang, X., … Yuan, Z. (2016). Aqueous two-phase system (ATPS): an overview and advances in its applications. *Biological Procedures Online*, *18*(1), 18. https://doi.org/10.1186/s12575-016-0048-8

Ishimoto, R., Sugimoto, M., & Kawai, F. (2001). Screening and characterization of trehalose-oleate hydrolyzing lipase. *FEMS Microbiology Letters*, *195*(2), 231–235. https://doi.org/10.1016/S0378-1097(01)00016-7

Iyer, P. V., & Ananthanarayan, L. (2008). Enzyme stability and stabilization-Aqueous and non-aqueous environment. *Process Biochemistry*, *43*(10), 1019–1032. https://doi.org/10.1016/j.procbio.2008.06.004

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*, 315–51. https://doi.org/10.1146/annurev.micro.53.1.315

Ji, X., Chen, G., Zhang, Q., Lin, L., & Wei, Y. (2015). Purification and characterization of an extracellular cold-adapted alkaline lipase produced by psychrotrophic bacterium Yersinia enterocolitica strain KM1. *Journal of Basic Microbiology*, *55*(6), 718–728. https://doi.org/10.1002/jobm.201400730

Ji, X., Li, S., Wang, B., Zhang, Q., Lin, L., Dong, Z., & Wei, Y. (2015). Expression, purification and characterization of a functional, recombinant, cold-active lipase (LipA) from psychrotrophic Yersinia enterocolitica. *Protein Expression and Purification*, *115*, 125–131. https://doi.org/10.1016/j.pep.2015.08.002

Jiang, H. L., Yang, J. L., & Shi, Y. P. (2017). Optimization of ultrasonic cell grinder extraction of anthocyanins from blueberry using response surface methodology. *Ultrasonics Sonochemistry*, *34*, 325–331. https://doi.org/10.1016/j.ultsonch.2016.06.003

Jiang, Y., Xia, H., Yu, J., Guo, C., & Liu, H. (2009). Hydrophobic ionic liquids-assisted polymer recovery during penicillin extraction in aqueous two-phase system. *Chemical Engineering Journal*, *147*(1), 22–26. https://doi.org/10.1016/j.cej.2008.11.012

Jones, L. A., Prabel, J. B., Glennon, J. J., Copeland, M. F., & Kavlock, R. J. (1993). Extraction of Phenol and Its Metabolites from Aqueous Solution. *Journal of Agricultural and Food Chemistry*, *41*(5), 735–741. https://doi.org/10.1021/jf00029a011

Jurado, E., Garcia-Roman, M., Luzon, G., Altmajer-Vaz, D., & Jimenez-Perez, J. L. (2011). Optimization of lipase performance in detergent formulations for hard surfaces. *Industrial and Engineering Chemistry Research*, *50*(20), 11502–11510. https://doi.org/10.1021/ie201486a

Kaushik, J. K., & Bhat, R. (1998). Thermal stability of proteins in aqueous polyol solutions: Role of the surface tension of water in the stabilizing effect of polyols. *Journal of Physical Chemistry B*, *102*(98), 7058–7066. https://doi.org/10.1021/jp981119l

Kaushik, V., & Roos, Y. H. (2007). Limonene encapsulation in freeze-drying of gum Arabic-sucrose-gelatin systems. *LWT - Food Science and Technology*, *40*(8), 1381–1391. https://doi.org/10.1016/j.lwt.2006.10.008

Ketnawa, S., Benjakul, S., Ling, T. C., Martínez-Alvarez, O., & Rawdkuen, S. (2013). Enhanced recovery of alkaline protease from fish viscera by phase partitioning and

its application. *Chemistry Central Journal*, *7*(1), 79. https://doi.org/10.1186/1752-153X-7-79

Ketnawa, S., Rungraeng, N., & Rawdkuen, S. (2017). Phase partitioning for enzyme separation : An overview and recent applications. *International Food Research*, *24*(February), 1–24.

Kislik, V. S. (2012). *Solvent Extraction: Classical and Novel Approaches*. *Journal of Chemical Information and Modeling*. Amsterdam: Elsevier.

Krishna, S. H., Srinivas, N. D., Raghavarao, K. S. M. S., & Karanth, N. G. (2002). Reverse micellar extraction for downstream processing of proteins/enzymes. *Advances in Biochemical Engineering/biotechnology*, *75*, 119–183.

Kuepethkaew, S., Sangkharak, K., Benjakul, S., & Klomklao, S. (2017). Optimized synthesis of biodiesel using lipase from Pacific white shrimp (Litopenaeus vannamei) hepatopancreas. *Renewable Energy*, *104*(12), 139–147. https://doi.org/10.1016/j.renene.2016.12.014

Kumari, A., Verma, V. V., & Gupta, R. (2012). Comparative biochemical characterization and in silico analysis of novel lipases Lip11 and Lip12 with Lip2 from Yarrowia lipolytica. *World Journal of Microbiology and Biotechnology*, *28*(11), 3103–3111. https://doi.org/10.1007/s11274-012-1120-4

Laachari, F., El Bergadi, F., Sayari, A., Elabed, S., Mohammed, I., El Harchali, H., & Ibnsouda, S. K. (2015). Bacillus pumilus suşundan elde edilen yeni termostabil lipazın biyokimyasal karakterizasyonu. *Turkish Journal of Biochemistry*, *40*(1), 8–14. https://doi.org/10.5505/tjb.2015.44227

Lee, S. Y., Khoiroh, I., Ling, T. C., & Show, P. L. (2016). Aqueous Two-Phase Flotation for the Recovery of Biomolecules. *Separation & Purification Reviews*, *45*(1), 81–92. https://doi.org/10.1080/15422119.2015.1007147

Lee, S. Y., Khoiroh, I., Ling, T. C., & Show, P. L. (2017a). Enhanced recovery of lipase derived from Burkholderia cepacia from fermentation broth using recyclable ionic liquid/polymer-based aqueous two-phase systems. *Separation and Purification Technology*, *179*, 152–160. https://doi.org/10.1016/j.seppur.2017.01.047

