

Thesis

Extraction and Purification of Green Polymers Using Aqueous Two-Phase Extraction (ATPE)

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ABSTRACT

Due to the increasing concern towards depleting petroleum resource and pollutions done by conventional plastics, polyhydroxyalkanoates (PHAs) which have diverse structure variability and rapid biodegradation have gained increasing attentions as potential replacement for plastics. However, PHAs have a much higher production cost compared to that of conventional plastics due to expensive carbon source and downstream processing. Aqueous two-phase extraction (ATPE) outshines other PHAs purification techniques by having the advantages of providing a mild environment for bioseparation, being green and non-toxic, and easily scaled-up. Henceforth, this research aimed to study on the purification and recovery of PHAs using thermoseparation-based ATPE.

Firstly, cloud point extraction technique which based on thermoseparating polymers (TSP)/water two phase system was employed. The recovery yield of 94.8 % and purification factor (PF) of 1.41 fold was achieved under the conditions of 20 wt/wt % ethylene oxide-propylene oxide (EOPO) with 3900 g/mol MW and 10 mM of NaCl addition at thermoseparating temperature of 60 °C.

TSPs have also been coupled with ammonium sulfate to form a two-phase system. Under the condition of 14 wt/wt % of both EOPO 3900 and $(NH_4)_2SO_4$ at pH 6, yield and purity up to 72 % and 60 % can be achieved. Without the need of additional TSPs top-up, recycling and reutilization of phase-forming polymers can be done at least twice with satisfying yield and PF.

Using two-level full factorial design, the statistical analysis demonstrated that the phosphate and thermoseparating polymer concentration are the most significant parameters due to their individual influence and synergistic interaction between them on all response variables. Utilizing the two significant parameters, the purification and recovery of PHAs were further optimized using central composite design and achieved

recovery yield as high as 99.9 %. The optimum PF of 1.431 fold was obtained at 17.12 wt/wt % of phosphate salts and 18.52 wt/wt % of EOPO 3900. Using the addition of fresh phase-forming components, a total of 4 successive purification cycles with satisfying yield and PF were demonstrated.

Utilizing the carbon source screened by the preliminary integrated economic and environmental assessment developed, the feasibility of extractive bioconversion of PHAs under the influence of different parameters was studied. The strategy successfully achieved a yield and PF of 97.6 % and 1.36 fold respectively under the condition of 5 wt/wt % EOPO 3900 concentration, 30 °C fermentation temperature and pH 6. The scaling-up to 2 L bioreactor proved that the scale-up of ATPE can be predicted reliably from laboratory-scale experimental data.

In the final section, the evaluation of economic and environmental performance of two processes (which are with and without thermoseparating ATPE as primary purification step) were performed. With the basis of 9,000 tons PHAs production per year and 7,920 operating hours, the process with thermoseparating ATPE as primary purification step standout in terms of both economic and environmental performance. PHA production cost of 5.77 US$/kg with a payback period of fewer than 4 years and ROI of 25.2 % was achieved.

The research proved that thermoseparating ATPE is a powerful and potential technique for PHAs purification and recovery as the technique showed a much higher PHAs recovery yield (approximately 2 times) in comparison with the literature (Divyashree and Shamala, 2010). In conclusion, this opens promising standpoints for utilizing thermoseparating ATPE as the primary step in the isolation and purification of PHAs from fermented broth.

Keywords: Aqueous two-phase extraction, Bioseparation, Extractive bioconversion, Polyhydroxyalkanoates, Process simulation, Thermoseparating polymer

Nomenclature

Acronyms

ANOVA	Analysis of Variance
AP	Acidification or Acid Rain Potential
ATP	Aquatic Toxicity Potential
ATPE	Aqueous Two-Phase Extraction
ATPS	Aqueous Two-Phase System
CCD	Central Composite Design
CP	Cloud Point
CPE	Cloud Point Extraction
DO	Dissolved Oxygen
DOE	Design of Experiment
EB	Environmental Burden
EO	Ethylene Oxide
EOPO	Ethylene Oxide-Propylene Oxide
GC	Gas Chromatography
GWP	Global Warming Potential
HM-EOPO	Hydrophobically Modified EOPO
HTPE	Human Toxicity Potential By Either Inhalation or Dermal Exposure

HTPI	Human Toxicity Potential by ingestion
IRR	Internal Rate of Return
LCA	Life Cycle Analysis
LCST	Lower Critical Solution Temperature
NPCM	Non-PHA Cell Mass
NREU	Non-Renewable Energy Use
ODP	Ozone Depletion Potential
OFAT	One-factor-at-a-time
P(3HB)	Poly(3-hydroxybutyrate)
PEI	Potential Environmental Impact
PEG	Polyethylene Glycol
PF	Purification Factor
PHAs	Polyhydroxyalkanoates
PO	Propylene Oxide
POP	Photochemical Oxidation or Smog Formation Potential
RSM	Response Surface Method
SDS	Sodium Dodecyl Sulfate
SPI	Sustainable Process Index
TAC	Total Annual Cost
TLL	Tie-Line Length

TSPs	Themoseparating Polymers
TTP	Terrestrial Toxicity Potential
WAR	Waste Reduction Algorithm
WCO	Waste Cooking Oil

Chemical Ions

ClO_4^-	Chlorate ion
I^-	Iodide ion
PO_4^{3-}	Phosphate ion
SCN^-	Thiocyanate ion
SO_4^{2-}	Sulfate ion

Symbols

λ_o	Degree of satisfaction	dimensionless
$Conc_E$	PHA Concentration of Extract	g/L
$Conc_T$	PHA Concentration of Top Phase	g/L
M_{PHA}	Mass of PHA	g
M_{Sample}	Total Mass of Dried Sample Used	g
MW	Molecular Weight	Da
R^2	Coefficient of Determination	dimensionless
R^2_{adj}	Adjusted Coefficient of Determination	dimensionless
R^2_{pred}	Predicted Coefficient of Determination	dimensionless

V_B	Bottom Phase Volume	ml
V_E	Extract Volume	ml
V_r	Volume Ratio	dimensionless
V_T	Top Phase Volume	ml
Y	Recovery Yield	%

Published Works

Journal paper:

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Yoong Kit Leong, John Chi-Wei Lan, Hwei-San Loh, Tau Chuan Ling, Chien Wei Ooi and Pau Loke Show, 2017. Cloud-point extraction of green-polymers from Cupriavidus necator lysate using thermoseparating-based aqueous two-phase extraction. Journal of Bioscience and Bioengineering, 123(3), 370-375.

Yoong Kit Leong, John Chi-Wei Lan, Hwei-San Loh, Hon Loong Lam, Tau Chuan Ling, and Pau Loke Show, 2017. Economic and environmental analysis of PHAs production process. Clean Technologies and Environmental Policy, 19(7), 1941-1953.

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Review paper:

Yoong Kit Leong, Pau Loke Show, Chien Wei Ooi, Tau Chuan Ling and John Chi-Wei Lan, 2014. Current trends in polyhydroxyalkanoates (PHAs) biosynthesis: Insights from the recombinant *Escherichia coli*. Journal of Biotechnology, 180, 52-65.

Yoong Kit Leong, John Chi-Wei Lan, Hwei-San Loh, Tau Chuan Ling, Chien Wei Ooi and Pau Loke Show, 2016. Thermoseparating Aqueous Two-Phase System: Recent trends and mechanisms.Journal of Separation Science, 39, 640-647.

Conference Paper:

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YK Leong, PL Show, SWS Loh and JCW Lan, 2015. Technological Aspects of Drying in Green Polymer Biosynthesis. Paper presented at the 8th Asia Pacific Drying Conference, Kuala Lumpur, Malaysia.

Yoong Kit Leong, Pau Loke Show, John Chi-Wei Lan, Hwei-San Loh, 2015. Thermoseparating Aqueous Two Phase System: Recent trends and applications. Paper presented at the 18th Conference of Process Integration, Modelling and Optimisation for Energy Saving and Pollution Reduction, Kuching, Malaysia.

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Chapter 1

INTRODUCTION AND BACKGROUND

1.1 CONVENTIONAL PETROCHEMICAL-BASED PLASTICS

The term "Plastic Age" has often been used to refer the present period of human history (Cozar et al., 2014). Due to their impressive diversity, durability, and lightweight, plastics are intensively involved in our daily life, from stationery, packaging, personal hygiene products, machinery housings, disposable utensils, furniture, and automobile to accessories (Castilho et al., 2009). At present, fossil fuel accounts for approximately 80 % of the raw material for all polymeric materials produced. In the year of 2014, approximately 311 million tons of conventional petrochemical-based plastics were manufactured (Gumel et al., 2012, PlasticsEurope, 2015). Following the rapid growth of plastic production and usage, the concerns such as waste management, non-renewable resource reserve, and environmental impact have become a current and pressing issue. Countries such as Japan, U.S.A. and members of European Union have enforced legislations and established policies to reduce the consumption of synthetic plastic (Chanprateep, 2010).

Plastics most attractive attribute, durability has also become their most troublesome characteristic as they are very stable and can last for hundreds to thousands of years. Consequently, they accumulate in the natural environment and this not only damages the beauties of forest and cities but also causes the destruction of local flora and fauna (Ojumu and Solomon, 2004). Due to uncontrolled dumping of plastics into the water body (8 million tons per year) (Jambeck et al., 2015), massive floating masses of plastic waste present in every sea and ocean in the world, with a third of it accumulated in the notorious Great Pacific Garbage Patch (Cozar et al., 2014). The uprising solid waste disposal cost, the release of potential health hazards such as hydrogen cyanide and hydrogen chloride from incineration, and secondary pollution (such as odor, soil pollution and seepage of leachate into the groundwater) caused by landfill have made synthetic plastic a waste management issue. Although recycling and reusing do improve the material utilization, the idea of recycling plastic does not seem attractive as categorizing of the wide range of unwanted plastic material is

already a very labor intensive and time-consuming process (Khanna and Srivastava, 2005, Castilho et al., 2009). Also, recycling of plastics cannot be done indefinitely as the quality of the materials deteriorates and it will become waste eventually. Therefore, there is a pressing demand for the alternative of conventional plastics and bioplastics have gained much attraction from industries and researchers.

1.2 BENEFITS AND CHALLENGES OF BIOPLASTICS

There are numerous advantages of replacing conventional petrochemical-based plastics with bioplastics. Not only reducing the use of crude oil, this will also drastically reduce the emissions of CO_2 and other greenhouse gasses, leading to a reduced carbon footprint for plastics. Due to the volatility of crude oil in recent years, it can be a guarantee of material supply in manufacturing bioplastics for non-crude oil producing countries to reduce dependence on other oil producing countries. Contributed by the rising concern towards environmental issues, bioplastics-based products are much more acceptable for the eco-conscious consumers. Assuming the appropriate end of life management option is applied, utilization of bioplastics that are either biodegradable or compostable can help minimize waste disposal for landfill. Furthermore, some bioplastics such as polylactic acid (PLA) provide the chance of cradle to cradle lifecycles as there is a variety of choices to recycle and incorporate them into raw materials for new products. Compared to conventional plastics, wide range of bioplastics provide a much more diversify properties and have some important technical advantages, such as more tactile and softer with less "oily" surface feel, less likely to pass on a taste to the product, have greater water vapor permeability, and can be made more transparent and clearer (Conor et al., 2013).

On the other hands, there are also some challenges and obstacles present in wide applications of bioplastics. One of the main challenges is the expensive cost of bioplastics compared to that of conventional plastics, which can be two or three times pricier and

sometimes can even be up to tenfold its petrochemical counterpart. This is normally due to the high cost of feedstock and downstream processing together with a low production volume of bioplastics. Also, there is always an argument of bioplastics versus food for crop utilization. Food security makes community concerns whether bioplastics will reduce the availability of land for food production, or encourage deforestation for more arable land. In addition, there is a need for legislations and standards to standardize and protect the terminologies which including 'biodegradable', 'compostable' or 'biobased'. Clear labeling of different bioplastic types and consumer education will facilitate widespread utilization of bioplastics as well (Conor et al., 2013). The advantages and disadvantages of using bioplastics are summarized and compared in Table 1.1.

Table 1.1 Advantages and disadvantages of using bioplastics

Advantages	Disadvantages
Reducing the use of crude oil	Expensive cost
Reduce the emissions of other greenhouse gasses	Food security issue
A guarantee of material supply in manufacturing bioplastics for non-crude oil producing countries	Needs for legislations and standards to standardize and protect the terminologies
Much more acceptable for the eco-conscious consumers	
Minimize waste disposal for landfill	
Can be recycled and incorporated into raw materials for new products	
Provide more diversify properties and have important technical advantages	

1.3 POLYHYDROXYALKANOATEs (PHAs)

PHAs are thermoplastics which are biocompatible and completely biodegradable, enantiomerically pure, non-toxic, exhibit piezoelectricity (which helps in wound healing and stimulates bone growth), water-insoluble, inert, indefinitely stable in air, has high structural diversity and good processability on equipment (Venkateswar Reddy et al., 2012, Rai et al., 2011). Due to its properties, PHAs have high potential in medical field's applications, such as orthopaedic (screws, scaffolds for cartilage engineering, bone graft substitutes), for cardiovascular system devices (blood vessel replacements, heart valves, cardiovascular stents, atrial and pericardial septal repair patches), wound management (skin substitutes, sutures, dressings, dusting powders), urological stents, nano- and microspheres for controlled drug delivery and others (Castilho et al., 2009, Keshavarz and Roy, 2010).

There are some properties that make PHAs outshine other biodegradable polyesters are their rapid degradation (3 to 9 months) aerobically and anaerobically without the need of special environment conditions and large variability in structure with extensive material properties (Keshavarz and Roy, 2010, Mumtaz et al., 2010). They are degraded by different kinds of microorganisms into carbon dioxide and water under aerobic condition or methane under anaerobic condition (Jindal and Tiwari, 2013). Currently, Zeneca is the industrial manufacturer of P(3HB) and copolymers of 3-hydroxybutyrate with the trade name of Biopol (Lemos et al., 1998).

However, production of PHAs is much more expensive than conventional petrochemical-based plastics. Thus, a cost-effective recovery method for PHA is essential for this type of biopolymers to stay competitive among others bio- and synthetic plastics. The most conventional and most extensively used method in the laboratory to recover PHA is by using solvent extraction due to its rapidity and simplicity (Valappil et al., 2007, Noel, 1962, Lafferty and Heinzle, 1978, Fiorese et al., 2009, Traussnig et al., 1990). Not mentioning this

method consumes a large amount of volatile and toxic solvents such as chloroform, it disrupts the native orders of PHA granules' polymer chains.

Therefore, researchers have put in efforts to develop more techniques for high-efficiency PHA recovery in both laboratory and industrial scale. These methods include chemical digestion (Lakshman and Shamala, 2006, Valappil et al., 2007, Berger et al., 1989, Ramsay et al., 1990, Lee et al., 1993, Choi and Lee, 1999a, Dong and Sun, 2000, Chen et al., 2001), selective dissolution of non-PHA cell mass (NPCM) by protons (Yu and Chen, 2006), enzymatic treatment (Kapritchkoff et al., 2006, Lakshman and Shamala, 2006, Kathiraser et al., 2007), mechanical disruption (Tamer et al., 1998, Ghatnekar et al., 2002, Divyashree et al., 2009), supercritical fluid (Hejazi et al., 2003), using cell fragility (Divyashree and Shamala, 2010), air classification (Noda, 1998), floatation techniques (van Hee et al., 2006, Ibrahim and Steinbüchel, 2009), aqueous two-phase systems (Divyashree et al., 2009) and use of gamma irradiation (Divyashree and Shamala, 2009).

1.4 AQUEOUS TWO-PHASE SYSTEM (ATPS)

An aqueous two-phase system, ATPS is a system of two immiscible phases formed when two structurally different polymers or an inorganic salt and one polymer are mixed in water beyond a critical concentration (Diamond and Hsu, 1992). Besides utilization as an analytical and preparation tool in biochemistry (Kepka et al., 2003, Rosa et al., 2010), ATPSs also serves as a primary recovery step for isolation and purification of partially removing the target product from impurities or substrates, hence reducing the subsequent downstream processing volume. ATPS has many advantages compared to conventional purification technique, such as mild environment for bioseparation, green and non-toxic as well as ability to scaled-up easily.

Depending on their physicochemical properties and the system variables, proteins and other bioproducts will partition between the top and bottom phases. The main factors that affect the partitioning behavior on biomaterials in ATPS are the physicochemical properties of the proteins, such as size, net charge and relative hydrophobicity (Persson et al., 1999a), and further influenced by intermolecular electrostatic attraction (long range) and van der Waals force (short range) (Kammoun et al., 2009).

Conventional ATPSs are composed of polyethylene glycol (PEG)/dextran which formed because of polymer-polymer incompatibility (Leuchau et al., 2011, Kammoun et al., 2009), and PEG/salt which formed because of the salting out effect on the polymer, usually by sulfate (SO42-) or phosphate (PO43-) (Li et al., 2002a). The PEG/Dextran systems are conventionally used for laboratory-scale separations of cells, cell particle, membranes and macromolecules (Walter et al., 1985). Due to the prohibiting cost of dextran (Ng et al., 2013), starch derivatives which have similar phase-forming properties and more cost-efficient, such as hydroxypropyl starch, have been researched on as a substitute. PEG/salt systems which are generally used in industrial-scale enzyme extraction provides a broad range of hydrophobic differences between the two phases of ATPS.

However, there are some critical downsides for both of these ATPSs. For polymer/salt system, an amphiphilic protein which has low solubility tends to aggregate in water solutions (Persson et al., 1998). The main drawback for both polymer/polymer and polymer/salt systems is that the target bioproducts are accumulated in either the polymer-rich phase or salt-rich phase, both leading to difficulty in subsequent purification steps (Kepka et al., 2004). These systems require back-extraction into salt-rich bottom phase, or additional and complicated operations (such as ion-exchange chromatography, diafiltration, crystallization or ultrafiltration) to remove the phase-forming components from target products (Dembczyński et al., 2010). Furthermore, recycling of salts, which are bottom phase-forming components, cannot be done effectively. This is because salt recycling requires large amounts

of polymers/chemicals, leading to environmental pollution and high operating costs (Li et al., 2013).

Therefore, advanced and improved ATPSs have been developed using surfactants (Moradi and Yamini, 2012), alcohols, anionic liquid (Li et al., 2015), micellar compounds and thermoseparating polymers (Silvério et al., 2013). The thermoseparating polymers, TSPs were firstly discussed in the early 1990s where they are able to form ATPS together with dextran (Berggren et al., 1995). The common TSPs used are random, diblock, and triblock copolymers of hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO), thus, named EOPO copolymers. These polymers have a wide range of application as their EO/PO ratio and molecular weight can be greatly varied (Hasmann et al., 2008). Other examples of TSPs include poly(N-isopropylacrylamide), poly(vinylcaprolactam), ethyl-hydroxyethylcellulose and non-ionic surfactants (Persson et al., 2000b).

1.5 PROBLEM STATEMENT

- Is thermoseparating ATPE a feasible process for PHAs purification and recovery and can it be scaled-up to industrial scale?
- One of the main advantages of thermoseparating ATPE is the phase-forming polymer can be recycled and reutilized, hence minimize the production cost. Therefore, can the phase-forming polymer for thermoseparating ATPE be recycled during PHAs purification and recovery process?
- As stated previously, high cost of carbon source has become an obstacle in wide application of PHAs. Therefore, it is important to find a method to select an optimum carbon source for extractive bioconversion of PHAs.
- How is the economic and environmental performance of PHAs purification and recovery by thermoseparating ATPE compared to other purification strategies?

1.6 OBJECTIVES

- To determine the optimum condition of PHAs purification and recovery by thermoseparating ATPE with recovery yield more than 95 % and purification factor more than 1.4 fold.
- To determine the performance of recycling and reutilization of phase-forming polymer for at least 2 cycles and compare different recycling strategies
- To develop integrated economic and environmental assessment for PHAs biosynthesis
- To perform extractive bioconversion of PHAs and scale-up to bioreactor scale
- To evaluate the economic and environmental performance of PHAs production using thermoseparating ATPE

1.5 RESEARCH FLOW

In the first chapter, introduction to the two main elements of this research, which are polyhydroxyalkanoates (PHAs) and aqueous two-phase extraction (ATPE) has been made. The advantages of green polymers and the challenges met for its wide application have been discussed. Following that, the in-depth literature review of PHAs and ATPE has been performed. Different types of PHAs, purification techniques as well as challenges and perspectives have been included, while the mechanisms behind ATPE and integration techniques have been studied. Further into the third chapter, the principal experimental methodologies as well as bacteria strain and chemicals which will be utilized throughout the research have been explained. Furthermore, different strategies of cell disruption and characterization of PHAs have been discussed.

In the first part of the research (chapter 4), purification and recovery of PHAs using two different ATPE techniques, which are cloud point extraction (CPE) and thermoseparating

polymer/salt ATPE have been investigated using one-factor-at-a-time optimization strategy on key ATPE influencing parameters. Subsequently, thedesign of experiment has been performed on thermoseparating polymer/salt ATPE to obtain the global optimum for purification condition of PHAs utilizing two-level full factorial design, method of thepath of steepest ascent and response surface methodologyin chapter 5. Different strategies of recycling and reutilization of phase-forming components have been carried out in these two chapters.

In chapter 6, extractive bioconversion of PHAs via thermoseparating ATPE, which is an advanced technique which integrates upstream fermentation and downstream purification have been studied and scaled-up. Prior to that, a preliminary integrated economic and environmental evaluation technique have been developed to select the suitable carbon source for the purpose of extractive bioconversion. In the final part of the research, the process flowsheet of industrial-scale of PHAs purification and recovery by thermoseparating ATPE have been drawn and simulated using engineering software. Other than that, the evaluation of the economic and environmental performance of thermoseparating ATPE have been performed and compared with other conventional PHAs purification technique. Finally, the conclusion of the research has been drawn and some future works on the research have been proposed. Figure 1.3 summarize the flow of the research to provide a clearer picture to the readers.

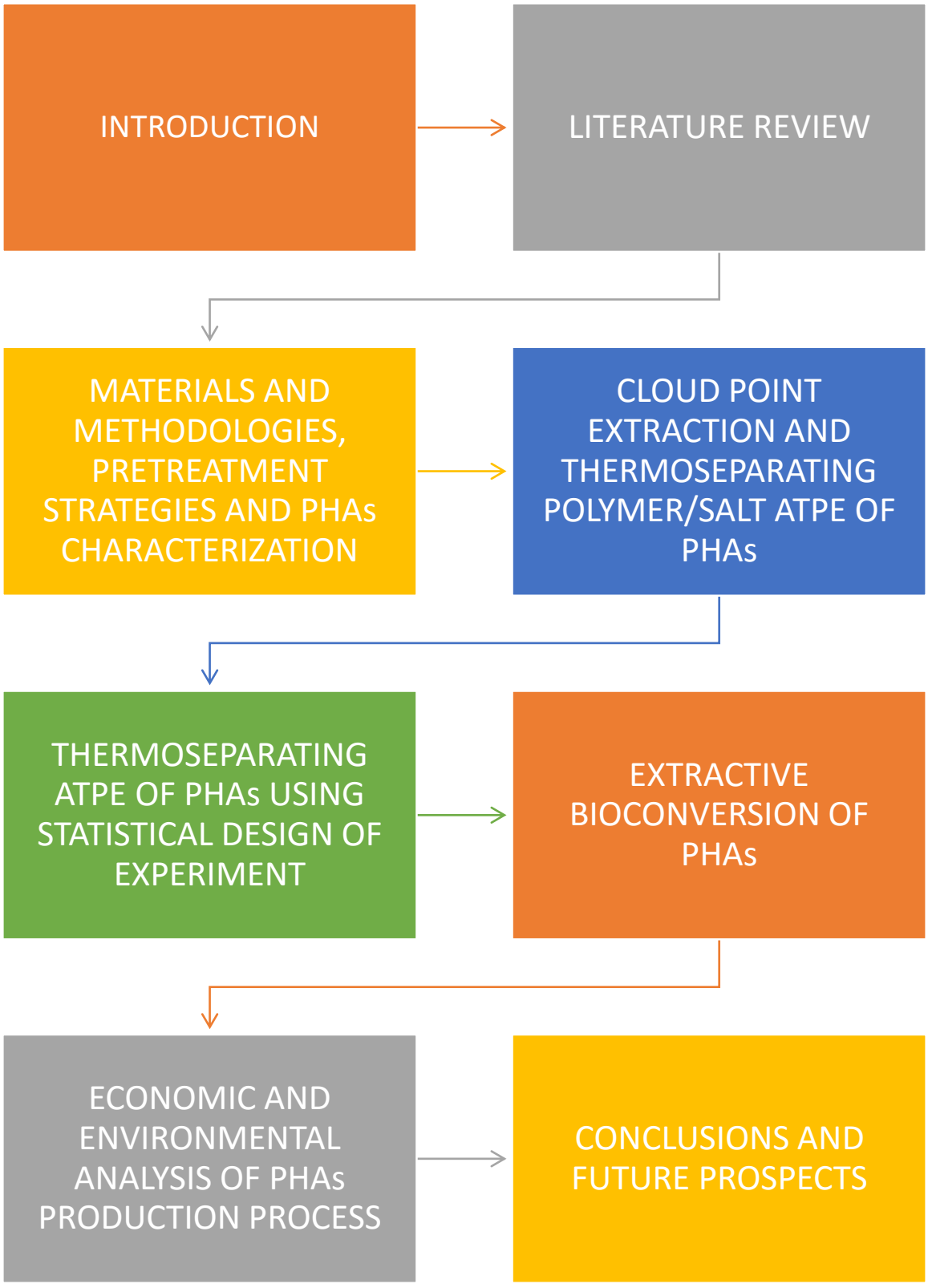


Figure 1.3: Research Flow Diagram

Chapter 2

LITERATURE REVIEW

2.1 WHAT IS BIOPLASTICS?

What are bioplastics? Following the definition is given by European Bioplastics, bioplastic is a plastic material that is biodegradable, biobased or features both properties (Bioplastics, 2016, Conor et al., 2013). The biobased material is defined by American Society for Testing and Materials (ASTM) as "an organic material in which carbon is derived from a renewable resource via biological processes. Biobased materials include all plant and animal mass derived from CO_2 recently fixed via photosynthesis, per definition of a renewable resource". U.S. Department of Agriculture (USDA) has been suggested by Institute of Local Self-Reliance (ILSR) to set 50 % biobased content as the minimum threshold for goods to be considered as biobased. When claiming a product as biobased, the unit (biobased mass content, biobased carbon content), the measuring method and a percentage should be clearly specified. Most companies used the method of U.S. standard ASTM 6866 (corresponding 14C method supported by the European standard CEN/TS 16137) (Conor et al., 2013). As defined by Biodegradable Products Institute (BPI), a biodegradable material is "where under the right conditions the microbes in the environment can break down the material and use it as an energy and food source, leaving no remaining residuals or by-products except water, carbon dioxide, and compost" (Institute, 2015). It must be remembered that biobased plastics are not necessary biodegradable and vice versa.

Bioplastics can be made from a wide range of natural resources, such as potatoes, corn, tapioca, rice, wood cellulose, palm fiber, bagasse and wheat fiber. In the 1850's, the first industrial bioplastic, parkesine which was made from cellulose was produced (Conor et al., 2013). Bioplastic can be categorized into three groups following its definition, where the first group is fully or partly bio-based, but not degradable plastics with the examples of bio-based polyethylene (PE), polytrimethylene terephthalate (PTT), polyethylene terephthalate (PET) or technical polymers like polyamides. The second group is fossil resource-based plastics which are biodegradable, including polydioxanone (PDO/PDS), polyvinyl alcohol (PVA),

poly(ε-caprolactone) (PCL) and poly(butylene adipate-co-terephthalate) (PBAT). Biodegradable bioplastics can be further divided into anaerobically digestible or compostable bioplastics and bioplastics that degrade in specific receiving environment (soil, marine or fresh water) after uncontrolled disposal (through loss or littering) (Conor et al., 2013). The third and final group are plastics that are both biobased and biodegradable, such as poly(butylene succinate) (PBS), polylactide (PLA), polyhydroxyalkanoates (PHAs), starch blends and cellulose (Bioplastics, 2016). Figure 2.1 depicts the group categorization of bioplastics. From the year 2005 to 2010, there is 22% average annual growth rate over 5 years (Akaraonye et al., 2010). Contribute to approximately 0.5 % of the current global production capacity, the worldwide production of bio-plastics is roughly 1400 kilotons per annum (PlasticsEurope, 2015). By the year of 2018, the expected productions of these "green plastics" are roughly $5 billion worth of the growth of about 35% (Gumel et al., 2012).

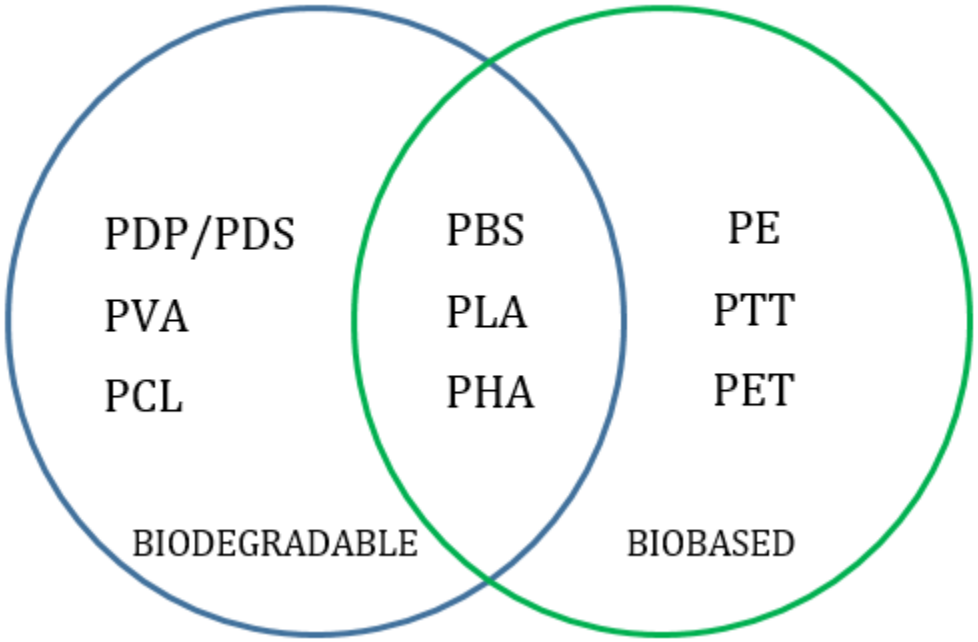


Figure 2.1 Group categorization of bioplastics

2.2 POLYHYDROXYALKANOATES (PHAs)

First discovery and characterization of PHA polymers were made by Maurice Lemoigne, a French microbiologist in 1920s which is poly(3-hydroxybutyrate) [P(3HB)] in *Bacillus megaterium* (Keshavarz and Roy, 2010). PHAs serve as intracellular carbon and/or energy storage reserve or as electron sink in various microorganisms, accumulated under unfavorable growth condition which is a deprivation of nutrients such as oxygen, nitrogen, phosphorus, sulfur, iron or magnesium with a supply of excess carbon source. The Gram negative and Gram positive bacteria (over 300 species) that accumulate PHA polymers as intracellular inclusions are from genera of *Necator, Cupriavidus, Ralstonia, Pseudomonas, Aeromonas, Bacillus, Alcaligenes, Enterobacter* and *Rhodobacteras* well as some cyanobacteria and halophiles (Philip et al., 2007, Gumel et al., 2013). Nevertheless, some bacteria are able to synthesise PHAs without the requirement of nutrients limitation, such as *A. eutrophus, A. vinelandii UWD, Alcaligenes latus*, recombinant *E. coli* (Keshavarz and Roy, 2010).

Based on the constituent number of carbon atoms in the monomer units, PHAs can be classified into two main groups. The first group is short-chain-length (scl) PHAs which are PHA consisting of C_3-C_5 atoms produced by bacteria such as *A. latus* and *Cupriavidus necator* (previously known as *Wautersia eutropha* or *Ralstonia eutropha*). In the other hand, those consist of C_6-C_{14} atoms are medium-chain-length (mcl) PHAs, which accumulated by bacteria such as *Aeromonas caviae* and *Pseudomonas putida* (Laycock et al., 2013). There are also PHA copolymers with examples of scl-copolymers [e.g. P(3HB-co-4HB), P(3HB-co-3HV)], mcl-copolymers [e.g. P(3HHx-xo-3HO)], and scl-mcl-copolymers which consisting both scl- and mcl- monomers [e.g. P(3HB-co-3HHx)] (Akaraonye et al., 2010). Figure 2.2 depicted the general structure of polyhydroxyalkanoates.

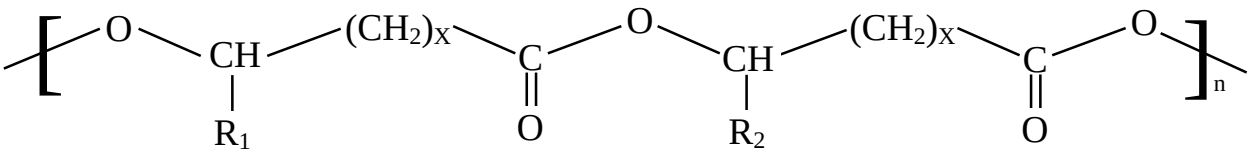


Figure 2.2. General structure of polyhydroxyalkanoates (R_1/R_2 = alkyl groups C_1-C_{13}, X = 1-4, n = 100-30000) (modified from (Akaraonye et al., 2010))

Generally, molecular weight (MW) of polymer PHAs fall in the range of 2 x 10^5 to 3 x 10^6 Da which is dependent on host microorganism, substrate composition, thestate of inoculum, culture condition and downstream processing techniques (Rai et al., 2011). The physical properties of PHA polymers such as glass transition temperature, melting temperature and crystallinity are greatly influenced by the side chain length and its functional group (Akaraonye et al., 2010). PHA polymers exist as discrete cytoplasmic inclusions with the size of normally 0.2 ± 0.5 mm in diameter and appear as electron-dense bodies under transmission electron microscopy. Due to their high refractivity, it can be visualized using aphase contrast light microscope. Stained with Sudan Black B, it clearly shows that the inclusions are of lipid nature. Other than that, PHAs display strong orange fluorescence when stained by oxazine dye Nile Blue A at 460 nm excitation wavelength (Khanna and Srivastava, 2005).

In screening the microorganism for large scale PHA biosynthesis, there are some key factors that need to be considered are such as growth rate, the capability to feed on inexpensive carbon source, cost of substrate, quantity, and quality of PHA synthesized, the maximum polymer yield from the substrate, PHA productivity and downstream processing cost (Chanprateep, 2010). Even though there are more than 300 types of microorganisms are able to accumulate PHA polymers, only a few are able to synthesize sufficient PHA for industrial scale production, which are *C.necator, Methylobacterium organophillum, Pseudomonas olovorans, A. latus, Protomonas extorquens, Azotobacter vinelandii, Paracoccus denitrificans* and recombinant *Escherichia coli*(Valappil et al., 2007, Chanprateep, 2010).

2.2.1 Short-chain-length (scl) PHAs

In recent times, development of novel scl-PHAs with enhanced mechanical and thermal properties that have potential desired properties for a wide range of applications from daily use to thebiomedical field.