Lee, S. Y., Khoiroh, I., Ling, T. C., & Show, P. L. (2017b). Enhanced recovery of lipase derived from Burkholderia cepacia from fermentation broth using recyclable ionic liquid/polymer-based aqueous two-phase systems. *Separation and Purification Technology*, *179*, 152–160. https://doi.org/10.1016/j.seppur.2017.01.047

Lee, S. Y., Show, P. L., Ling, T. C., & Chang, J.-S. (2017). Single-step disruption and protein recovery from Chlorella vulgaris using ultrasonication and ionic liquid buffer aqueous solutions as extractive solvents. *Biochemical Engineering Journal*, *124*(2017), 26–35. https://doi.org/10.1016/j.bej.2017.04.009

Leinonen, H. (1996). Stress Corrosion Cracking and Life Prediction Evaluation of Austenitic Stainless Steels in Calcium Chloride Solution. *CORROSION*, *52*(5), 337–346. https://doi.org/10.5006/1.3292121

Li, M., & Dong, H. (2010). The investigation on the aqueous two-phase floatation of lincomycin. *Separation and Purification Technology*, *73*(2), 208–212. https://doi.org/10.1016/j.seppur.2010.04.002

Li, X. L., Zhang, W. H., Wang, Y. D., Dai, Y. J., Zhang, H. T., Wang, Y., … Lu, F. P. (2014). A high-detergent-performance, cold-adapted lipase from Pseudomonas stutzeri PS59 suitable for detergent formulation. *Journal of Molecular Catalysis B: Enzymatic*, *102*, 16–24. https://doi.org/10.1016/j.molcatb.2014.01.006

Li, Z., Teng, H., & Xiu, Z. (2010). Aqueous two-phase extraction of 2,3-butanediol from fermentation broths using an ethanol/ammonium sulfate system. *Process Biochemistry*, *45*(5), 731–737. https://doi.org/10.1016/j.procbio.2010.01.011

Li, Z., Teng, H., & Xiu, Z. (2011). Extraction of 1,3-propanediol from glycerol-based fermentation broths with methanol/phosphate aqueous two-phase system. *Process Biochemistry*, *46*(2), 586–591. https://doi.org/10.1016/j.procbio.2010.10.014

Lin, Y. K., Show, P. L., Yap, Y. J., Tan, C. P., Ng, E., Ariff, A. B., … Ling, T. C. (2015). Direct recovery of cyclodextringlycosyltransferase from Bacillus cereus using aqueous two-phase flotation. *Journal of Bioscience and Bioengineering*, *120*(6), 684–689. https://doi.org/10.1016/j.jbiosc.2015.04.013

Linko, Y. Y., & Wu, X. Y. (1996). Biocatalytic production of useful esters by two forms of lipase from Candida rugosa. *Journal of Chemical Technology and Biotechnology*, *65*(2), 163–170. https://doi.org/10.1002/(SICI)1097-4660(199602)65:2<163::AID-JCTB416>3.0.CO;2-C

Lopes, A. S., Garcia, J. S., Catharino, R. R., Santos, L. S., Eberlin, M. N., & Arruda, M. A. Z. (2007). Cloud point extraction applied to casein proteins of cow milk and their identification by mass spectrometry. *Analytica Chimica Acta*, *590*(2), 166–172. https://doi.org/10.1016/j.aca.2007.03.043

Lopes, M. D. F., Cunha, a E., Clemente, J. J., Carrondo, M. J., & Crespo, M. T. (1999). Influence of environmental factors on lipase production by Lactobacillus plantarum. *Applied Microbiology and Biotechnology*, *51*(2), 249–54. https://doi.org/10.1007/s002530051389

López-Serrano, P., Wegman, M. A., Van Rantwijk, F., & Sheldon, R. A. (2001). Enantioselective enzyme catalysed ammoniolysis of amino acid derivatives. Effect of temperature. *Tetrahedron Asymmetry*, *12*(2), 235–240. https://doi.org/10.1016/S0957-4166(01)00023-4

Lu, Y., Yu, M., Tan, Z., & Yan, Y. (2016). Phase equilibria and salt effect on the aqueous two-phase system of polyoxyethylene cetyl ether and sulfate salt at three temperatures. *Journal of Chemical and Engineering Data*, *61*(6), 2135–2143. https://doi.org/10.1021/acs.jced.6b00097

Luque-García, J. L., & Luque De Castro, M. D. (2004). Ultrasound-assisted Soxhlet extraction: An expeditive approach for solid sample treatment - Application to the extraction of total fat from oleaginous seeds. *Journal of Chromatography A*, *1034*(1–2), 237–242. https://doi.org/10.1016/j.chroma.2004.02.020

Luque de Castro, M. D., & García-Ayuso, L. E. (1998). Soxhlet extraction of solid materials: An outdated technique with a promising innovative future. *Analytica Chimica Acta*, *369*(1–2), 1–10. https://doi.org/10.1016/S0003-2670(98)00233-5

Malekabadi, S., Badoei-dalfard, A., & Karami, Z. (2018). Biochemical characterization of a novel cold-active, halophilic and organic solvent-tolerant lipase from B. licheniformis

KM12 with potential application for biodiesel production. *International Journal of Biological Macromolecules*, *109*, 389–398. https://doi.org/10.1016/j.ijbiomac.2017.11.173

Mapari, S. A. S., Meyer, A. S., & Thrane, U. (2006). Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. *Journal of Agricultural and Food Chemistry*, *54*(19), 7027–7035. https://doi.org/10.1021/jf062094n

Mathiazakan, P., Shing, S. Y., Ying, S. S., Kek, H. K., Tang, M. S. Y., Show, P. L., … Ling, T. C. (2016). Pilot Scale Aqueous Two-Phase Floatation for Direct Recovery of Lipase Derived from Burkholderia cepacia strain ST8. *Separation and Purification Technology*, *171*(2016), 206–213. https://doi.org/10.1016/j.seppur.2016.07.017

Md Sidek, N. L., Tan, J. S., Abbasiliasi, S., Wong, F. W. F., Mustafa, S., & Ariff, A. B. (2016). Aqueous two-phase flotation for primary recovery of bacteriocin-like inhibitory substance (BLIS) from Pediococcus acidilactici Kp10. *Journal of Chromatography B*, *1027*, 81–87. https://doi.org/10.1016/j.jchromb.2016.05.024

Méndez-Sánchez, D., Ríos-Lombardía, N., Gotor, V., & Gotor-Fernández, V. (2015). Asymmetric synthesis of azolium-based 1,2,3,4-tetrahydronaphthalen-2-ols through lipase-catalyzed resolutions. *Tetrahedron: Asymmetry*, *26*(14), 760–767. https://doi.org/10.1016/j.tetasy.2015.06.006

Mercer, P., & Armenta, R. E. (2011). Developments in oil extraction from microalgae. *European Journal of Lipid Science and Technology*, *113*(5), 539–547. https://doi.org/10.1002/ejlt.201000455

Mhetras, N. C., Bastawde, K. B., & Gokhale, D. V. (2009). Purification and characterization of acidic lipase from Aspergillus niger NCIM 1207. *Bioresource Technology*, *100*(3), 1486–1490. https://doi.org/10.1016/j.biortech.2008.08.016

Miljevic, B., Hedayat, F., Stevanovic, S., Fairfull-Smith, K. E., Bottle, S. E., & Ristovski, Z. D. (2014). To sonicate or not to sonicate PM filters: Reactive oxygen species generation upon ultrasonic irradiation. *Aerosol Science and Technology*, *48*(12), 1276–1284. https://doi.org/10.1080/02786826.2014.981330

Mittal, R., Tavanandi, H. A., Mantri, V. A., & Raghavarao, K. S. M. S. (2017). Ultrasound assisted methods for enhanced extraction of phycobiliproteins from marine macro-algae, Gelidium pusillum (Rhodophyta). *Ultrasonics Sonochemistry*, *38*, 92–103. https://doi.org/10.1016/j.ultsonch.2017.02.030

Mo, Q., Liu, A., Guo, H., Zhang, Y., & Li, M. (2016). A novel thermostable and organic solvent-tolerant lipase from Xanthomonas oryzae pv. oryzae YB103: screening, purification and characterization. *Extremophiles*, *20*(2), 157–165. https://doi.org/10.1007/s00792-016-0809-y

Moreira, a. B. R., Perez, V. H., Zanin, G. M., & de Castro, H. F. (2007). Biodiesel synthesis by enzymatic transesterification of palm oil with ethanol using lipases from several sources immobilized on silica-PVA composite. *Energy and Fuels*, *21*(6), 3689–3694. https://doi.org/10.1021/ef700399b

Mukherjee, S., Mukhopadhyay, S., Pariatamby, A., Hashim, M. A., Redzwan, G., & Sen Gupta, B. (2015). Optimization of pulp fibre removal by flotation using colloidal gas

aphrons generated from a natural surfactant. *Journal of the Taiwan Institute of Chemical Engineers*, *53*, 15–21. https://doi.org/10.1016/j.jtice.2015.02.037

Naganagouda, K., & Mulimani, V. H. (2008). Aqueous two-phase extraction (ATPE): An attractive and economically viable technology for downstream processing of Aspergillus oryzae ??-galactosidase. *Process Biochemistry*, *43*(11), 1293–1299. https://doi.org/10.1016/j.procbio.2008.07.016

Naimi-Jamal, M. R., Hamzeali, H., Mokhtari, J., Boy, J., & Kaupp, G. (2009). Sustainable Synthesis of Aldehydes, Ketones or Acids from Neat Alcohols Using Nitrogen Dioxide Gas, and Related Reactions. *ChemSusChem*, *2*(1), 83–88. https://doi.org/10.1002/cssc.200800193

Najafpour, G. D. (2015). Gas and Liquid System (Aeration and Agitation)∗∗. In *Biochemical Engineering and Biotechnology* (pp. 51–102). Elsevier. https://doi.org/10.1016/B978-0-444-63357-6.00003-1

Nandini, K. E., & Rastogi, N. K. (2009). Reverse micellar extraction for downstream processing of lipase: Effect of various parameters on extraction. *Process Biochemistry*, *44*(10), 1172–1178. https://doi.org/10.1016/j.procbio.2009.06.020

Nandini, K. E., & Rastogi, N. K. (2010). Separation and purification of lipase using reverse micellar extraction: Optimization of conditions by response surface methodology. *Biotechnology and Bioprocess Engineering*, *15*(2), 349–358. https://doi.org/10.1007/s12257-009-0091-2

Nascimento, T. P., Sales, A. E., Porto, C. S., Brandão, R. M. P., de Campos-Takaki, G. M., Teixeira, J. A. C., … Converti, A. (2016). Purification of a fibrinolytic protease from Mucor subtilissimus UCP 1262 by aqueous two-phase systems (PEG/sulfate). *Journal of Chromatography B*, *1025*, 16–24. https://doi.org/10.1016/j.jchromb.2016.04.046

Nfor, B. K., Ahamed, T., van Dedem, G. W., van der Wielen, L. A., van de Sandt, E. J., Eppink, M. H., & Ottens, M. (2008). Design strategies for integrated protein purification processes: challenges, progress and outlook. *Journal of Chemical Technology & Biotechnology*, *83*(2), 124–132. https://doi.org/10.1002/jctb.1815

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*, 723–726. https://doi.org/10.1016/j.biortech.2013.05.087

Ojha, K. S., Mason, T. J., O'Donnell, C. P., Kerry, J. P., & Tiwari, B. K. (2017). Ultrasound technology for food fermentation applications. *Ultrasonics Sonochemistry*, *34*, 410–417. https://doi.org/10.1016/j.ultsonch.2016.06.001

Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., … Goldman, A. (1992). The α / β hydrolase fold. *"Protein Engineering, Design and Selection," 5*(3), 197–211. https://doi.org/10.1093/protein/5.3.197

Ooi, C. W., Hii, S. L., Kamal, S. M. M., Ariff, A., & Ling, T. C. (2011). Extractive fermentation using aqueous two-phase systems for integrated production and purification of extracellular lipase derived from Burkholderia pseudomallei. *Process Biochemistry*, *46*(1), 68–73. https://doi.org/10.1016/j.procbio.2010.07.014

Ooi, C. W., Tey, B. T., Hii, S. L., Ariff, A., Wu, H. S., Lan, J. C. W., … Ling, T. C. (2009). Direct

purification of Burkholderia Pseudomallei lipase from fermentation broth using aqueous two-phase systems. *Biotechnology and Bioprocess Engineering*, *14*(6), 811–818. https://doi.org/10.1007/s12257-008-0306-y