2.2.1.1 Poly [(R)-3-hydroxybutyrate] [P(3HB)]

As the most ubiquitous PHA synthesized by a broad range of phototropic and chemotrophic bacteria in nature, production of poly[(R)-3-hydroxybutyrate] [P(3HB)] has been extensively studied as a model PHA in the development of cultivation strategies (Hiroe et al., 2013). P(3HB) has some useful properties, such as water insolubility, moisture resistance, optical purity and good oxygen impermeability. However, it is highly crystalline and its processing by injection molding is challenging due to its high melting point. Other than that, P(3HB) has higher tensile strength and Young's modulus than polypropylene, but much lower elongation to break (6%) compared to that of polypropylene (400%) (Ojumu et al., 2004). There are three sequential enzymatic steps in most of the P(3HB)-producing bacteria, starting from 2 acetyl-coenzyme A (CoA) moieties condensed by β-ketothiolase (*phaA*) to form acetoacetyl-CoA, which its reduction catalyzed by an NADPH-dependent acetoacetyl-CoA reductase (*phaB*) becoming D-(-)-3-hydroxybutyryl-CoA, and finally 3HB-CoA monomers linked up by P(3HB) synthase (*phaC*) to a growing chain via ester bond formation.

Researchers and industries have employed different strategies to improve the quality and quantity of P(3HB) synthesized and reduce the overall operation cost. Several strategies have been used to promote P(3HB) production such as oxygen limitation during fed-batch culture, asmall quantity of oleic acid and amino acid addition for cultivation in defined medium (Li et al., 2009). Strategies of utilizing unwanted by-products as acarbon source have becoming a trend as thecost of carbon source contributes to major of PHAs production cost. As the by-product in biodiesel synthesis, glycerol has a drastically fall in its price due to

therapid growth of biodiesel industry and treated as pollutant waste that should be disposed of (Andreessen et al., 2010, Nikel et al., 2010). Therefore, it has gain attractions to become a very potential carbon source for PHA biosynthesis. The influence of*phaP*on P(3HB) production and microbial growth have been studied using glycerol as sole carbon source. It is found that *phaP*enhanced both the total biomass weight and the PHA content as it serves as a barrier between the cytoplasmic components and hydrophobic polymer (de Almeida et al., 2007). Other low-value byproducts such as corn steep liquor and milk whey have been designed and evaluated as the primary carbon and nitrogen sources for the production of P(3HB) as well.

Recently, P(3HB) biosynthesis under anaerobic fermentation has gained attractions as a low-cost PHA production strategy due to higher P(3HB) conversion rate, robust control strategies, simpler reactor design and mild operating conditions. Under oxygen limitation, microorganisms undergo mixed acid fermentation and produce lactate,acetate, formate, succinate and ethanol (or H_2 and CO_2) as by-products (Jian et al., 2010). In addition to that, researchers have also working on strategies on manipulating environmental factor of P(3HB) production, such as addition of casein amino acids (CAS) as supplement (Nikel et al., 2008), effect of aeration when utilizing glucose and glycerol as carbon sources (de Almeida et al., 2010) and influence of culture temperature on molecular weight (Mw) of P(3HB) synthesized (Agus et al., 2010)(Tomizawa et al., 2011).

From polymerization studies, it is suggested that the major factors controlling the molecular weight (MW) of P(3HB) are a hypothetical chain transfer agent and PHA synthase's activity. Native PHA-synthesizing bacteria such as *C. necator* produce P(3HB) with average MW ranging from 0.5 to 1.0 MDa. On the other hand, metabolically engineered *E. coli* is capable to synthesis P(3HB) with a very high Mw ranging from 3×10^6to20×10^6[in gel permeation chromatography (GPC) analysis using polystyrene as standard] which defined as ultra-high-molecular-weight P(3HB) [UHMW-P(3HB)]. It is suitable for making strong fibers

or films by a cold- or hot-drawing procedure (Hiroe et al., 2013). The fiber or films made from UHMW-P(3HB) have excellent mechanical properties improved by stretching (Agus et al., 2006b), for example, extension to break and tensile strength of 112% and 237 MPa respectively, depending on processing conditions. Furthermore, the material properties of UHMW-P(3HB) can be further enhanced by annealing treatment of stretched films (Park et al., 2005). Nonetheless, high-level accumulation of UHMW-P(3HB) in abacterial cell is hampered due to the trade-off between PHA polymer's yield and its molecular weight (Hiroe et al., 2012). Also, lack of efficient synthesis system gives rise to difficulty in its commercialization and application.

In order to overcome these, several strategies have been utilized to improve the biosynthesis of UHMW-P(3HB), such as optimizing the plasmid vector (Kahar et al., 2005, Agus et al., 2006a), IPTG induction (Agus et al., 2006a), PHA synthase (*phaC*), insertion of point mutation to *phaC*, cultivation conditions (Agus et al., 2006b), balance between PHA synthase abundance and monomer supply (Hiroe et al., 2012), host bacterial strain (Hiroe et al., 2013, Agus et al., 2006b), and *phaC*activator (Hiroe et al., 2013). Microorganisms such as recombinant *E. coli*cultured at optimal growth condition of pH 6.0 can produces P(3HB) with extremely high molecular weight (20 MDa), which termed as supra-molecular-weight-P(3HB)(Hiroe et al., 2012, Agus et al., 2006b, Kahar et al., 2005, Agus et al., 2006a). It has superior mechanical properties compared to normal P(3HB), such as 237 MPa and 112% for tensile strength and elongation at break respectively. Therefore, it is useful PHA material for processed into thebiodegradable strong fiber and also drug carrier.

2.2.1.2 Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)[P(3HB-co-3HV)]

Homopolymer PHBs are too brittle and stiff for many applications due to its high crystallinity. Incorporation of other monomerssuch as 3-hydroxyvalerate can help to reduce the brittleness andcompensate processability of P(3HB) (Singh and Mohanty, 2007). Moreover,

P(3HB-co-3HV) copolymers are more impermeable togas compared to polypropylene and polyethylene (Salehizadeh and Van Loosdrecht, 2004). Owe to it enhanced mechanical andthermal properties, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)], the copolymer of3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) gainsmuch commercial interest (Chen et al., 2011). The applications ofcopolymer P(3HB-co-3HV) are including plastic beverage bottles,film, as a creating on paper, food packaging, disposable personalhygiene, and potential in biomedical applications (Ojumu and Solomon, 2004). By adjusting the processing conditions, the copolymer consisting of 3HV mole fraction more than 20% can be used to makefibers and films (Philip et al., 2007). In the 1980s, this copolymerhas been commercialized, though the wide applications havebeen hindered by the high production costs (Nikel et al., 2006).Currently, there are two commercial brands of P(3HB-co-3HV),which are BiopolTMand BiocycleTM(Mumtaz et al., 2010).

Desired mechanical and thermal properties can be achieved byvarying the 3HV mole fraction in the copolymer. The higher the3HV content in P(3HB-co-3HV), the lower the crystallinity whichresulted in increased flexibility, strength and elongation to break(Nduko et al., 2012). The melting temperature reduces as well andthis enables the copolymer undergoes thermal processing without degradation, resulted in widening the processing window ofP(3HB-co-3HV). In the other hand, this copolymer has some majordrawbacks which are slow crystallization rate and growth of interlamellar secondary crystallization during storage. Nonetheless, the 3HV mole fraction in the copolymers cannot be easily controlled, and synthesis of P(3HB-co-3HV) consisting>40 wt% 3HV monomer content are generally rare using usual fermentation method (Yang et al., 2012b).

Propionate which serves as the direct precursor of propionyl-CoA is pricey(Yang et al., 2012b) and resulted in thebiosynthesis of P(3HB-co-3HV) expensive compared to P(3HB). As a result, a strategy to produce P(3HB-co-3HV) through threonine biosynthetic pathway using inexpensive, unrelated carbon sources has been developed (Chen et al., 2011). To further

enhance the efficiency of the limiting step, threonine deamination, the overexpression of threonine deaminase, *ilvA* from *Corynebacterium glutamicum* among other bacteria could significantly increase the 3HV mole fraction.

On the other hand, astrategy such as adjusting propionate concentration during fermentation process has been used to obtain the desired 3HV contents in P(3HB-co-3HV). However, this strategy has some drawbacks such as the toxicity of propionate to microbe cell at high concentration decreases the conversion efficiency of propionate to 3HV (Liu et al., 2009), inexpensive carbon sources may not consist of correct carbon composition and it cannot be easily scaled up (Wong et al., 2008). As a result, an alternative strategy which is changing the critical gene's induction level (e.g. *prpE*) has been proposed. Factors influencing the P(3HB-co-3HV) accumulation and 3HV content have also been investigated to produce copolymers with high 3HV monomer composition (Yang et al., 2012a). As one of the commercialized PHAs, the large-scale production of P(3HB-co-3HV)with 3HV content more than 20% is desirable due to its properties. Various strategies such as varying propionate concentration and adjusting IPTG induction to obtain copolymers with 3HV content, though the strategies have their own drawbacks. More researches can be done on theproduction of P(3HB-co-3HV) using low-cost substrate or by mixed culture fermentation as well as create copolymers with interesting properties.

2.2.1.3 Other scl-PHAs

Poly(3-hydroxypropionoate) [P(3HP)] is a polymer which combines the properties of poly(2-hydroxypropionoate) [P(2HP)], which also known as polylactic acid and P(3HB). Having best mechanical properties among all known PHA (Meng et al., 2012, Tripathi et al., 2013), its exceptional tensile strength, high ductility and rigidity has increasingly gained interests (Wang et al., 2012a).

As 4HB is known to enhance theelasticity of PHA, copolymer P(3HP-co-4HB) has potential to give desirable and usefulproperties. Although higher concentrations of PDO and BDO lead to increasing biomass weight, but thetoxicity of high PDO or BDO concentrationcaused asubstantial reduction in both cell density and PHAs contentespecially when thetotal concentration was over 20 g/l. The monomercompositions of 4HB and 3HP in copolymer P(3HP-co-4HP) couldbe fixed by adjusting the ratio of PDO:BDO. For example, a PDO:BDOratio of 1:1 resulted in P(3HP-co-4HB) of 30 mol% 4HB, whilecopolymer with amole fraction of 87% 4HB is synthesized when theratioof 1:10 is used. Dependent on its monomer composition, copolymerP(3HP-co-4HB) is only fully transparent when consisting mole fraction of 67% 4HB. Copolymers P(3HP-co-4HB) have abetter elongationat break compared to P(3HP) as expected and constant meltingtemperature from 62 to 65 °C.Polymer microstructure development, such as block copolymersis an innovative method to develop novel and desired properties(Philip et al., 2007). Block copolymers haveadvantages of high MWwithout property detrimental aging problem and new added physical properties as polymer microstructureis introduced in biological PHA systems. P(3HP)-b-P(4HB) produced are proven to have superior properties over P(3HP-co-4HB)in terms of melting temperature, tension strength, yield strength,and Young's modulus. The comparisons between random, blockand blend copolymers P(3HP-co-4HB) with homopolymers P(3HP)and P(4HB) are shown in Table 2.1.

By developing new PHA synthase and combining with wide substrate range for 2-hydroxy acid, biosynthesis of polylactic acid (PLA) has made a significant development in PHA history asits diversity is further expanded (Gao et al., 2011). By adjustingcrystallinity and molecular weight, lactate-based PHAs exhibiteddifferent thermal and mechanical properties that have potential in thewide range of applications such as food, biomedical, and generalpurpose application (Taguchi et al., 2008). As the chemical processes are energy-consuming and required the use of dangerousmetal catalysts, thus, it is desired to develop a one-step biosynthesis process, a "microbial factory" to produce the polymer. These

biopolymers have glass transition temperature and melting temperature around 2–14 °C and 157–168 °C respectively. Asa new member in PHAs family, there are still a lot of hidden potential in lactate-based polymers that can be explored. Therefore,more researches can be done on theinvention of novel polymers withunique properties, biosynthesis of polymers using low-cost substrate and/or by mixed culture fermentation, maximize the yieldand productivity of polymers, simpler and more economical downstream processing and others. These works should be done to makethe large-scale production of lactate-based PHA polymers possible.

Table 2.1: Comparison between random, block and blend copolymers P(3HP-co-4HB) with homopolymers P(3HP) and P(4HB)

PHA type	Molecular weights		Thermal properties		Mechanical properties				References
	M_W (10^5)	PDI	T_m (°C)	T_g (°C)	σ_y (MPa)	σ_{mt} (MPa)	ε_b (%)	E (MPa)	
P(3HP)	1.63	1.48	78.13	-17.85	33.83 ± 0.76	21.54 ± 1.10	497.6 ± 6.20	2889.9 ± 698.80	(Meng et al., 2012)
P(4HB)	3.89	1.17	61	-47	13.77 ± 1.41	34.66 ± 0.98	696.6 ± 43.60	180.9 ± 59.20	
P(3HP-co-25% 4HB)	2.59	1.32	62.74	-31.28	1.70 ± 0.18	6.36 ± 0.76	962.90 ± 20.50	14.50 ± 0.40	
P(3HP-co-38% 4HB)	2.80	1.30	63.46	-36.14	0.92 ± 0.03	0.54 ± 0.04	1611.0 ± 19.30	4.40 ± 0.10	
Blend P3HP/P4HB	4.87	2.83	57.14, 78.10	-16.15, -47.03	10.83 ± 2.95	13.79 ± 2.94	94.69 ± 99.14	190.18 ± 34.08	(Tripathi et al., 2013)
P3HP-b-29% P4HB	5.48	2.52	54.97, 67.60	-20.20, -46.19	20.04 ± 3.0	44.71 ± 6.74	876.70 ± 85.11	177.03 ± 18.76	
P3HP-b-37% P4HB	5.47	2.62	53.41, 66.80	-21.58, -45.19	7.40 ± 0.35	25.32 ± 4.48	1031.51 ± 129.16	113.21 ± 14.61	

2.2.2 Medium-chain-length (mcl) PHAs

Conventional mcl-PHA is biodegradable rubber and elastomer with low degree of polymerization (MW normally below 100 kDa), low melting temperature (40–60 °C) and low glass transition temperature (around −40 °C) but also low tensile strength (Liu et al., 2011, Muhr et al., 2013). The mechanical and thermal properties of mcl-PHA copolymers which consist of more than 15% 3HDD is significantly better than those of less than 15% 3HDD (Liu et al., 2011). Nevertheless, copolymers of mcl-PHA are poorly manageable because of their multiple monomer structures which are hard to regulate (Ma et al., 2009). Due to their high elasticity and low crystallinity, they are suitable materials for biodegradable elastomer and rubber, which is desired for novel applications in cosmetics, paint formulations, other coatings, medical devices and tissue engineering (Park and Yup Lee, 2004). Moreover, chiral monomers from depolymerization of mcl-PHAs can be used to synthesize vitamins, antibiotics, and other bioactive compounds (Jiang et al., 2006).

Medium-chain-length (mcl) PHAs generally consist of 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD) (Ma et al., 2009). The composition of mcl-PHA's co-monomer depends mainly on the culture conditions, carbon source, and PHA formation metabolic pathways (Li et al., 2007). These metabolic pathways are presented in Fig. 2.2. These productions of mcl-PHA are following three different metabolic routes which are the degradation of fatty acids β-oxidation (main metabolic pathways of fatty acid), de novo fatty acid biosynthesis route (non-related carbon sources), and finally, chain elongation where acyl-CoA extended by acetyl-CoA (Kim et al., 2007). Those fatty acid metabolism's intermediates which function as main precursors for mcl-PHAs are 3-ketoacyl-CoA, (R)-3-hydroxyacyl carrier protein (ACP), (S)-3-hydroxyacyl-CoA and enoyl-CoA.

Mcl-PHAs productions in various carbon and nitrogen sources such as glucose, glycerol, sucrose, maltose, fish peptone, special peptone and protease peptone have been studied

(Sujatha and Shenbagarathai, 2006). Agricultural co-products such as whey, fats and oils have the potential as low-cost substrate for PHA production. The study by Fonseca and Antonio concluded that vegetable oils with shorter fatty acids and higher saturation degree should be used in order to get optimum mcl-PHAs production (Fonseca and Antonio, 2006). Thorough literature study has shown the potential and flexibility of microorganisms utilizing a wide range of both related and unrelated inexpensive carbon sources for mcl-PHA synthesis(Park and Yup Lee, 2004, Fonseca and Antonio, 2006, Kang et al., 2011, Wang et al., 2012b). This further reduces the production cost and open up the possibility of mcl-PHAs largescale production.