Ooi, C. W., Tey, B. T., Hii, S. L., Kamal, S. M. M., Lan, J. C. W., Ariff, A., & Ling, T. C. (2009). Purification of lipase derived from Burkholderia pseudomallei with alcohol/salt-based aqueous two-phase systems. *Process Biochemistry*, *44*(10), 1083–1087. https://doi.org/10.1016/j.procbio.2009.05.008

Ozkurt, I. (2009). Qualifying of safflower and algae for energy. *Energy Education Science and Technology Part A: Energy Science and Research*, *23*(2), 145–151. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-84861954605&partnerID=tZOtx3y1

Pakhale, S. V., & Bhagwat, S. S. (2016). Purification of serratiopeptidase from Serratia marcescens NRRL B 23112 using ultrasound assisted three phase partitioning. *Ultrasonics Sonochemistry*, *31*, 532–538. https://doi.org/10.1016/j.ultsonch.2016.01.037

Pakhale, S. V., Vetal, M. D., & Rathod, V. K. (2013). Separation of Bromelain by Aqueous Two Phase Flotation. *Separation Science and Technology*, *48*(6), 984–989. https://doi.org/10.1080/01496395.2012.712596

Parmar, R., & Majumder, S. K. (2013). Microbubble generation and microbubble-aided transport process intensification-A state-of-the-art report. *Chemical Engineering and Processing: Process Intensification*, *64*(2013), 79–97. https://doi.org/10.1016/j.cep.2012.12.002

Patel, R. N. (2002). Microbial/enzymatic synthesis of chiral intermediates for pharmaceuticals. *Enzyme and Microbial Technology*, *31*(6), 804–826. https://doi.org/10.1016/S0141-0229(02)00186-2

Pchelintsev, N. A., Adams, P. D., & Nelson, D. M. (2016). Critical parameters for efficient sonication and improved chromatin immunoprecipitation of high molecular weight proteins. *PLoS ONE*, *11*(1), 1–11. https://doi.org/10.1371/journal.pone.0148023

Phong, W. N., Le, C. F., Show, P. L., Chang, J.-S., & Ling, T. C. (2016). Extractive disruption process integration using ultrasonication and aqueous two-phase system for proteins recovery from Chlorella sorokiniana. *Engineering in Life Sciences*, *0*, 1–13. https://doi.org/10.1002/elsc.201600133

Phong, W. N., Show, P. L., Teh, W. H., Teh, T. X., Lim, H. M. Y., Nazri, N. S. binti, … Ling, T. C. (2017). Proteins recovery from wet microalgae using liquid biphasic flotation (LBF). *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2017.05.165

Pratuangdejkul, J., & Dharmsthiti, S. (2000). Purification and characterization of lipase from psychrophilic Acinetobacter calcoaceticus LP009. *Microbiological Research*, *155*(2), 95–100. https://doi.org/10.1016/S0944-5013(00)80043-9

Prins, A., & van't Riet, K. (1987). Proteins and surface effects in fermentation: foam, antifoam and mass transfer. *Trends in Biotechnology*, *5*(11), 296–301. https://doi.org/10.1016/0167-7799(87)90080-1

Raja, S., Murty, V. R., Thivaharan, V., Rajasekar, V., & Ramesh, V. (2012). Aqueous Two Phase Systems for the Recovery of Biomolecules – A Review. *Science and*

Technology, *1*(1), 7–16. https://doi.org/10.5923/j.scit.20110101.02

Ramakrishnan, V., Goveas, L. C., Suralikerimath, N., Jampani, C., Halami, P. M., & Narayan, B. (2016). Extraction and purification of lipase from Enterococcus faecium MTCC5695 by PEG/phosphate aqueous-two phase system (ATPS) and its biochemical characterization. *Biocatalysis and Agricultural Biotechnology*, *6*, 19–27. https://doi.org/10.1016/j.bcab.2016.02.005

Rathi, P., Saxena, R. K., & Gupta, R. (2001). A novel alkaline lipase from Burkholderia cepacia for detergent formulation. *Process Biochemistry*, *37*(2), 187–192. https://doi.org/10.1016/S0032-9592(01)00200-X

Ravanfar, R., Tamadon, A. M., & Niakousari, M. (2015). Optimization of ultrasound assisted extraction of anthocyanins from red cabbage using Taguchi design method. *Journal of Food Science and Technology*, *52*(12), 8140–8147. https://doi.org/10.1007/s13197-015-1880-6

Ray, A. (2012). Application of Lipase in Industry. *Asian Journal of Pharmaceutical Technology*, *2*(2), 33–37.

Reinehr, C. O., Treichel, H., Tres, M. V., Steffens, J., Brião, V. B., & Colla, L. M. (2017). Successive membrane separation processes simplify concentration of lipases produced by Aspergillus niger by solid-state fermentation. *Bioprocess and Biosystems Engineering*, *40*(6), 843–855. https://doi.org/10.1007/s00449-017-1749-3

Reschke, T., Brandenbusch, C., & Sadowski, G. (2014). Modeling aqueous two-phase systems: I. Polyethylene glycol and inorganic salts as ATPS former. *Fluid Phase Equilibria*, *368*, 91–103. https://doi.org/10.1016/j.fluid.2014.02.016

Reschke, T., Brandenbusch, C., & Sadowski, G. (2015). Modeling aqueous two-phase systems: III. Polymers and organic salts as ATPS former. *Fluid Phase Equilibria*, *387*, 178–189. https://doi.org/10.1016/j.fluid.2014.12.011

Rito-Palomares, M., & Lyddiatt, A. (2002). Process integration using aqueous two-phase partition for the recovery of intracellular proteins. *Chemical Engineering Journal*, *87*(3), 313–319. https://doi.org/10.1016/S1385-8947(01)00241-8

Rivera, I., Robles, M., Mateos-Díaz, J. C., Gutierrez-Ortega, A., & Sandoval, G. (2017). Functional expression, extracellular production, purification, structure modeling and biochemical characterization of Carica papaya lipase 1. *Process Biochemistry*, *56*, 109–116. https://doi.org/10.1016/j.procbio.2017.02.009

Rodríguez-Durán, L. V., Spelzini, D., Boeris, V., Aguilar, C. N., & Picó, G. A. (2013). Partition in aqueous two-phase system: Its application in downstream processing of tannase from Aspergillus niger. *Colloids and Surfaces B: Biointerfaces*, *101*, 392–397. https://doi.org/10.1016/j.colsurfb.2012.07.023

Rodríguez-Pérez, C., Mendiola, J. ., Quirantes-Piné, R., Ibañez, E., & Segura-Carretero, A. (2016). Green downstream processing using supercritical carbon dioxide, CO2-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from Moringa oleifera leaves. *The Journal of Supercritical Fluids*, *116*, 90–100. https://doi.org/10.1016/j.supflu.2016.05.009

Romdhane, I. B. Ben, Fendri, A., Gargouri, Y., Gargouri, A., & Belghith, H. (2010). A novel

thermoactive and alkaline lipase from Talaromyces thermophilus fungus for use in laundry detergents. *Biochemical Engineering Journal*, *53*(1), 112–120. https://doi.org/10.1016/j.bej.2010.10.002

Romero, C. M., Pera, L. M., Loto, F., Vallejos, C., Castro, G., & Baigori, M. D. (2012). Purification of an organic solvent-tolerant lipase from Aspergillus niger MYA 135 and its application in ester synthesis. *Biocatalysis and Agricultural Biotechnology*, *1*(1), 25–31. https://doi.org/10.1016/j.bcab.2011.08.013

Rooze, J. (2012). *Cavitation in gas-saturated liquids*. Eindhoven: Technische Universiteit Eindhoven. https://doi.org/10.6100/IR732583

Rosa, P. A. J., Azevedo, A. M., Ferreira, I. F., Sommerfeld, S., Bäcker, W., & Aires-Barros, M. R. (2009). Downstream processing of antibodies: Single-stage versus multi-stage aqueous two-phase extraction. *Journal of Chromatography A*, *1216*(50), 8741–8749. https://doi.org/10.1016/j.chroma.2009.02.024

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*(16), 2296–2305. https://doi.org/10.1016/j.chroma.2009.11.034

Sae-leaw, T., & Benjakul, S. (2018). Lipase from liver of seabass (Lates calcarifer): Characteristics and the use for defatting of fish skin. *Food Chemistry*, *240*(February 2017), 9–15. https://doi.org/10.1016/j.foodchem.2017.07.089

Salabat, A. (2001). The influence of salts on the phase composition in aqueous two-phase systems: Experiments and predictions. *Fluid Phase Equilibria*, *187–188*, 489–498. https://doi.org/10.1016/S0378-3812(01)00569-6

Salabat, A., Moghadam, S. T., & Far, M. R. (2010). Liquid-liquid equilibria of aqueous two-phase systems composed of TritonX-100 and sodium citrate or magnesium sulfate salts. *Calphad-Computer Coupling of Phase Diagrams and Thermochemistry*, *34*(1), 81–83. https://doi.org/DOI 10.1016/j.calphad.2009.12.004

Salameh, M. A., & Wiegel, J. (2010). Effects of Detergents on Activity, Thermostability and Aggregation of Two Alkalithermophilic Lipases from Thermosyntropha lipolytica. *Open Biochem J*, *4*, 22–28. https://doi.org/10.2174/1874091X01004010022

Samarakoon, K., & Jeon, Y. J. (2012). Bio-functionalities of proteins derived from marine algae - A review. *Food Research International*, *48*(2), 948–960. https://doi.org/10.1016/j.foodres.2012.03.013

Sankaran, R., Show, P. L., & Chang, J.-S. (2016). Biodiesel production using immobilized lipase: feasibility and challenges. *Biofuels, Bioproducts and Biorefining*, *10*(6), 896–916. https://doi.org/10.1002/bbb.1719

Sankaran, R., Show, P. L., Lee, S. Y., Yap, Y. J., & Ling, T. C. (2018). Integration process of fermentation and liquid biphasic flotation for lipase separation from Burkholderia cepacia. *Bioresource Technology*, *250*, 306–316. https://doi.org/10.1016/j.biortech.2017.11.050

Santos-Ebinuma, V. C., Teixeira, M. F. S., Pessoa, A., & Jauregi, P. (2016). Separation of natural colorants from the fermented broth of filamentous fungi using colloidal gas aphrons. *Separation and Purification Technology*, *163*, 100–108.

https://doi.org/10.1016/j.seppur.2016.02.037

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. https://doi.org/10.1016/j.ces.2006.10.025

Saxena, R. K., Sheoran, A., Giri, B., & Davidson, W. S. (2003). Purification strategies for microbial lipases. *Journal of Microbiological Methods*, *52*(1), 1–18. https://doi.org/10.1016/S0167-7012(02)00161-6

Sebba, F. (1962). *Ion flotation*. Amsterdam, New York: Elsevier Pub. Co.

Selvakumar, P., Ling, T. C., Covington, A. D., & Lyddiatt, A. (2012). Enzymatic hydrolysis of bovine hide and recovery of collagen hydrolysate in aqueous two-phase systems. *Separation and Purification Technology*, *89*, 282–287. https://doi.org/10.1016/j.seppur.2012.01.046

Selvakumar, P., Ling, T. C., Walker, S., & Lyddiatt, A. (2010). A practical implementation and exploitation of ATPS for intensive processing of biological feedstock: A novel approach for heavily biological feedstock loaded ATPS. *Separation and Purification Technology*, *75*(3), 323–331. https://doi.org/10.1016/j.seppur.2010.08.022

Sengupta, R., Chakraborty, S., Bandyopadhyay, S., Dasgupta, S., Mukhopadhyay, R., Auddy, K., & Deuri, a S. (2007). A Short Review on Rubber / Clay Nanocomposites With Emphasis on Mechanical Properties. *Engineering*, *47*, 21–25. https://doi.org/10.1002/pen

Shah, S., Sharma, S., & Gupta, M. N. (2004). Biodiesel preparation by lipase-catalyzed transesterification of Jatropha oil. *Energy and Fuels*, *18*(1), 154–159. https://doi.org/10.1021/ef030075z