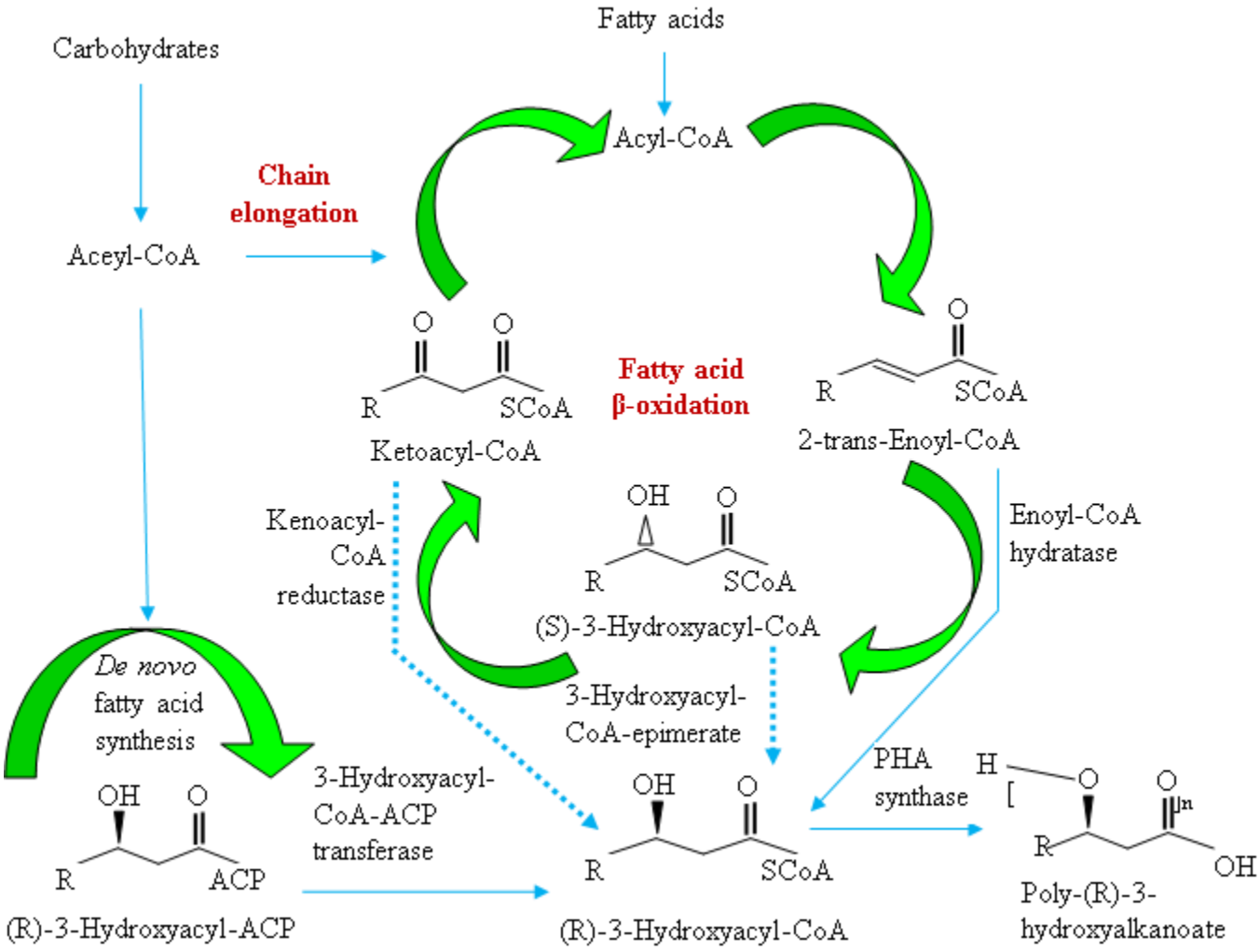


Figure 2.2. Metabolic pathway for mcl-PHA biosynthesis (modified from (Kim et al., 2007))

2.2.3 Short-chain-length-medium-chain-length (scl-mcl) PHAs

The P(3HB) polymer backbone incorporate with mcl monomer units enhances the combine the properties of both scl-PHAs and mcl-PHAs with improved mechanical and thermal properties which are suitable for a wide range of commercial applications (Park and Yup Lee, 2004, Li et al., 2011). The PHA copolymer's properties are greatly influenced by the type, ratio and distribution of the comonomer units. Scl-mcl PHA copolymers with a high mole fraction of short-chain-length monomer composition (3HB) and low mole fraction of medium-chain-length monomer composition possess similar mechanical properties to those of low-density polyethylene (LDPE) (Davis et al., 2008). One of the examples is copolymer P(3HB-co-3HHx) which contains less than 20% mole fraction of 3HHx (Lu et al., 2003). However, the biosynthesis of scl-mcl PHA copolymers meets obstacles such as lack of adequate monomer supplying enzymes and PHA synthases (Nomura et al., 2005). As scl-mcl-PHAs provide a versatile range of mechanical and thermal properties that have the potential for many applications, intensive researches should be done to obtain the polymers with desired properties and further study for the purpose of economical large scale production.

2.2.4 Isolation and recovery of PHAs

Table 2.2 presents various methods for separation and purification of PHAs together with its advantages and disadvantages.

544 Table2.2: Recovery methods of PHA with its advantages and disadvantages (modified from
545 (Jacquel et al., 2008, Kunasundari, 2011))

Recovery methods	**Advantages**	**Disadvantages**
Solvent extraction	Elimination of endotoxin High purity Negligible or no polymer degradation Useful for medical application Higher molecular weight PHA	Break PHA granules morphology Hazards associated with halogenated solvents High capital and operating cost Low recovery
Chemical digestion by surfactant	Treatment of high cell densities Negligible or no polymer degradation	Low purity Wastewater treatment required
Chemical digestion by sodium hypochlorite	Higher purity	Severe reduction in molecular weight of PHA
Sequential surfactant-hypochlorite digestion	Limited degradation Low operating cost compared to solvent extraction Rapid recovery and simple process	Combined cost of surfactant and sodium hypochlorite Wastewater treatment required

Dispersion by sodium hypochlorite and solvent extraction	High purity Negligible or no polymer degradation	Consumption of large volume of volatile and toxic solvent Not environmental friendly Higher recovery cost
Surfactant-chelate	High purity Convenient operation Low environmental pollution	Large volume of wastewater Slight polymer degradation
Selective dissolution of NPCM by protons	High recovery High purity Low operating costs	Severe reduction in molecular weight if the process parameters are not controlled stringently
Enzymatic digestion	Mild operation condition Good recovery	Complex process High cost of enzymes
Bead mill	No chemicals used Less contamination Not susceptible to blockages No micronization of PHA granules	Require several passes Long processing time Various process parameters have to be controlled precisely
High-pressure homogenization	No chemicals used Less contamination	Poor disruption rates for low biomass levels

		Severe micronization of PHA granules Depends on both process and microbial physiological parameters Possible for thermal degradation of desired products Formation of fine cellular debris that interfere with downstream processing
Supercritical fluid (SCF)	Simple and rapid Low cost Low toxicity	Dependent on process parameters Frequent need for clean up Difficulties in extracting polar analytes Difficulties in dealing with natural samples Low recovery
Cell fragility	Simple recovery methods Mild extraction conditions	Need to balance cell wall softening and cell wall integrity
Self-flotation	Cost effective as additional steps could be avoided	Consumption of large volume of toxic and volatile solvents

Air classification	High purity	Low recovery
Dissolved air-flotation	No chemicals used Less contamination	Require several consecutive floatation steps
Aqueous two-phase extraction (ATPE)	Short processing time Low material cost Low energy consumption Good resolution High yield and relatively high capacity	Dependent on process parameters Issue of robustness and reproducibility Absence of commercial kits to evaluate ATPE at bench scale Poor understanding of the mechanism
Spontaneous liberation	No extracting chemicals needed	Low recovery
Gamma irradiation	Retention of solvent solubility due to the low degree of cross-linking No chemicals used Low contamination	Length of irradiation time High initial investment cost

546

547 **2.2.5 Challenges and perspective**

548 Generally, in producing bioplastics that are random copolymers, there are still some
549 challenges in obtaining desired proportion of monomers which highly influenced the
550 mechanical and thermal properties. Using the knowledge accumulated from previous research,

an ideal bacteria strain which is economically feasible for large-scale PHAs production can be designed. More work should be done in high cell density cultivation method coupled with PHA biosynthesis from low-value by-product or mixedculture fermentation to further improve theeconomic competitiveness of the bioplastics. Other than that, environmental factor, such as temperature, pH, dissolved oxygen (DO), by-product inhibition and nutrient concentration can be further optimized to improve the bacteria growth rate and PHA content as well as obtain desired mechanical and thermal properties. As mentioned previously, novel PHA biopolymers should be further researched for awider range of application and to be able for large-scale production. Table 2.3 presents a list of challenges in current PHA productionwith corresponding proposed solutions.

573 Table 2.3: List of challenges in current PHA production with corresponding proposed
574 solutions

Challenges	Proposed solutions
High production cost of PHA compared to conventional petrol-based plastics	• More work should be done on high cell density cultivation method coupled with PHA biosynthesis from low-value by-product or mixedculture fermentation • Genetically modify microorganisms to utilize wider range of carbon source more efficiently
Unsatisfactory growth rate and PHA content for large-scale production	Further optimize environmental factors, such as temperature, pH, dissolved oxygen (DO), by-product inhibition and nutrient concentration
Limited range of application	Explore the hidden potential of novel PHA biopolymers

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2.3 AQUEOUS TWO-PHASE SYSTEMS (ATPS)

In 1896, aqueous two-phase systems (ATPSs) has been introduced to the world by Beijerinck, however, it only starts attract interest from researchers and industries in the 1950s due to Andersson's work (Ferreira et al., 2008). Up to the present time, various studies have been performed on ATPSs from different combinations of polymer and polymer or polymer and salt. The application ranges of ATPS are wide and includes proteins, microorganisms, nucleic acids, animal and plant cells (Walter et al., 1985), amino acids (Liu et al., 2012), lipids (Sharpe, 1985), antibiotics (Jiang et al., 2009), antibodies (Azevedo et al., 2009), small organic species (Huddlestion et al., 2002), metal ions (Rogers et al., 1996, Bauer and Rogers, 1996), as well as micro and nano-solid particulates (Dembczyński et al., 2010). ATPS has also been further applied on the delignification of paper pulp processing (Willauer et al., 2000, Li et al., 2001) and as green reaction media (Chen et al., 2005). Table 2.4 presented some examples of ATPE that can be used for protein or bioproduct partitioning, however, the list is not meant to be exhaustive.

Not mentioning the phase-forming polymers which have enzyme-stabilizing properties, ATPS which consists of high water content (70-90 % wt/wt) provides a mild environment for separation of sensitive bioproducts without causing denaturation (Ng et al., 2012), while high interfacial contact area facilitated efficient mass transfer due to low interfacial tension. Compared to conventional solvent extraction techniques which consume a huge amount of volatile and toxic solvents, the phase-forming components of ATPS are generally safe, non-flammable, non-toxic and relatively environmental-friendly (Li and Peeples, 2004). In contrast to chromatography techniques that require a long processing time and high operation cost, applications of ATPSs on purification of both high- and low-value products give satisfactory purity and yield, presenting the robustness, flexibility and potential universal application of this technique (Silvério et al., 2013). Furthermore, phase separation can be done selectively and rapidly under room temperature with relatively little energy inputs (Nilsson et al., 2002).

603 Apart from the ability to handle high capacity and reduce the volume of subsequent
604 purification steps (Rabelo et al., 2004), large-scale purification using ATPS can be easily and
605 reliably predicted from laboratory data (Moreira et al., 2013). Thus, ATPS is a viable solution
606 for the industrial demand of cost-effective and highly efficient large-scale bioseparation
607 technology with short processing time (Ferreira et al., 2008).

608 The partitioning of biomolecules can be manipulated by changing the types, molecular
609 mass or concentration of polymers; types and concentrations of ions in the system (Leuchau
610 et al., 2011); pH; and the addition of salts (Persson et al., 1998). Strategies such as alteration
611 of amino acids composition (Berggren et al., 1999), peptide tags addition (Berggren et al.,
612 2000) by genetic engineering, pH altering (Persson et al., 1999b) and addition of co-solutes
613 (such as non-ionic surfactants or salts) (Cunha et al., 1998) can be used to direct or enhance
614 the partitioning of biomaterials to the desired phase. For example, large monovalent ions such
615 as SCN^-, I^- and ClO_4^- are used to promote the partitioning of hydrophobic positively charged
616 molecules to the hydrophobic phase (Johansson et al., 1997), while partitioning of
617 hydrophobic negatively charged molecules to the hydrophobic phase can be enhanced by
618 addition of salt with hydrophobic cation such as triethylammonium phosphate (Persson et al.,
619 1998).

620

621

622

623 Table 2.4: List of ATPS systems

A. Polymer-polymer-water systems	
Polymer	Polymer
Polyethylene glycol	Dextran
	Starch derivatives (methylcellulose, hydroxyethyl cellulose, etc)
	Ficoll
	Polyvinyl alcohol
Thermoseparating polymers (EOPO, UCON, HM-EOPO)	Dextran
	Starch derivatives
Starch derivatives	Dextran
Ficoll	Dextran
Polyvinyl alcohol	Dextran
B. Polymer-low molecular weight solute-water systems	
Polyethylene glycol	Salt (Potassium phosphate, etc)
	Amino acid
	Mono/ Dissacharide
	Peptide/ Protein
Thermoseparating polymers	Salt
Polyvinyl alcohol	Butylcellosolve

2.3.1 Thermoseparating-based aqueous two-phase extraction (ATPE)

EOPO random copolymers have been used to form ATPSs with potassium phosphate, sodium or ammonium sulfate, dextran and other starch derivatives as the replacements of PEG (Dembczyński et al., 2010) where much liquid-liquid equilibria have been studied and the phase diagrams were determined. Other than easy phase separation and good results of biomolecules partitioning (Hasmann et al., 2008), a thorough literature review shows that a lower amount of phase-forming components is required to form the two phases for thermoseparating ATPS, thus, the risks of both precipitation of proteins and salting out are minimized (Rabelo et al., 2004, Show et al., 2012a). Besides, most protein recovery techniques are not suitable for larger molecules (>100 nm in size) such as plasmid DNA, the application of thermoseparating polymer-based ATPS has been researched and revealed the huge potential for the large-scale application (Kepka et al., 2004).

Besides coupling with other phase-forming components to form ATPS for the purpose of two-stage extraction, aqueous solutions of TSP can also be used for bioseparation (known as cloud point extraction, CPE) accounting to volume exclusion effect (Kepka et al., 2003). Using the concept of temperature-induced phase separation, TSPs has decreased solubility in its aqueous solution and form two phases (water and polymer phases) eventually after heated beyond a critical point, known as lower critical solution temperature (LCST) or cloud point (CP) (Li et al., 2002a). In these systems, the top phase has almost 100% water content, while the bottom phase contains EOPO with a concentration of 40-60% (Persson et al., 1999b). The concept of thermoseparation-based ATPS is shown in Figure 1.2. This feature simplifies the extraction of the target product and makes the recycling and reutilizing of polymers possible without the need for additional third component or extra separation steps. This makes bioseparation more cost-effective and environmental-friendly, which is suitable for the industrial-scale application and aids in further purification steps as well (Kepka et al., 2004).

The CP of TSPs aqueous solution can be easily identified by the solution turbidity (Rabelo et al., 2004). For example, the CP of a 10 wt/wt % solution of $EO_{20}PO_{80}$, $EO_{30}PO_{70}$ and $EO_{50}PO_{50}$ is 30 °C, 40 °C and 50 °C, respectively (Persson et al., 2000b). The commonly used polymer in ATPS, PEG, is also a TSP. However, LCST above 100 °C (low-molecular-mass PEG has CP as high as 180 °C) is unsuitable for the most case of thermoseparation for biomaterials [18]. A novel TSP, hydrophobically modified EOPO (HM-EOPO), presented by Johansson and his coworkers (Johansson et al., 1999) has low CP around 12°C at 3 % wt/wt, which means that thermoseparation can be done near room temperature. The behavior of polymers in solution depends on the EO/PO ratio, as well as block size, molecular mass, and concentration of EO and PO (Rabelo et al., 2004). Given that CP decreases with increasing hydrophobicity (i.e. increase of PO content), CP further depends on the concentration of copolymer (Persson et al., 1998). Strategies such as making the polymer more hydrophobic or addition of hydrophilic salt (e.g. sodium sulfate), acids/bases (Persson et al., 1999b) or ionic surfactants (e.g. sodium dodecyl sulfate (SDS)) (Cunha et al., 1998) have been used to lower the CP of TSPs and promote partitioning of target products.

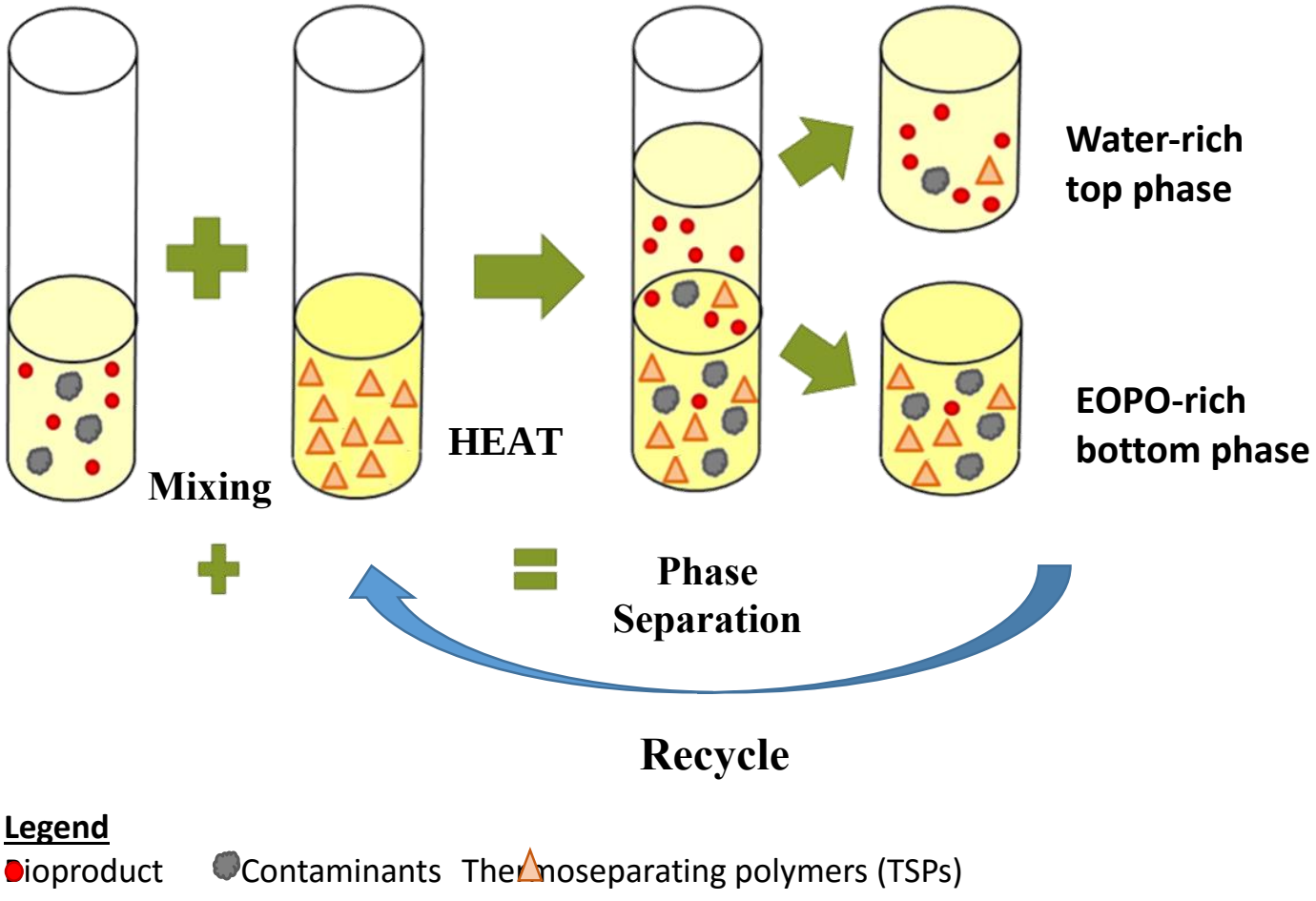


Figure 1.2: Concept of thermoseparation-based ATPS

2.3.1.1 Influence of EO:PO Ratio

Following the decrease in EO/PO ratio, EOPO copolymers that have a higher PO content become more hydrophobic, more viscous and have a lower CP as PO monomers have a longer hydrocarbon chain (Berggren et al., 1995). They also form stronger hydrophobic interaction with proteins with high hydrophobicity. On the other hand,gellification can be observed in copolymers, that contain a high EO %, such as copolymers with 50 % and 80 % EO content (Rabelo et al., 2004). Also, the LCST of TSP with 50% EO content is not affected significantly by polymer concentration (Rabelo et al., 2004). A TSP with 50 % PO content was found that

is more suitable for partitioning of cyclodextrin glycosyltransferase as the polymer phase provides the greatest solubility, thus avoiding the precipitation in interphase (Ng et al., 2012).