Sharma, P., Sharma, N., Pathania, S., & Handa, S. (2017). Purification and characterization of lipase by Bacillus methylotrophicus PS3 under submerged fermentation and its application in detergent industry. *Journal of Genetic Engineering and Biotechnology*, *15*(2), 369–377. https://doi.org/10.1016/j.jgeb.2017.06.007

Sharma, R., Chisti, Y., & Chand, U. (2001). Production, Purification, Characterization, and Applications of Lipases. *Biotechnology Advances*, *19*, 627–662. https://doi.org/10.1016/S0734-9750(01)00086-6

Sharma, S., & Kanwar, S. S. (2014). Organic Solvent Tolerant Lipases and Applications. *The Scientific World Journal*, 1–15. https://doi.org/10.1155/2014/625258

Shin, Y. O., & Vera, J. H. (2002). Solubilization limit of lysozyme into DODMAC reverse micelles. *Biotechnology and Bioengineering*, *80*(5), 537–543. https://doi.org/10.1002/bit.10413

Show, P. L., Ooi, C. W., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K., … Ling, T. C. (2013a). Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt. *Separation and Purification Technology*, *110*, 112–118. https://doi.org/10.1016/j.seppur.2013.03.018

Show, P. L., Ooi, C. W., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K., … Ling, T. C. (2013b).

Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt. *Separation and Purification Technology*, *110*, 112–118. https://doi.org/10.1016/j.seppur.2013.03.018

Show, P. L., Tan, C. P., Anuar, M. S., Ariff, A., Yusof, Y. A., Chen, S. K., & Ling, T. C. (2011). Direct recovery of lipase derived from Burkholderia cepacia in recycling aqueous two-phase flotation. *Separation and Purification Technology*, *80*(3), 577–584. https://doi.org/10.1016/j.seppur.2011.06.013

Show, P. L., Tan, C. P., Shamsul Anuar, M., Ariff, A., Yusof, Y. A., Chen, S. K., & Ling, T. C. (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*(2012), 226–233. https://doi.org/10.1016/j.biortech.2011.09.131

Sinha, J., Dey, P. K., & Panda, T. (2000). Aqueous two-phase: the system of choice for extractive fermentation. *Applied Microbiology and Biotechnology*, *54*(4), 476–486. https://doi.org/10.1007/s002530000342

Sivakumar, V., Anna, J. L., Vijayeeswarri, J., & Swaminathan, G. (2009). Ultrasound assisted enhancement in natural dye extraction from beetroot for industrial applications and natural dyeing of leather. *Ultrasonics Sonochemistry*, *16*(6), 782–789. https://doi.org/10.1016/j.ultsonch.2009.03.009

Sivaramakrishnan, R., & Incharoensakdi, A. (2016). Purification and characterization of solvent tolerant lipase from Bacillus sp. for methyl ester production from algal oil. *Journal of Bioscience and Bioengineering*, *121*(5), 517–522. https://doi.org/10.1016/j.jbiosc.2015.09.005

Snellman, E. A., Sullivan, E. R., & Colwell, R. R. (2002). Purification and properties of the extracellular lipase, LipA, of Acinetobacter sp. RAG-1. *European Journal of Biochemistry*, *269*(23), 5771–5779. https://doi.org/10.1046/j.1432-1033.2002.03235.x

STINSON, S. C. (1995). Fine And Intermediate Chemicals Makers Emphasize New Products and Processes. *Chemical & Engineering News*, *73*(29), 10–26. https://doi.org/10.1021/cen-v073n029.p010

Szydłowski, J., & Szykuła, M. (1999). Isotope effect on miscibility of acetonitrile and water. *Fluid Phase Equilibria*, *154*(1), 79–87. https://doi.org/10.1016/S0378-3812(98)00438-5

Tan, C. H., Chen, C. Y., Show, P. L., Ling, T. C., Lam, H. L., Lee, D. J., & Chang, J. S. (2016). Strategies for enhancing lipid production from indigenous microalgae isolates. *Journal of the Taiwan Institute of Chemical Engineers*, *63*, 189–194. https://doi.org/10.1016/j.jtice.2016.02.034

Tan, C. H., Show, P. L., Ooi, C. W., Ng, E. P., Lan, J. C. W., & Ling, T. C. (2015). Novel lipase purification methods - a review of the latest developments. *Biotechnology Journal*, *10*(1), 31–44. https://doi.org/10.1002/biot.201400301

Tan, J. S., Abbasiliasi, S., Lin, Y. K., Mohamed, M. S., Kapri, M. R., Kadkhodaei, S., … Ariff, A. B. (2014). Primary recovery of thermostable lipase 42 derived from recombinant

Escherichia coli BL21 in aqueous two-phase flotation. *Separation and Purification Technology*, *133*(2014), 328–334. https://doi.org/10.1016/j.seppur.2014.06.048

Tan, Z. J., Li, F. F., & Xu, X. L. (2013). Extraction and purification of anthraquinones derivatives from Aloe vera L. using alcohol/salt aqueous two-phase system. *Bioprocess and Biosystems Engineering*, *36*(8), 1105–1113. https://doi.org/10.1007/s00449-012-0864-4

Tan, Z., Song, S., Han, J., Wang, Y., Lu, Y., & Yan, Y. (2013). Optimization of partitioning process parameters of chloramphenicol in ionic liquid aqueous two-phase flotation using response surface methodology. *Journal of the Iranian Chemical Society*, *10*(3), 505–512. https://doi.org/10.1007/s13738-012-0184-2

Tao, Y., Cai, H., Li, W., & Cai, B. (2015). Ultrafiltration coupled with high-performance liquid chromatography and quadrupole-time-of-flight mass spectrometry for screening lipase binders from different extracts of Dendrobium officinale. *Analytical and Bioanalytical Chemistry*, *407*(20), 6081–6093. https://doi.org/10.1007/s00216-015-8781-4

Thömmes, J., & Etzel, M. (2007). Alternatives to chromatographic separations. *Biotechnology Progress*, *23*(1), 42–45. https://doi.org/10.1021/bp0603661

Tonova, K., & Lazarova, Z. (2008). Reversed micelle solvents as tools of enzyme purification and enzyme-catalyzed conversion. *Biotechnology Advances*, *26*(6), 516–532. https://doi.org/10.1016/j.biotechadv.2008.06.002

Tsai, W. H., Chuang, H. Y., Chen, H. H., Wu, Y. W., Cheng, S. H., & Huang, T. C. (2010). Application of sugaring-out extraction for the determination of sulfonamides in honey by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A*, *1217*(49), 7812–7815. https://doi.org/10.1016/j.chroma.2010.10.008

Tubio, G., Nerli, B. B., Pic??, G. A., Ven??ncio, A., & Teixeira, J. (2009). Liquid-liquid equilibrium of the Ucon 50-HB5100/sodium citrate aqueous two-phase systems. *Separation and Purification Technology*, *65*(1), 3–8. https://doi.org/10.1016/j.seppur.2008.01.018

Tubtimdee, C., & Shotipruk, A. (2011). Extraction of phenolics from Terminalia chebula Retz with water-ethanol and water-propylene glycol and sugaring-out concentration of extracts. *Separation and Purification Technology*, *77*(3), 339–346. https://doi.org/10.1016/j.seppur.2011.01.002

U.S. Energy information administration. (2015). *Monthly biodiesel production report*. Retrieved from http://www.eia.gov/biofuels/biodiesel/production/

Ülker, S., & Karaoğlu, Ş. A. (2012). Purification and characterization of an extracellular lipase from Mucor hiemalis f. corticola isolated from soil. *Journal of Bioscience and Bioengineering*, *114*(4), 385–390. https://doi.org/10.1016/j.jbiosc.2012.04.023

Undurraga, D., Markovits, A., & Erazo, S. (2001). Cocoa butter equivalent through enzymic interesterification of palm oil midfraction. *Process Biochemistry*, *36*(10), 933–939. https://doi.org/10.1016/S0032-9592(00)00260-0

Unni, K. N., Priji, P., Sajith, S., Faisal, P. A., & Benjamin, S. (2016). Pseudomonas aeruginosa strain BUP2, a novel bacterium inhabiting the rumen of Malabari goat,

produces an efficient lipase. *Biologia*, *71*(4). https://doi.org/10.1515/biolog-2016-0057

Valsaraj, K. T., Thoma, G. J., Thibodeaux, L. J., & Wilson, D. J. (1991). Nonfoaming adsorptive bubble separation processes. *Separations Technology*, *1*(5), 234–244. https://doi.org/10.1016/0956-9618(91)80018-U

Van Rantwijk, F., Lau, R. M., & Sheldon, R. A. (2003). Biocatalytic transformations in ionic liquids. *Trends in Biotechnology*, *21*(3), 131–138. https://doi.org/10.1016/S0167-7799(03)00008-8

van Winssen, F. A., Merz, J., Czerwonka, L.-M., Schembecker, G., & Dortmund, T. (2014). Application of the Tunable Aqueous Polymer-Phase Impregnated Resins-Technology for protein purification. *Separation and Purification Technology*, *136*, 123–129. https://doi.org/10.1016/j.seppur.2014.08.030

Velmurugan, P., Kamala-Kannan, S., Balachandar, V., Lakshmanaperumalsamy, P., Chae, J. C., & Oh, B. T. (2010). Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. *Carbohydrate Polymers*, *79*(2), 262–268. https://doi.org/10.1016/j.carbpol.2009.07.058

Wang, B., Ezejias, T., Feng, H., & Blaschek, H. (2008). Sugaring-out: A novel phase separation and extraction system. *Chemical Engineering Science*, *63*(9), 2595–2600. https://doi.org/10.1016/j.ces.2008.02.004

Wang, H. X., Wu, H., Ho, C. T., & Weng, X. C. (2006). Cocoa butter equivalent from enzymatic interesterification of tea seed oil and fatty acid methyl esters. *Food Chemistry*, *97*(4), 661–665. https://doi.org/10.1016/j.foodchem.2005.04.029

Wang, M., Yuan, W., Jiang, X., Jing, Y., & Wang, Z. (2014). Disruption of microalgal cells using high-frequency focused ultrasound. *Bioresource Technology*, *153*, 315–321. https://doi.org/10.1016/j.biortech.2013.11.054

Wang, Y., Hu, X., Han, J., Ni, L., Tang, X., Hu, Y., & Chen, T. (2016). Integrated method of thermosensitive triblock copolymer–salt aqueous two phase extraction and dialysis membrane separation for purification of lycium barbarum polysaccharide. *Food Chemistry*, *194*, 257–264. https://doi.org/10.1016/j.foodchem.2015.08.016

Wang, Y., Xu, X. hui, Han, J., & Yan, Y. sheng. (2011). Separation/enrichment of trace tetracycline antibiotics in water by [Bmim]BF4-(NH4)2SO4 aqueous two-phase solvent sublation. *Desalination*, *266*(1–3), 114–118. https://doi.org/10.1016/j.desal.2010.08.010

Wasserscheid, P., & Keim, W. (2000). Ionic Liquids—New “Solutions” for Transition Metal Catalysis. *Angewandte Chemie*, *39*(21), 3772–3789. https://doi.org/10.1002/1521-3773(20001103)39:21<3772::AID-ANIE3772>3.0.CO;2-5

Wingfield, P. (1998a). Protein Precipitation Using Ammonium Sulfate. *Current Protocols in Protein Science*, *53*(4), A.3F.1-A.3F.8. https://doi.org/10.1002/0471140864.psa03fs13

Wingfield, P. (1998b). Protein Precipitation Using Ammonium Sulfate. In *Current Protocols in Protein Science* (p. A.3F.1-A.3F.8). Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/0471140864.psa03fs13

Won, K., & Sun Bok Lee. (2001). Computer-aided control of water activity for lipase-catalyzed esterification in solvent-free systems. *Biotechnology Progress*, *17*(2), 258–264. https://doi.org/10.1021/bp000154i

Wood, D. W. (2014). New trends and affinity tag designs for recombinant protein purification. *Current Opinion in Structural Biology*, *26*(1), 54–61. https://doi.org/10.1016/j.sbi.2014.04.006

Wu, X., Joyce, E. M., & Mason, T. J. (2011). The effects of ultrasound on cyanobacteria. *Harmful Algae*, *10*(6), 738–743. https://doi.org/10.1016/j.hal.2011.06.005

Xia, J., Ni, L., Han, J., Wang, Y., Li, Y., Li, Y., & Tian, Y. (2016). Simultaneous aqueous two-phase flotation of sodium chlorophyllin and removal of sugars from saponified solution of bamboo leaves. *Chemical Engineering and Processing: Process Intensification*, *101*(2016), 41–49. https://doi.org/10.1016/j.cep.2015.12.014

Xing, J. min, & Li, F. fang. (2012). Chiral separation of mandelic acid by temperature-induced aqueous two-phase system. *Journal of Chemical Technology and Biotechnology*, *87*(3), 346–350. https://doi.org/10.1002/jctb.2720

Yagmurov, E. R., Kozlov, G. V., & Pushkarev, M. A. (2017). LIPASE PURIFICATION : THE REVIEW OF CONVENTIONAL AND NOVEL METHODS. *Journal of Hygienic Engineering and Design Review*, *20*, 60–69.