2.3.1.2 Influence of EOPO Molecular Weight (Mw)

The hydrophobicity of the polymer increase following the Mw due to increase in hydrophobic area to hydrophilic group ratio. Besides requiring lower concentration to form ATPS for the copolymer of high Mw (Ng et al., 2012), random EOPO copolymers with high Mw result in lower LCST and provide highly efficient temperature-induced phase separation together with recovery of target bioproducts (Rabelo et al., 2004). The Mw of EOPO also has a significant influence on the partitioning of alarge protein (Johansson et al., 1998), with the example of partition coefficient (K) increase with copolymer MW in bromelain recovery (Rabelo et al., 2004). In partitioning of L-asparaginase in PEO-PPO-PEO/phosphate system, purification factor (PF) generally showed an increasing trend for increasing triblock copolymer molar mass, whereas there is no specific trend for K though those with higher molar mass gave a higher value (Zhu et al., 2007). Comparing between EOPO 970 and 3900, the former needed higher concentrations of EOPO to form two phases that might cause precipitation at the interphase and volume exclusion effect, which causes negative impacts on K and the purification factor(Ng et al., 2012).

2.3.1.3 Influence of Tie-Line Length (TLL)

The TLL of a system can be modified by altering either phase-forming component concentration or both. In conventional ATPE, it is well known that partitioning of biomolecules become more extreme following the increasing concentration of phase-forming components (Albertsson, 1986) with examples such as amylase (Li et al., 2002a), endo-polygalacturonase (Pereira et al., 2003), human immunoglobulin G (Ferreira et al., 2008) and lysozyme

(Dembczyński et al., 2010) in thermoseparating ATPE as long as volume-exclusion effect does not take place. This is due to entropic effect which causes partition of target biomolecules to the other phases plays a major role in the systems containing high polymer concentration. On the other hand, in the system with low polymer concentration, electrochemical force becomes the dominating factor for polymer/salt system, whereas entropic driving force is greatest for polymer/polymer system (Nilsson et al., 2002).

However, being more hydrophobic than conventional PEG which has astabilizing effect, ahigh concentration of TSP might interfere with enzyme activity thus inhibit it (Moreira et al., 2013), or cause precipitation at the interphase and introduce volume excursion effect (Ng et al., 2012). On the other hand, high salt concentration interact and subsequently modify the protein charges, leading to protein dehydration (Ng et al., 2012). The partitioning of some bioparticles such as plasmid DNA (Kepka et al., 2004) is very sensitive to slight changes in theconcentration of phase-forming polymer. During theinvestigation of laccase extraction, the shortest TLL of four systems which composed of UCON with citrate and formate salts were chosen as least phase-forming components are required and have lowest viscosities (Lladosa et al., 2012).

2.3.1.4 Influence of Volume Ratio (Vr)

To minimize the downstream processing volume and production cost, the subsequent step after designing a primary purification ATPE is the volume reduction of bioproduct-containing phase. In theory, Vr can be adjusted by altering the phase compositions along the same tie-line without affecting the partitioning behavior of the biomolecules. Following thereduction in top phase volume, there is more free-volume in bottom phase which provides better removal of contaminant to the desired phase. However, low V_r reduces the top phase's free volume and might limit the solubility of bioproducts, thus require a certain degree of compromising (Ng et al., 2012, Show et al., 2012a). In both small- (5 g) and large-scale (5

kg) recovery of apolipoprotein A-1_{Milano} from *Escherichia coli*, the system volume is reduced by 30 % (Persson et al., 1998). In theseparation of plasmid DNA from a protein mixture with RNA, the total protein in top phase is reduced by 23 % with volume reduction of approximately fourfold and RNA removal up to 80 % (Kepka et al., 2004).

2.3.1.5 Influence of Temperature

Due to the strong influence of polymer concentrations on each phase, partitioning of biomaterials in thermoseparating system is strongly influenced by temperature. The low CP of thermoseparating polymers is advantageous as the risk of denaturation in temperature-induced phase separation is minimized. Although less salt concentration is required to form biphasic system at high temperature (Pereira et al., 2003), literature had reported that high temperature may induce aggregation and precipitation of biomolecules (such as human immunoglobulin G, especially for system with high concentration(Ferreira et al., 2008)), or even inhibit the enzyme activity (such as bromelain (Rabelo et al., 2004)).

Temperature plays an even greater role in bioseparation utilizing ATPE formed by TSPs as it affects the partition behavior of protein during term separation. In thermoseparation, the polymer concentration of polymer-rich phase become higher with increasing temperature which leads to areduction in free space per unit volume, causing more proteins partition to the water phase (Johansson et al., 1999). A system's entropy dominates with increased thermoseparation temperature which favors less extreme partitioning for bioproducts with high hydrophobicity (Johansson et al., 1997). Apolipoprotein A-1 partition strongly to the polymer-rich phase at 22 – 32 °C during thermoseparation of HM-EOPO/water system (Johansson et al., 1999). In some ATPE systems, phase inversion occurs at atemperature higher than room temperature, such as 30 °C. For example, phase inversion at 30 °C is desired for extraction of endo-polygalacturonase in UCON/sulfate system as it partition preferably to the bottom phase (Pereira et al., 2003).

771

772 2.3.1.6 Influence of pH

773 Normally influenced by the nature of phase-forming salt or/and adjusted by adding
774 appropriate amounts of strong acid and base solution, system pH plays a crucial role in
775 thermoseparation-based ATPE as it modifies the phase-forming polymer concentration and
776 influence partitioning of the target product. Literature also reported that alkaline salt is more
777 effective in recovery of certain proteins, and vice versa (Chen et al., 2014). Some bioproducts
778 have optimal activity within therange of pH, especially enzymes, such as fungal laccase (acidic
779 pH range) (Moreira et al., 2013). Besides that, system pH generally has no effect on the CP
780 of TSPs, with the exception of cationic hydrophobically modified ethylene oxide polymer (HM-
781 EOPO) which CP increased rapidly at pH < 9 (Jonsson and Johansson, 2003). The hydrophobic
782 interaction dominates at pH around pI of biomolecules due to zero net charge of proteins
783 (Berggren et al., 1995), while electrostatic forces show agreater effect at other pH(Show et
784 al., 2012b). Table 2.5 presented a list of bioproducts with their optimal extraction pH in
785 thermoseparating ATPE.

786

787 2.3.1.7 Influence of Salt Addition

788 Salt addition plays a significant role in thepartitioning of protein in thermoseparation-
789 based ATPE. One important thing to noted down is that the salt concentration should be at
790 least tenfold higher compared to the buffer to obtain a dominating effect in an ATPE(Kepka
791 et al., 2004). Due to thediverse affinity of ions for the different phases where effects
792 contributed by anions is stronger compared to cations, theaddition of cosolutes such as salts
793 into system serve as "counter-ions" which enhance the partition of bioproducts to the
794 preferred phase. The Hofmeister series serve as a rule of thumb for relative hydrophobicity
795 of particular ion (Berggren et al., 1995). The electrical potential difference between the two
796 phases (Donnan potential) formed by partitioning of the cations and anions between the two

phases will generate an electrochemical driving force and subsequently influence the partitioning of biomolecules and/or other charged particles in the system (Kepka et al., 2004). With increasing anion hydrophobicity by theaddition of large monovalent ions such as Et_3NH^+, Cl^- and ClO_4^-, the partition coefficient increased for a positively charged biomolecule, while decreased for a negatively charged biomolecule(Berggren et al., 1995). However, it must remember that amphiphilic proteins, which has low solubility, tends to precipitate at high salt concentration (Persson et al., 1998).

Partitioning of salts and their influence in partitioning of some model proteins such as BSA and lysozyme have been investigated in the systems such as TSP/polymer systems (Berggren et al., 1995) and TSP/water systems (Johansson et al., 1999), TSP/TSP systems (Persson et al., 1999a). Besides that, theinfluence of salts on thepartitioning of peptides in UCON/water system (Johansson et al., 1997) and TSP/dextran system (Berggren et al., 2000) have been investigated as well. It was

Table 2.5: Summary of bioproducts with their respective optimal extraction pH

Bioproducts (pI)	System	Optimal pH	Reference
BSA (5.0)	HM-EOPO/water	7	(Johansson et al., 1999)
Ciprofloxacin	EOPO/phosphate	11	(Chen et al., 2014)
Cyclodextrin glycolsyltransferase (6.9)	EOPO 3900/phosphate	7	(Ng et al., 2012)
Fruit bromelain (4.6)	PEO-PPO-PEO/water	6	(Rabelo et al., 2004)
L-asparaginase (4.9)	PEO-PPO-PEO/phosphate	5	(Zhu et al., 2007)

Laccase	UCON/phosphate	4.6	(Moreira et al., 2013)
Lipase (6.3)	EOPO 3900/water	8.5	(Show et al., 2012b)
Lipase (6.3)	EOPO 3900/phosphate	7	(Show et al., 2012a)
Lysozyme (10.7)	EOPO/phosphate	9	(Dembczyński et al., 2013)

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812

found that the difference in top phase-forming polymer's hydrophobicity has little influence on salt partitioning (Johansson et al., 1999). Phosphate and borates salts were employed in thepartitioning of plasmid DNA with the former giving a better result, while LiCl showed no effect (Kepka et al., 2004). Studies showed that phosphate addition increase the partition coefficient of bromelain, but the PF has been reduced which might due to thesalting-out effect on total protein in the system (Rabelo et al., 2004). Using Box-Behnken design, the addition of NaCl has been concluded as the most significant factor in lysozyme recovery by thermoseparation-based ATPE(Dembczyński et al., 2013).

Furthermore, salts addition such will promote the hydrophobicity difference between the phases, causing a decrease in CP due to strengthened hydrophilicity of water-rich top phase (Cunha et al., 1998). The fall in CP following the rise in salt concentration can be explained by the reduced counter-ion entropy due to decrease in thedifference of ion concentration between the two phases with increasing salt addition to the system (Jonsson and Johansson, 2003). The literature reported that there is only insignificant variation in LCST in the range of phosphate salts addition (15 to 100 mM) (Rabelo et al., 2004). Cunha et al (Cunha et al., 1998) investigated the influence of salt addition on thecomposition of TSP/water ATPE system and found that concentration of polymer in bottom phase increase with salt added. Lastly, there are studies showed that polymer recovery increased with theaddition of 10 mM chloride, phosphate or sulfate salt, however, there are no significant differences that were found when different types of salts were utilized (Persson et al., 2000b).

2.3.1.8 Affinity partitioning

Covalent binding of thermoseparating copolymer phase with affinity ligands can assist in directing the partition of target products to the desired phase. Triethylene glycol-diglutaric acid, a free ligand with carboxyl functional group was utilized to direct human immunoglobulin G partition to UCON-rich phase due to electrostatic interactions between the negatively

charged carboxylic group and positively charged human immunoglobulin G (Ferreira et al., 2008). Higher partition coefficient and purity together with recovery yield were achieved across a range of UCON with different Mw, but thereduction in performance can be observed from those with higher Mw which due to both increasing hydrophobicity and volumeexclusion effect. It was also reported that ligand's influence become more significant at high dextran concentration. Not only does that ligand addition not complicate the recycling process, it can be recycled and reutilized as well, leading to a more efficient and cost-effective recovery process (Ferreira et al., 2008).

2.3.1.9 Recycling of EOPO copolymers

As mentioned before, thermoseparation-based ATPE has outstanding advantages over conventional ATPE as the polymers can be recycled and reutilized due to its characteristic of thermoseparating into one water phase and one polymer-rich phase as the temperature exceed CP. Not only being a greener and cheaper downstream processing technique, separation of target products exclusively in water phase also makes the recovery more efficient.

Polymer recycling had been studied in depth by Persson et al (Persson et al., 2000b). It is reported that copolymer with higher PO content can be separated from water more efficiently, whereas polymer recovery is better with acopolymer of higher Mw. Besides that, polymer recovery increased can be up to 90 % with thermoseparation temperature in a non-linear way and 10 to 15 minutes appeared to be the threshold where recovery of thecopolymer is no longer increased. The recycling studies were performed with lysozyme and BSA using $EO_{50}PO_{50}$/Reppal PES 100 system and the proteins showed constant partition behavior throughout the four cycles even with salt addition (Persson et al., 2000b). The recovery and recycling of thermoseparating copolymers are influenced by the geometry of the vessel and heat transfer capacity as well (Persson et al., 2000a).

In most of the recycling studies, the yield and purity fall following the recycling cycles due to increasing amount of contaminants in bottom phase after each cycle, thus, recommending that the EOPO copolymer can only be recycled and reused few times with example such as cyclodextrin glycolsyltransferase (twice) (Ng et al., 2012), ciprofloxacin (twice) (Chen et al., 2014). However, it appears that the recycling can be performed continuously as long as thefresh salt phase is used after every few recycling since constant polymer recovery was achieved throughout the studies. There are exceptions in both lysozyme (Dembczynski et al., 2010) and lipase (Show et al., 2012a) extraction studies which showed that the parameters such as purity, yield and Vr are quite stable, regardless of high accumulation of contaminant protein in bottom phase throughout four consecutive recoveries. Besides, it had also been shown that recycling of both phases are possible when the ATPE is formed by two different TSPs and can be recycled at least 3 times without significant decrease in yield and purity (Persson et al., 1999a).

Table 2.6 summarizes the recent applications of thermoseparation-based ATPE on separation of biomaterials with their respective recovery efficiency and recycling properties.

888 Table 2.6: Summary of recent application of thermoseparation-based ATPE on bioseparation

Bioproduct	System	Recovery efficiency	Polymer Recycling	Reference
α-amylase	EOPO 2500/sulfate	91 % yield / 3.31 PF	N.A.	(Li and Peeples, 2004)
CGTase	EOPO 3900/phosphate	87 % yield / 13.1 PF	2 times	(Ng et al., 2012)
Ciprofloxacin	EOPO L31/K_2HPO_4	97.7 % extraction efficiency	2 times	(Chen et al., 2014)
Endo-polygalacturonase	UCON/sulfate	Up to 99 % yield / 10 PF	N.A.	(Pereira et al., 2003)
Human immunoglobulin G	UCON/dextran	82 % yield / 100 % purity	N.A.	(Ferreira et al., 2008)
Laccase	UCON/KH_2PO_4	134 % yield / 1.31 PF	N.A.	(Moreira et al., 2013)
Laccase	UCON/K_2HPO_4	K = 0.272	N.A.	(Silvério et al., 2013)
Lipase	EOPO 3900/phosphate	99 % yield / 14 PF	3 times	(Show et al., 2012a)
Lipase	EOPO 3900	99 % yield / 14 PF	N.A.	(Show et al., 2012b)
Lysozyme	$EO_{50}PO_{50}$/K_2HPO_4	85 % yield / 16.9 PF	3 times	(Dembczynski et al., 2010, Dembczyński et al., 2010)

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2.3.2 Extractive Bioconversions

Extractive bioconversion (also known as extractive fermentation or extractive cultivation) which integrates fermentation together with downstream processing (clarification, concentration and partial purification) using a continuous process has gained increasing interests (Show et al., 2012b, Canales et al., 2009, Li et al., 2002a, Chavez-Santoscoy et al., 2010, Sinha et al., 2000, Ng et al., 2013, Zijlstra et al., 1998, Kuboi et al., 1995, Li et al., 2000, Kulkarni et al., 1999, Umakoshi et al., 1996, Pandey and Banik, 2011, Ooi et al., 2011, Kwon et al., 1995, Banik et al., 2003). The main principle of extractive fermentation using ATPE is to promote the partitioning of the target product in one phase, while cell and substrates accumulated in the other phase (Ooi et al., 2011). Literature have shown that TSPs has surfactant effect on cell membrane's permeability which enhances enzyme secretion (Show et al., 2012b). Thermoseparation-based extractive cultivation has been applied on the biosynthesis of starch (Li et al., 2002a, Li et al., 2002b) and lipase (Show et al., 2012b), while was proposed to be studied on laccase (Silvério et al., 2013). Preliminary studies were normally performed to obtain the suitable ATPE system for extractive bioconversion (Li et al., 2002a, Silvério et al., 2013).