Yan, Y., Li, X., Wang, G., Gui, X., Li, G., Su, F., … Liu, T. (2014). Biotechnological preparation of biodiesel and its high-valued derivatives: A review. *Applied Energy*, *113*(2014), 1614–1631. https://doi.org/10.1016/j.apenergy.2013.09.029

Yang, H., Goja, A. M., Cui, M., & Li, C. (2013). Aqueous Two-Phase Extraction Advances for Bioseparation. *Journal of Bioprocessing and Biotechniques*, *44172*(10), 2155–9821. https://doi.org/10.4172/2155-9821.1000140

Yang, J., Guo, D., & Yan, Y. (2007). Cloning, expression and characterization of a novel thermal stable and short-chain alcohol tolerant lipase from Burkholderia cepacia strain G63. *Journal of Molecular Catalysis B: Enzymatic*, *45*(3–4), 91–96. https://doi.org/10.1016/j.molcatb.2006.12.007

Yang, L., Huo, D., Hou, C., He, K., Lv, F., Fa, H., & Luo, X. (2010). Purification of plant-esterase in PEG1000/NaH2PO4 aqueous two-phase system by a two-step extraction. *Process Biochemistry*, *45*(10), 1664–1671. https://doi.org/10.1016/j.procbio.2010.06.018

Yang, W., He, Y., Xu, L., Zhang, H., & Yan, Y. (2016). A new extracellular thermo-solvent-stable lipase from Burkholderia ubonensis SL-4: Identification, characterization and application for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic*, *126*, 76–89. https://doi.org/10.1016/j.molcatb.2016.02.005

Yang, Z., Tan, Z., Li, F., & Li, X. (2016). An effective method for the extraction and purification of chlorogenic acid from ramie (Boehmeria nivea L.) leaves using acidic ionic liquids. *Industrial Crops and Products*, *89*, 78–86. https://doi.org/10.1016/j.indcrop.2016.05.006

Ye, P., Xu, Z. K., Che, A. F., Wu, J., & Seta, P. (2005). Chitosan-tethered poly(acrylonitrile-co-maleic acid) hollow fiber membrane for lipase immobilization. *Biomaterials*, *26*(32), 6394–6403. https://doi.org/10.1016/j.biomaterials.2005.04.019

Yeh, K.-L., & Chang, J.-S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga Chlorella vulgaris ESP-31: Implications for biofuels. *Biotechnology Journal*, *6*(11), 1358–1366. https://doi.org/10.1002/biot.201000433

Yong, S. K., Lim, B. H., Saleh, S., & Tey, L. H. (2016). Optimisation, purification and characterisation of extracellular lipase from Botryococcus sudeticus (UTEX 2629). *Journal of Molecular Catalysis B: Enzymatic*, *126*, 99–105. https://doi.org/10.1016/j.molcatb.2016.02.004

Zarai, Z., Ali, M. B., Fendri, A., Louati, H., Mejdoub, H., & Gargouri, Y. (2012). Purification and biochemical properties of Hexaplex trunculus digestive lipase. *Process Biochemistry*, *47*(12), 2434–2439. https://doi.org/10.1016/j.procbio.2012.10.004

Zaslavsky, Boris, Y. (1994). *Aqueous two-phase partitioning: physical chemistry and bioanalytical applications.* CRC Press.

Zhang, C., Huang, K., Yu, P., & Liu, H. (2012). Sugaring-out three-liquid-phase extraction and one-step separation of Pt(IV), Pd(II) and Rh(III). *Separation and Purification Technology*, *87*, 127–134. https://doi.org/10.1016/j.seppur.2011.11.032

Zhang, H. Y., Wang, X., Ching, C. B., & Wu, J. C. (2005). Experimental optimization of enzymic kinetic resolution of racemic flurbiprofen. *Biotechnology And Applied Biochemistry*, *42*, 67–71. https://doi.org/10.1042/BA20040163

Zhang, L., Wang, Y., & Wu, S. (2017). Sugaring-out strategy for counter-current chromatography isolation: podophyllotoxins and flavones from Dysosma versipellis as examples. *RSC Advances*, *7*(19), 11591–11600. https://doi.org/10.1039/C6RA27838H

Zhang, S., Sun, N., He, X., Lu, X., & Zhang, X. (2006). Physical properties of ionic liquids: Database and evaluation. *Journal of Physical and Chemical Reference Data*, *35*(4), 1475–1517. https://doi.org/10.1063/1.2204959

Zhao L.; Liu, D., X. . W. (2007). Effect of several factors on peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis. *Journal of Chemical Technology & Biotechnology*, *82*(May), 1115–1121. https://doi.org/10.1002/jctb

Zhuang, J., Yang, W., Chen, X., & Jiao, F. (2014). Enantioseparation of Phenylsuccinic Acid Enantiomers Using Aqueous Two-Phase Flotation and Their Determination by HPLC and UV Detection. *Chromatographia*, *77*(9–10), 679–685. https://doi.org/10.1007/s10337-014-2668-y

Zouari, N., Miled, N., Cherif, S., Mejdoub, H., & Gargouri, Y. (2005). Purification and characterization of a novel lipase from the digestive glands of a primitive animal: The scorpion. *Biochimica et Biophysica Acta - General Subjects*, *1726*(1), 67–74. https://doi.org/10.1016/j.bbagen.2005.08.005