In extractive fermentation, choice of phase-forming components requires careful consideration as ahigh concentration of salts will inhibit cell growth, while dextran, on the other hand, promote cell growth but is economically unfavorable for thelarge-scale application (Ooi et al., 2011). Microorganisms and enzymes need to stay in a range of pH and temperature for optimal bioconversion. Medium viscosity which influenced by TSP's EO:PO ratio, concentration or molecular mass is an important element due to thelimitation of oxygen mass transfer by increased viscosity, leading to retarded cell growth and fall in bioproduct formation (Show et al., 2012b). Besides that, it needs to be noted down that agitation speed influence the productivity and partition behavior of biomaterial in extractive cultivation. High shaking speeds promote the production of target bioproducts due to enhanced rate of oxygen

mass transfer and cell growth, but also forming homogeneous cultivation medium as a result of intermixing between two phases leading to reduced partitioning efficiency (Pandey and Banik, 2011). Bioreactor scaling-up studies further proving that agitation serves as an important role in extractive bioconversion involving highly viscous phase-forming components such as EOPO (Show et al., 2012b). Extractive cultivation studies can be fitted with kinetic models such as Michaelis-Menten kinetic model to get a better understanding the kinetic mechanism behind the bioconversion (Li et al., 2002b).

2.2.3 Integration with other purification techniques

Other than integration with upstream fermentation to form extractive bioconversion, thermoseparating ATPE were also often integrated with other purification techniques, such as high-pressure homogenizer (Zhu et al., 2007), cross-flow membrane (Dembczyński and Białas, 2013) and HPLC/UV (Chen et al., 2014) to improve the performance of downstream processing and reduce the total operation cost.

For conventional extraction and separation of intracellular bioproducts, cell disruption is normally done by (bio)chemical or mechanical means. To minimize adsorption on cell debris during cell disruption as well as eliminate the centrifugation and storage process, in-situ ATPE integrated with high-pressure homogenizer was performed for extraction of L-asparaginase. A higher yield (73.3 %) and improved specific activity (94.8 U/mg) with a shorter total process time are obtained through this integrated process compared to that of conventional ATPE (52.1 % and 78.6 U/mg respectively) (Zhu et al., 2007).

As the use of centrifugation for theindustrial-scale process is uneconomical, thermoseparation-based ATPE coupled with cross-flow membrane separation technique was investigated for pilot-scale separation and recovery of lysozyme. This integrated process

provided specific activity and yield of 34 000 U/mg and 47.5 % with polymer recovery up to 83.9 % in single extraction step (Dembczyński and Białas, 2013).

Because of the limitation in traditional detection techniques such as high-performance capillary electrophoresis and spectrofluorimetry due to their high detection limit and complex pretreatment, thermoseparation-based ATPE coupled with HPLC/UV detection system have been proposed for separation and concentration of ciprofloxacin. The optimized ATPE was performed on real samples, such as milk, egg, and shrimp and presented satisfactory results following the maximum residual limits developed by the Europian Union (Chen et al., 2014).

2.3.4 Challenges and perspectives

The efficiency and effectiveness of ATPEare very dependent on process parameters, such as tie-line length, volume ratio, pH, temperature, additions of salts or ligands and others. In thermoseparating ATPE, asides from high yield, elements such as high purity, high concentration factor, thesimplicity of product recovery, cheap chemical cost and environmental friendliness should be taken into consideration as well (Dembczyński et al., 2010). More efforts should be put into develop novel thermoseparating polymers with unique characteristics and study different types/combinations of abiphasic system. Mathematical models can be established to generalize correlations between different parameters and predict the partitioning behavior of bioproducts in the system. Other than that, poor understanding of the mechanisms behind ATPE and issue regarding reproducibility and robustness also hinders the wide application in industry. Kinetics of bioseparation and underlying mechanisms can be further studied and simplified into simpler concepts. Commercial kits used to assess and evaluate ATPE at bench scale should also be researched and developed.

Chapter 3

MATERIALS AND METHODS, PRETREATMENT STRATEGIES AND CHARACTERIZATION OF PHAs

3.1 Introduction

This chapter focused on describing materials such as bacteria strain and chemicals which will be used throughout the research. Furthermore, common experimental procedures and techniques, including the production of polyhydroxyalkanoates (PHAs), quantification by gas chromatography (GC), partitioning behavior of PHAs with governing equations were also described. Other than that, the chapter also studied different cell pretreatment strategies and performed characterization of PHAs.

3.2 Materials and Methods

3.2.1 Materials

C. necator H16 strain was kindly provided by Yuan Ze University, Taiwan. Poly(ethylene glycol-ran-propylene glycol) monobutyl ether with a molecular weight of 3900 g/mol (EOPO 3900), UCON HTF 14, benzoic acid and propanol were purchased from Sigma-Aldrich (M) Sdn Bhd (Selangor, Malaysia). EOPO 3900 and UCON HTF 14 are both TSPs with EO:PO ratio of 50:50, though EOPO 3900 has a higher molecular weight and is more hydrophobic. All the salts used in this study were obtained from LGC Scientific Sdn Bhd (Selangor, Malaysia). All the other chemicals used in this study were of analytical grade.

3.2.2 Production of PHAs

The bacterial cells were first cultured on agar plates at 30 °C for 24 hours. An inoculum was prepared in a 10 ml nutrient broths (8 g/L) by inoculating the medium with a single colony from the cultured agar plate. The culture was then grown aerobically at 30 °C for 24 hours. Subsequently, the inoculum (5 % v/v) was transferred to a 250 ml shake flask containing 100 ml operating volume of defined medium supplemented with glycerol of 30 g/L and yeast extract of 2 g/L. The inoculated fermentation medium was then incubated aerobically at 30 °C for 72 hours. The defined medium was composed of Na_2HPO_4-$7H_2O$, 6.7

g/L; KH_2PO_4, 1.5 g/L; $(NH_4)_2SO_4$, 2.5 g/L; $MgSO_4$-$7H_2O$, 0.2 g/L; and $CaCl_2$, 10 mg/L) and 0.5 % v/v of trace mineral solution (Na_2EDTA, 6.0 g/L; $FeCl_3$-$6H_2O$, 0.29 g/L; H_3BO_3, 6.84 g/L; $MnCl_2$-$4H_2O$, 0.86 g/L; $ZnCl_2$, 0.06 g/L; $CoCl_2$-$6H_2O$, 0.026 g/L; and $CuSO_4$-$5H_2O$, 0.002 g/L).

Without undergoing extra clarification or filtration step, the cultured broth of *C. necator* H16 was directly subjected to ultrasonic cell disruption with an ultrasonic processor (Hielscher, UP4005) at 30 kHz per cycle for 15 min to obtain intracellular PHAs. Disrupted biomass was stored in 500 ml graduated laboratory bottles.

3.2.3 Quantification of PHAs by gas chromatography (GC) analysis

For the determination of PHAs content, the GC method by Akaraonye et al. with minor modification was employed (Akaraonye et al., 2012). 2 ml of chloroform and 2 ml of propanol with 15% v/v of sulphuric acid were added to about 2 mg of dried samples. After incubation at 100 °C for 2 hours, the sample was cooled rapidly and 1 ml of water was then added to the sample to remove the sulphuric acid. The sample was then allowed to settle until separated into organic and aqueous phases. Then, 0.2 µl of the organic phase (bottom phase) was injected into Clarus 500 gas chromatograph (Perkin Elmer, United States) equipped with a DB-WAX capillary column (0.25 mm by 30 m; 0.25 µm film thickness). The initial oven temperature is set at 60 °C and held for 3 min, then increased to 210 °C at a rate of 10 °C/min and held for 3 min at the same temperature. The PHA content of the injected samples was determined by internal standard calibration using PHA reference compound and benzoic acid internal standard.

3.2.4 Partitioning behaviors of PHAs in ATPE

The partitioning of PHAs can be described by their respective partition coefficients (Kpa), which defined as the ratio of PHAs concentration in the top to that in the bottom phase:

$$Kpa = Conc_T / Conc_B \quad (3.1)$$

The purity, % was defined as the ratio between the mass of PHAs as quantified by GC and thetotal mass of dried sample used for the GC analysis:

$$Purity = (M_{PHA} / M_{Sample}) \times 100 \quad (3.2)$$

Where M_{PHA} is the mass of PHA (g) and M_{Sample} is total mass of dried sample used (g).

The phase volume ratio, V_r was defined as ratio between top phase volume and bottom phase volume:

$$V_r = V_T / V_B \quad (3.3)$$

Where V_T and V_B are top and bottom phase volume respectively.

Recovery Yield (Y), % of PHA in top phase was calculated as the ratio between PHA concentration in top phase and the initial PHA concentration in the extract:

$$Y = (Conc_T \times V_T) / (Conc_E \times V_E) \quad (3.4)$$

Where $Conc_T$ and $Conc_E$ are PHA concentration of top phase and extract respectively and V_E is extracted volume.

Purification factor (PF) was defined as the ratio of top phase PHA purity to the overall PHA purity:

$$PF = (\text{Top Phase PHA Purity}) / (\text{Overall PHA Purity}) \quad (3.5)$$

3.2.5 Different strategies of cell disruption

Three different cell disruption strategies, which are sonication by two different equipment and microwave were employed to study their efficiency of disrupting *C. necator* cells. Without undergoing extra clarification or filtration step, the cultured broth of *C. necator* H16 was directly subjected to ultrasonic cell disruption with an ultrasonic cell disruptor (Hielscher, UP4005) at 30 kHz per cycle and ultrasonic water bath (Elmasonic E30H, Germany) at different amplitudes for 5 min to obtain intracellular PHAs. For the study of cell disruption of the microwave, the cell culture was subjected to cell disruption in the microwave at a different time as well as for different heating power. After cell disruption, 1 mL of the crude cell was sampled and diluted to a suitable dilution (<300 CFU) before cultured in agar plate for 24 hours. Then, the number of viable bacteria is calculated.

3.2.6 PHAs characterization

Fourier Transform Infrared (FTIR) Spectroscopy. The functional groups, types of bonding, and identified components in the samples were determined by a Perkin-ElmerSpectrum RX1 (Massachusetts, U.S.). The infrared spectra of the samples were recorded in the range of frequency of 400 – 4000 cm^{-1}.

Thermo Gravimetric Analysis. Thermal stability of the PHA was investigated by Thermogravimetric analysis (TGA) using a TGA-DSC 1 Mettler Toledo (Greifensee, Switzerland) under 20 ml/min nitrogen atmosphere. The temperature range was from 30 ℃ to 800 ℃ with a heating rate of 10 ℃/min.

3.3 Results and Discussion

3.3.1 Different strategies of cell disruption – Sonication and microwave

Defined as sound wave transmitted at a frequency range beyond the upper audible limit of human hearing (usually above 20 kHz per second), ultrasonic wave has been used for cell disruption to kill harmful microorganisms or extract intracellular bioproducts. The examples from literature are such as human myelomonocytic lymphoma U937 cells (Feril Jr et al., 2003), phycocyanin (Furuki et al., 2003), bacteria cell (Wrigley and Llorca, 1992, Ordoñez et al., 1987, Guerrero et al., 2001, Lo´pez-Malo et al., 1999, Mañas et al., 2000), vegetal tissues (Toma et al., 2001), herbal extracts (Vinatoru, 2001), viruses (Taylor et al., 2002) and others. Other than cell disruption, utilization of sonication in extraction offers the benefits of improved penetration, mass transfer intensification and capillary effects (Toma et al., 2001). Therefore, in this study, two ultrasonic extraction equipment include ultrasonic cell disrupter and ultrasonic water bath have been used to study the effectiveness of ultrasonic wave in cell disruption. It should be noted that 400 W and 330 W are the maximum amplitude that can be provided by the ultrasonic cell disruptor and ultrasonic water bath in the study respectively, thus, affect the design of the experiment.

Table 3.1 a and b demonstrate the cell disruption efficiency of ultrasonic cell disruptor and ultrasonic water bath under the influence of different amplitude. Clearly shown in the results, ultrasonic cell disruptor generally offered a better cell disruption efficiency compared to ultrasonic water bath. For all three amplitudes, the ultrasonic cell disruptor achieved almost complete cell disruption, which means that amplitude of 120 W is sufficient to release PHAs from the cells. On the other hands, the ultrasonic water bath can only reached 80 % cell disruption efficiency for all three amplitudes.

Although both methods utilize ultrasonic wave to disrupt the cell membrane and thus, release the intracellular PHAs, there is a simple reason behind the difference of cell disruption efficiencies. The most fundamental difference between the two strategies is thelocation of

the source of theultrasonic wave. The source of theultrasonic wave by ultrasonic cell disruptor is located within the PHAs crude extract as the ultrasonic probe in inserted into the crude extract. This ensures the ultrasonic energy is fully utilized to disrupt the bacteria cell without losses. On the contrary, the PHAs crude extract receives anultrasonic wave from the surroundings when placed in an ultrasonic water bath. There is a loss of ultrasonic energy due to diffraction and absorption by the water medium or any impurities present when the wave traveled from the source to the crude extract through thewater.

Similarly, themicrowave has also been used in the same applications, which most studies focus on extraction of lipids from microalgae (Lee et al., 2010, Zheng et al., 2011, Guldhe et al., 2014, Grimi et al., 2014). Table 3.1 c and d demonstrate the results of cell disruption efficiency of microwave under influences of microwave time and heating power. Under the condition of constant 20 W heating power and 60 °C temperature, the cell disruption efficiency increase linearly by 7 % for every 60 s of microwave time increment. Following this linear relationship, a microwaving time of approximately 257 s (less than 5 minutes) is predicted to be required for complete cell disruption. On the contrary, a heating power in the range of 50 to 100 W for 60 s is required for themicrowave to completely disrupt the cell where the final temperature falls between 60 to 80 °C. Therefore, it can be concluded that both ultrasonic cell disruptor and microwave show great promise for efficient cell disruption.

Table 1a: Cell disruption efficiency of ultrasonic cell disruptor under the influence of different amplitude

Amplitude (W)	Cell disruption efficiency (%)
120	99.9
240	99.9
400	100.0

Table 1b: Cell disruption efficiency of ultrasonic water bath under the influence of different amplitude

Amplitude (W)	Cell disruption efficiency (%)
99	79.3
198	81.4
330	80.0

Table 1c. Summary of influence of microwave time on cell disruption efficiency at constant 20 W heating power and 60 °C temperature

Time (s)	Efficiency (%)
60	76.3
120	83.4
180	90.8

Table 1d. Summary of influence of heating power on cell disruption efficiency at constant 60 s microwave time

Heating Power (W)	Final Temperature (°C)	Efficiency (%)
50	60	91.8
100	80	100.0
150	100	100.0

3.3.2 PHAs characterization

Figure 3.1 demonstrates the FT-IR analysis result of PHAs synthesized by *R. eutropha* H16. The IR spectroscopic analysis provided further insights into the chemical structures of the polymer and reveal the monomeric units. The wave number (cm^{-1}) and percent light reflectance correspond to the function groups present in the PHAs. A very sharp reflectance observed at 1730 cm^{-1} indicated the presence of C=0 functional carbonyl bonding. The presence of carbonyl bonding at this wave number demonstrates a very strong presence of PHB in the produced structure. The sharp peak of carbonyl bonding also indicated that PHAs synthesized are of highly ordered crystalline nature (Cha et al., 2016). Similarly, FTIR confirms sharp peak of methyl (-CH_3) and ethyl (–CH_2) functional group at 1381 cm^{-1} and 1458 cm^{-1} respectively as well as band stretching between 1101 to 1282 $cm^{-1}$and 3437 to 3905 cm^{-1}illustrated presence of carbon oxygen stretch of the esters (C-O-C) and hydroxyl (-OH) functional group respectively (Cha et al., 2016, Nair et al., 2014). The strong band at 1230 cm^{-1} was predicted to be of an alkyl (-CH) stretch.

Figure 3.2 shows the result of thermogravimetric analyses (TGA) of PHAs produced by *R. eutropha* H16 during heating treatment from 30 ℃ to 800 ℃ under inert atmosphere (N_2). It was observed that the compound was stable up to 230 ℃. Then, the PHA gave a rapid thermal degradation between 230 and 290 ℃ which incongruent with values reported in the literature, such as 210 – 280 ℃ by Tanamool and his coworkers (Tanamool et al., 2011). The maximum thermal degradation temperature of the PHA is 269.19 ℃, which is similar to the literature of 259 to 283 ℃ (Cha et al., 2016).

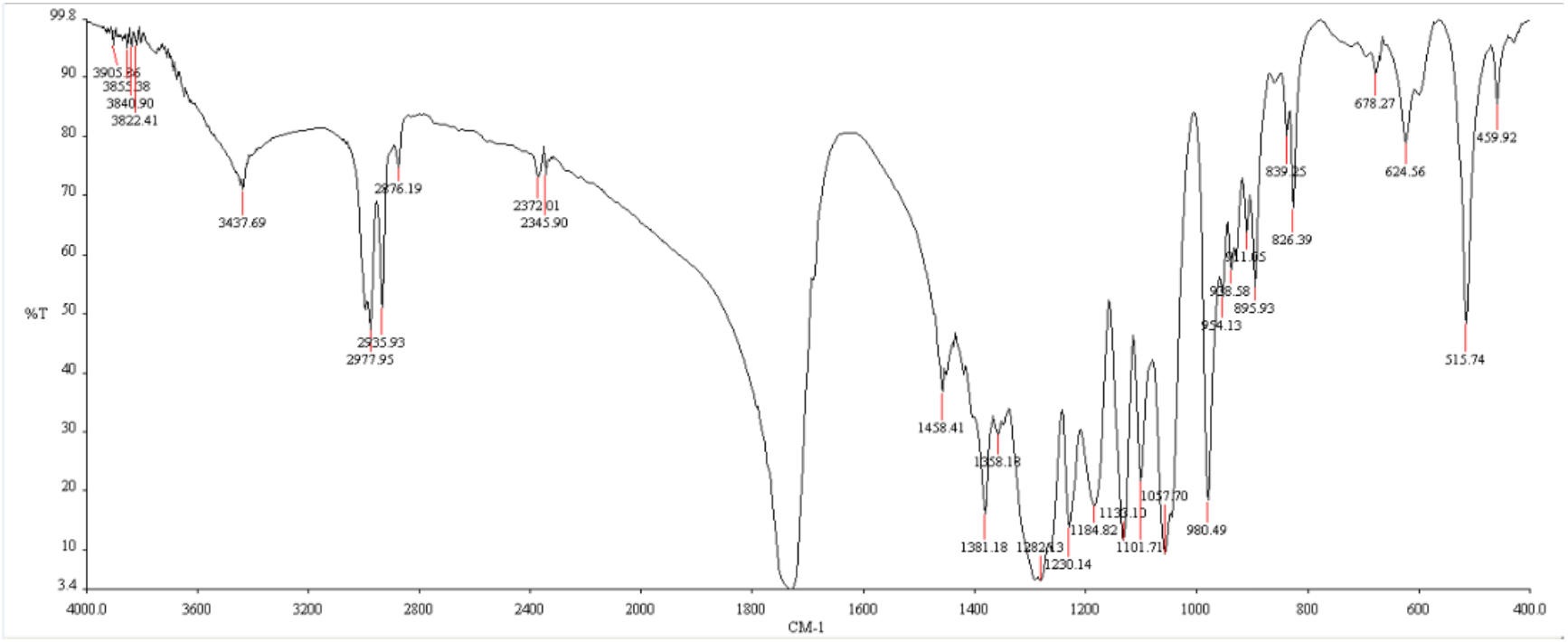


Figure 6.1 FTIR analysis of PHAs

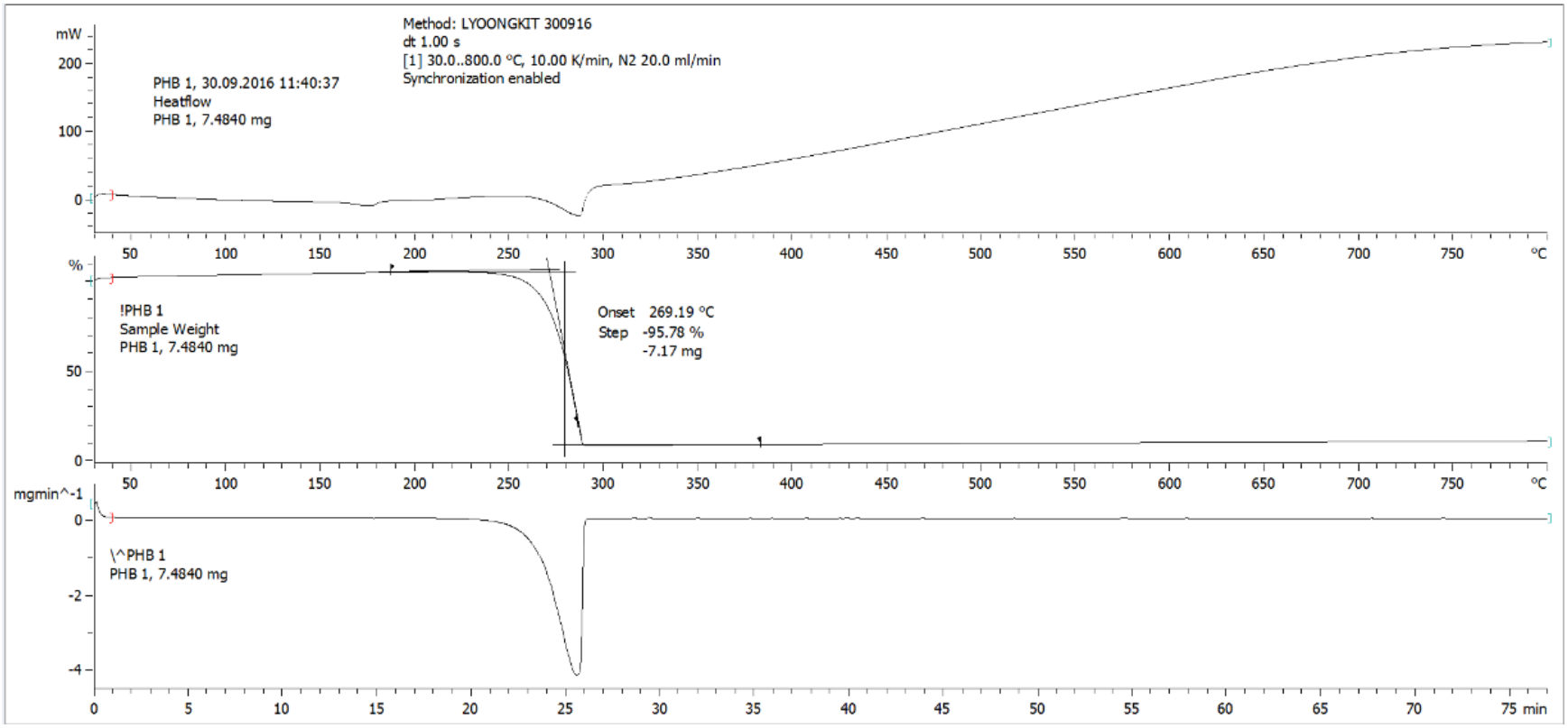


Figure 6.2TGA analysis of PHAs

3.4 Conclusion

Comparing between three different cell disruption techniques, ultrasonic cell disruptor and microwave demonstrated promising results of total cell disruption in a short amount of time. Biopolymer synthesized by *C. necator* H16 was confirmed by FTIR analysis demonstrated functional groups which include CH, CH_2, CH_3, C=O, C-O-C, and OH. The results have confirmed that the biopolymer produced by *C. necator* H16 is poly-3-hydroxybutyrate compound. The PHA produced has a maximum thermal degradation temperature of 269.19 ℃.

Chapter 4

CLOUD-POINT EXTRACTION VERSUS THERMOSEPARATING POLYMER/SALT ATPE OF GREEN-POLYMERS FROM *CUPRIAVIDUS NECATOR* LYSATE

4.1 INTRODUCTION

Firstly discussed in the early 1990s, thermoseparating polymers (TSPs) have been found to be able to replace conventional phase-forming polymers, such as poly(ethylene glycol)s (PEGs) to form aqueous two-phase systems (ATPSs) together with Dextran (Berggren et al., 1995, Hou et al., 2014). Being more hydrophobic than PEGs, TSP/Dextran, and TSP/salt systems have a wider range of hydrophobicity differences which can be employed to promote the partition selectivity of bioproducts (Abbasiliasi et al., 2014). Furthermore, a lower concentration of phase-forming components is required to form thermoseparating-based ATPSs, which not only reduces the risk for protein precipitation (Li and Peeples, 2004) and has a shorter phase separation time but also increasing the cost-competitiveness and environmental-friendliness of thermoseparating-based ATPE (Kepka et al., 2004).

Besides coupling with other phase-forming components to form ATPS for the purpose of aqueous two-phase extraction (ATPE), cloudpoint extraction (CPE) has been developed based on the concept of temperature-induced phase separation. CPE is done by heating TSP (aqueous) solution to a temperature few °C above lower critical solution temperature (LCST) or cloud point (CP) temperature which consequently induces thermoseparation (Moreira et al., 2013). The solution then becomes cloudy and separates into two phases, which are the top phase which contains mostly water and the bottom phase which consists of concentrated TSPs. Target products will then partitioned to either the water-rich top phase or the polymer-rich bottom phase, depending on size, hydrophobicity and charge of biomolecules as well as "volume-exclusion" effect. However, in this case, target products are preferably partitioned to the top phase, so that the polymer-rich bottom phase can be recycled and reutilized. It should be noted that polyethylene glycols (PEGs) which are commonly used for ATPS is also a type of thermoseparating polymer, however, it has an LCST above 100 °C (around 180 °C for those with lower molecular weight) which is unsuitable for thermo-based bioseparation as most biocomponents are sensitive to high temperature (Ng et al., 2012). Having a lower CP

which as low as 47 °C, TSPs are much more suitable for temperature-induced CPE of biomaterials (Moreira et al., 2013).

A thorough literature review has shown that the only reported work on PHAs partitioning using ATPE is by Divyashree and his coworkers (Divyashree et al., 2009). However, the work focused on separation of polyhydroxybutyrate-co-hydroxyvalerate [P(HB-co-HV)] from the hydrolyzed bacterial cells using conventional PEG/phosphate-based ATPE. Moreover, there is a lack of discussion on important ATPE-influencing parameters such as polymer's molecular weight, the concentration of phase-forming components, tie-line length (TLL), volume ratio (Vr) and others. Henceforth, factors like TSP types, the concentration of phase-forming components (both TSPs and salt), pH and effect of centrifugation have been studied in this chapter to achieve satisfactory PHAs yield and purity in thermoseparating polymer/salt ATPE. Furthermore, the isolation and purification of PHAs from *Cupriavidus necator* H16 utilizing CPE technique and thermoseparating polymer/salt ATPE were also studied for the first time in this chapter. For CPE study, the key influencing parameters such as type and concentration of thermoseparating polymer, salt addition, feedstock load, and thermoseparating temperature were investigated. The attempt of recycling and reutilizing the thermoseparating polymer for PHAs extraction has also been conducted.

4.2 MATERIALS AND METHODS

4.2.1 Partitioning of PHAs using CPE

ATPE system was prepared in a 50 ml centrifuge tube by mixing the thermoseparating polymer (in wt/wt %) into deionized water. The crude PHA feedstock was mixed well with aqueous solution of thermoseparating polymer (agitated in a vortex for 2 minutes at 2500 rpm) before the mixture was incubated in water bath at 65 °C for thermoseparation. After the stages of equilibration and phase separation, the top phase was carefully removed using

a pipette, while the bottom phase was then sampled through the interface. The samples from both phases and disrupted biomass are centrifuged and washed with deionized water for three times before left in the oven for overnight at 70 °C to be dried. The dried samples from both phases and disrupted biomass were analyzed for P(3HB) content using gas chromatography (GC). As for the recycling studies, a 40 g system was first prepared at an optimized temperature of thermoseparation. The top phase was removed and the mass of this phase was then determined. Later, the fresh water and feedstock were added to the recycled thermoseparating polymers and a new stage of ATPE repeats. All experiment works were done at least duplicate. Error bars were obtained by evaluating the difference between minimum/maximum and mean value.

4.2.2 Binodal phase diagram construction

The binodal curves were plotted using the turbidometric titration method, as described by Kaul (Kaul, 2000). The phase diagram depicts the equilibrium boundary between the single phase and multiple phases under a set condition. A mixture of $EO_{50}PO_{50}$ and salts of known concentrations are titrated with deionized water until the turbid solution become clear. The binodal curve was fitted into empirical equation suggested by Merchuk et al (1998), which as below:

$$Y = a\exp(b \times X^{0.5} - c \times X^3) \quad (4.1)$$

where X and Y aremassfraction of salt and $EO_{50}PO_{50}$ respectively.

4.2.3 Partitioning of PHAs in thermoseparating copolymer/salt ATPS

A 40 g ATPS was prepared by mixing the phase-forming components which are TSP and salt (in wt/wt %) with the crude feedstock. The mixture was then allowed to settle for phase separation. After the stages of equilibration and phase separation, the top phase was

carefully sampled using a pipette, while the bottom phase was then sampled through the interface. The samples from both phases and disrupted biomass are centrifuged and washed with deionized water for three times before left in the oven for overnight at 70 °C to be dried. The dried samples from both phases and disrupted biomass were analyzed for P(3HB) content using gas chromatography (GC). All experiment works were done at least duplicate. Error bars were obtained by evaluating the difference between minimum/maximum and mean value.

4.3 RESULTS AND DISCUSSION

4.3.1 Cloud point extraction (CPE) of PHA in thermoseparating ATPS

4.3.1.1 Cloud Point Curve

Two types of TSPs, UCON and EOPO 3900 were employed in this thermoseparation-based CPE of PHAs which both are of EO:PO 50:50 ratio. These two random copolymers were chosen due to the fact they were extensively used for thermoseparating ATPE in literature (Moreira et al., 2013, Dembczyński et al., 2010, Show et al., 2012a, Show et al., 2011, Chen et al., 2014, Dembczynski et al., 2010). Also, some literature suggested that the random copolymer with EO:PO ratio of 50:50 (such as EOPO 3900 and UCON HTF 14) are more suitable for biomolecule partitioning (Ng et al., 2012).Figure 4.1 presents the CP of thermoseparating polymers over a range of TSPs concentration (wt/wt %). It can be clearly observed that CP plummet until 5 wt/wt % of TSP concentration, then remains fairly constant afterward for both UCON and EOPO 3900. Although EOPO 3900 has lower CP at lower concentration (0 to 7.5 wt/wt %), both polymers have similar CP beyond 7.5 wt/wt % concentration.

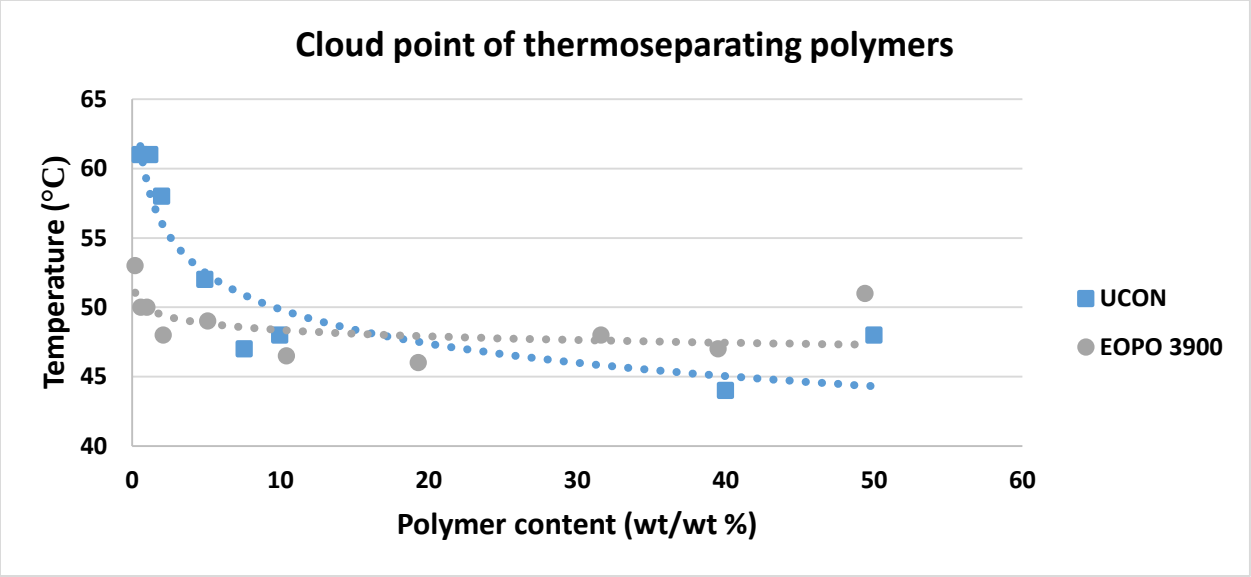


Figure 4.1: Cloud point of thermoseparating polymers over a range of polymer concentration (wt/wt %)

4.3.1.2 Effect of polymer concentration

The study of CPE of PHAs was done over a range of TSPs concentrations at thermoseparating temperature of 65 °C. The systems in CPE normally possess high V_r (i.e. large volume of water phase), ranging from 2 to 20. Figure 4.2 demonstrates the purification and recovery result of PHAs utilizing UCON- and EOPO 3900-based CPE. The recovery yield and PF showed a maximum of 87 % and 1.2 fold, respectively at 8 wt/wt % of UCON concentration. Other than that, the PHAs seem to partition preferably to the UCON-rich bottom phase in this system as most of the PF obtained were lower than 1. It was also clearly observed that the recovery yield of PHAs followed a similar trend as PF over a range of UCON concentrations. Both yield and PF have a decreasing trend from the maximum point at 8 wt/wt % to 13 wt/wt %, which increase afterward with the yield increased at a higher rate than PF. This increasing trend might due to the "excluded-volume effect" which showed a dominating effect forces both PHAs and contaminants partitioned to the water-rich top phase

following the reduction in "free-space volume" in bottom phase. In this case, PHAs extraction using UCON-based CPE did not show a promising result as the PHAs recovered in the top phase has a lower purity than that of initial extract, thus, is deemed inefficient and undesirable in the purification process.

On the other hand, for EOPO 3900 system, the recovery yield of PHAs was found to be generally higher than UCON system, where all yield was greater than 64.5 % and reached a maximum of 82.2 % at 20 wt/wt % of polymer concentration. The PHAs recovery yield obtained are much higher compared to that of literature which used conventional PEG/phosphate ATPE (51 %) (Divyashree et al., 2009). The high yield of PHAs compared to that of UCON is caused by the excluded volume effect due to the stronger hydrophobic effect of EOPO 3900, as the water phase is more entropically favorable for the biomolecules, compared to the polymer-rich phase (Dembczyński et al., 2010). The value obtained for PF over a range of TSP concentration was generally higher than 1, with the highest value of 1.3 achieved at 14 wt/wt % of EOPO concentration though it has a significantly lower yield (72.5 %) compared to that of 20 wt/wt %. Therefore, thesubsequent experiment of thermoseparation-based aqueous two-phase extraction (ATPE) of PHA has been chosen to be performed at 20 wt/wt % of EOPO 3900 concentration with the recovery of 82.2 % and PF of 1.29.

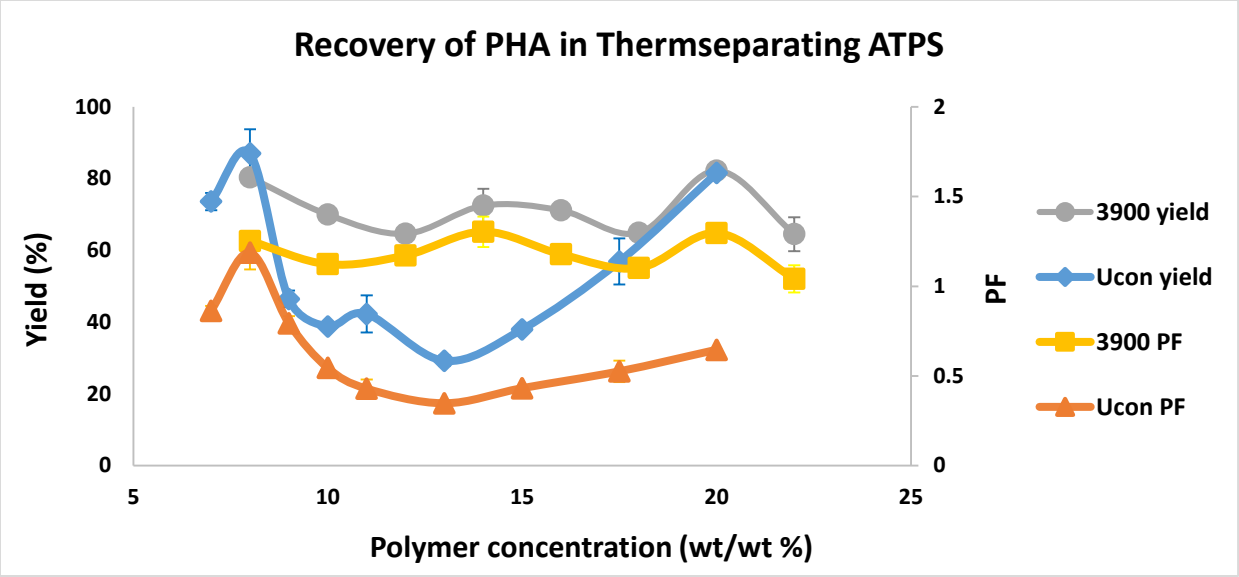


Figure 4.2: Results of PHA partitioning over a range of thermoseparating polymer concentrations

4.3.1.3 Effect of salt addition on PHAs partitioning

Salt addition plays an important role in the partitioning of protein by thermoseparation-based ATPE. Firstly, the addition of cosolutes such as salts which act as "counter-ions" can promote the partition of biomolecules to the targeted phase due to thediverse affinity of different ions for the two phases by which the anions has a stronger effect than cations. According to the Hofmeister series, the more hydrophobic (chaotropic) ions will partition preferably to the more hydrophobic phase. The addition of salts will cause the cation and anion to partition between the two phases. The electrostatic potential difference caused by the uneven partitioning of the ions will generate an electrochemical driving force and influence the partitioning of biomolecules and/or other charged particles in the system (Cunha et al., 1998).

Secondly, the addition of electrolytes such as salts will increase the hydrophobicity difference between the phases by strengthening the hydrophilicity of the water-rich top phase,

that in turn causing a decrease in CP. On the other hand, other cosolutes, such as ionic surfactants have opposite effect on CP with anionic surfactant have a greater impact than cationic surfactant (Cunha et al., 1998). However, literature showed that there is no significant change or little variation on LCST for the addition of phosphate salts (0 to 100 mM) (Rabelo et al., 2004).

Thirdly, there are also some studies focused on the effect of different salts on the recovery of TSPs(Persson et al., 2000b). These studies concluded a significant increase in polymer recovery from 69 % up to 92 % was observed with the addition of 10 mM sulfate, phosphate or chloride salt. It was also pointed out that there is no significant difference in the recovery of the polymer when utilizing different types of salts(Persson et al., 2000b).

In this study, three different salts have been selected to investigate their influence on PHAs partitioning, which are ammonium sulfate ($(NH_4)_2SO_4$), dipotassium phosphate (K_2HPO_4) and sodium chloride (NaCl). The hydrophobicity of the anions studied in this work is ranked in the following order: Cl^-> HPO_4^{2-}> SO_4^{2-}, while the hydrophobicity of the cations is Na^+> K^+> NH_4^+. The addition of salts is in the range of 0 to 100 mM concentration following the literature(Kepka et al., 2004, Berggren et al., 1995, Persson et al., 2000b).Table 4.1 presents the results of salt addition on PHA partitioning. At 10 mM of added salt concentration, every salt gave a higher yield and PF compared to that of control (i.e., without salt addition). However, this phenomenon changed as the salt concentration increased. In the case where the concentration of $(NH_4)_2SO_4$ increased, PHAs partitioned preferably into a TSP-rich bottom phase which may due to a slight fall in pH of the system, causing a decrease in PF and recovery yield. For K_2HPO_4, the yield reached the highest (87.9 %) at 50 mM of salt concentration, while greatest PF (1.26 fold) was obtained at 100 mM of salt concentration. It also can be observed that the whole range of 10 to 100 mM K_2HPO_4 addition gave higher recovery yield than control, which may due to the alkaline condition helps to release PHA granules more completely (Tamer and Moo-Young, 1998). On the other hand, PHAs partitioning showed a

lower yield and PF than control for most NaCl addition concentration except for 10 mM. Nevertheless, the addition of 10 mM NaCl gave both the highest yield (90.9 %) and PF (1.34) among all other salts across a range of concentrations. Thus, 10 mM NaCl was selected in further optimization of PHAs partitioning.

Table 4.1: Effects of salt addition on PHA partitioning

Salt type	Concentration (mM)	PF (fold)	Recovery Yield (%)
$(NH_4)_2SO_4$	10	1.21	90.6
	50	1.20	84.0
	100	1.17	75.9
K_2HPO_4	10	1.23	87.0
	50	1.03	87.9
	100	1.26	82.5
NaCl	10	1.34	90.9
	50	0.82	54.2
	100	0.80	53.5
Control		1.17	80.6

4.3.1.4 Effect of crude load on PHAs partitioning

In the study of PHAs isolation and purification utilizing CPE, PHAs crude feedstock was directly added to the thermoseparating ATPS after cell disruption without extra clarification or filtration steps, thus saving extra unnecessary purification cost. For the purpose of scaling-up, it is crucial to understand the limit of PHAs crude load that the system can process within a satisfying range of purity and yield. Without considering the mass of crude feedstock, the operating mass of the system throughout the study was maintained at 40 g.

Figure 4.3 presents the effect of PHAs crude load on the partitioning behavior over a range of crude load. The PF showed an increasing trend from 15 to 37.5 wt/wt %, and it dropped sharply afterward. It was observed that the optimum crude load for a 40 g system fall in the range of 35 to 40 wt/wt %. The partitioning of overall biomass to the top phase continued to increase as the crude load increased after 40 wt/wt %, however, the PHAs concentration at top phase increased at a comparatively lower rate (data not shown). This might due to three reasons. Firstly, the huge amount of large descending contaminant particles might have created a downward drag force which is stronger than the hydrophilic affinity between PHAs and the water phase. This directs the partitioning of biopolymer molecules towards the bottom phase. Following that, the second possible reason is that the large quantity of PHAs biomolecules may collide and aggregate together during thermoseparation, thus, forming larger and heavier particles which then fall to the bottom phase due to the gravitational force. The last conceivable explanation is that the reduction of free space in the water phase causing it can no longer support the massive amount of bioparticles and contaminants. Hence, the optimized crude load of 37.5 wt/wt % (yield = 92.9 %, PF = 1.39 fold) was chosen for further experimental works.

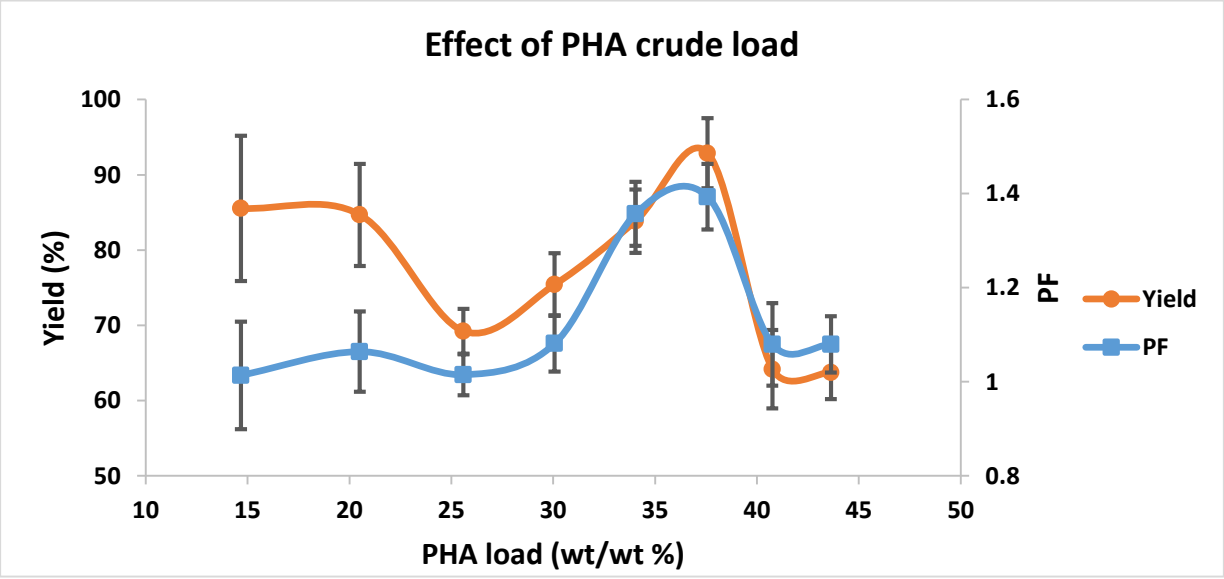


Figure 4.3: Effect of crude load on PHA partitioning

4.3.1.5 Effect of thermoseparating temperature on PHA partitioning

Cloud point (CP) temperature, also known as lowest critical solution temperature (LCST) is the temperature where the TSPs started to be "undissolved" from their aqueous solution. However, thermoseparation-based extraction is generally done at a temperature few degrees above the CP to facilitate phase separation, thus reducing the time required (Berggren et al., 1995). Moreover, the concentrations of the polymer in both phases are strongly influenced by the thermoseparation temperature. The polymer-rich phase becomes more saturated as the thermoseparating temperature increases, which reduces the free space for biomolecule per unit volume. This also causes the bioparticles to have an increase tendency to separate into the water phase. Other than that, the thermoseparating temperature also has a significant influence on bioseparation as high temperature may cause denaturation of protein, thus affecting the quality of bioproducts (Show et al., 2012b).

The system is composed of 20 wt/wt % EOPO 3900 with the addition of 10 mM NaCl. It was found that the biopolymers have partitioned preferably to the water-rich phase at all the investigated temperature. Figure 4.4 illustrates the effect of thermoseparating temperatures on the partitioning of PHA. The highest PF was obtained at 60 °C (1.42 fold) while thermoseparation at 75 °C gave the maximum yield (98.8 %). To avoid denaturation of biomolecules due to high temperature and reduce high heat energy consumption, 60 °C has been chosen as the most suitable thermoseparation temperature with the yield of 94.8 % and PF of 1.42 fold.

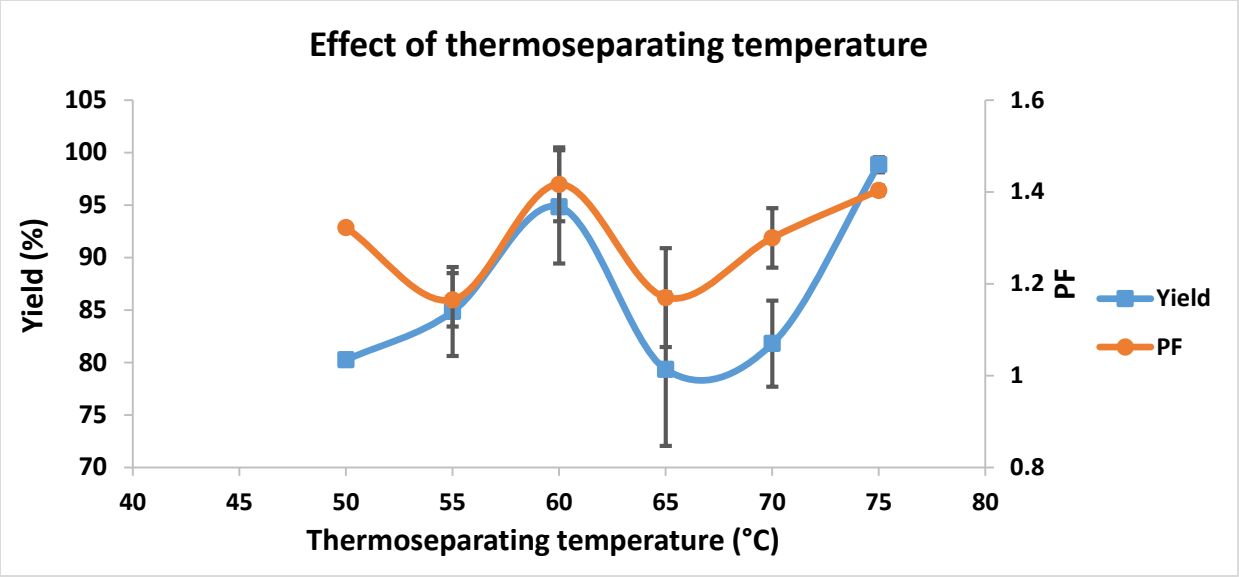


Figure 4.4: Effect of thermoseparating temperature on PHA partitioning

4.3.1.6 Repeated extraction and recycling of thermoseparating polymer in CPE

Repeated extraction has been suggested might be able to enhance biomolecule partitioning and improve the purity of final product. The results of repeated extraction for PHAs are tabulated in Table 4.2. The table clearly shows that there is a trade-off between the recovery yield and PF as yield drop while PF rise following the increasing times of repeated extraction. The fall in yield might be due to loss of PHAs into the bottom phase during repeated extractions and a sharp decline can be observed after second extraction. Therefore, there is a need of certain degree of compromise to obtain desired recovery yield and PF.

Table 4.2. Repeated extraction of PHAs

Extraction	Yield (%)	PF
First	96.7	1.45
Second	89.8	1.53
Third	72.6	1.60

In this thermoseparating-based ATPE, the biomolecules partitioned preferably to the water top phase and the EOPO 3900 can be recycled to form a new stage of ATPE. The recovery and recycling of the EOPO 3900 copolymers are dependent on the geometry of the vessel and heat transfer capacity. The vessel geometry affects the heating rate and sample losses in the interface. A narrow tubing not only promotes the heating rate but also reduces the loss of sample due to the smaller interfacial area (Persson et al., 2000b). Therefore, 50 ml centrifuge tube was used throughout the study for consistency purpose. Moreover, the EOPO 3900-rich bottom phase which contains the contaminants were directly recycled and used in the subsequent stage without going through any extra purification process and additional top-up of fresh polymer. After the polymer-rich bottom phase was collected and combined with fresh water top phase to form new ATPE, a new batch of feedstock was added to the system.

Table 4.3 summarizes the results of recycling and reutilization of EOPO 3900 in PHAs recovery. Based on Table 4.3, it was observed that the purity of PHAs decreased as the EOPO 3900 was being recycled (a reduction rate of approximately 2 to 3% for each cycle). This was due to the fact that the polymer-rich bottom phase had been increasingly saturated with the contaminants as the thermoseparating polymers being recycled and reused. However, in this case, the EOPO 3900 does not undergo further purification process to remove the contaminants and PHAs in the bottom phase. Moreover, "volume-exclusion effect" occurs due to saturation in bottom phase, promotes partitioning of both PHAs and contaminants to the top phase in the subsequent stage of PHAs extraction. It can also be observed that the overall yield does not follow a specific trend. Conclusion can be made that the EOPO can be recycled and reutilized for PHA partitioning up to at least twice with insignificant reduction in the recovery yield and purity of PHA. These results were satisfactory where three repeated extraction and purification of PHA was done while retaining similar partitioning behavior. Based on the experimental data, the PF obtained was predicted to reach a constant at 1.356

after the 5th repeated extraction. Taking in the factor there is only a decrease of 6.7 % in EOPO 3900 concentration after the first extraction, thus theoretically, the EOPO 3900 can be continuous recycled and reused until the concentration of thermoseparating polymer reaches a level too low which it can no longer support a thermoseparation system.

Table 4.3: Recycling and reutilization of EOPO 3900 in PHA partitioning

	Purity (%)	PF	Overall Yield (%)
First extraction	26.9	1.45	96.7
First recycling	26.1	1.41	86.9
Second recycling	25.5	1.38	95.9

4.3.2 Partitioning of PHAs using thermoseparating ATPE

As mentioned in the "Introduction" section, one of the main objectives of this chapter was to study the application of thermoseparating ATPE on PHAs purification utilizing TSP/salt system. Other than phase composition, the partitioning of biomolecules in thermoseparating ATPS depends on characteristics of target products, such as molecular size, hydrophobicity, molecular conformations and electrochemical properties. The partitioning of bioproducts in ATPS cannot easily be predicted due to an insufficient understanding of theexact mechanism behind it (Karkaş and Önal, 2012). Thus, key-influencing parameters such as the type of thermoseparating polymer, the concentration of phase-forming components, pH and effect of centrifugation have to be optimized systematically in order to develop a suitable and effective thermoseparating ATPE for PHAs partitioning.

4.3.2.1 Binodal phase diagram

Partitioning of PHAs was studied in 2 different ATPSs which consisted of two thermoseparating polymers, which are EOPO 3900 and UCON HTF 14 with ammonium sulfate (AS), $(NH_4)_2SO_4$. Due to the unique characteristic of TSP, they were chosen to form ATPS with salts instead of conventional poly(ethylene glycol), PEG. Moreover, a lower amount of copolymer is required to form ATPS as they were comparatively more hydrophobic due to the presence of propylene glycol monomer. Salts with multivalent anions such as SO_4^{2-} and HPO_4^{2-} are highly effective in forming ATPS due to their strong salting-out ability.

Presented in Figure 4.5 and 4.6 respectively, the binodal curves were fitted into the empirical equation suggested by Merchuk and his coworkers (Merchuk et al., 1998), which is $Y = a\exp(b \times X^{0.5} - c \times X^3)$ where X and Y are wt/wt % of salt and TSPs respectively. From the diagrams, it can be clearly observed that a higher concentration of salt and polymer is required to form biphasic system for UCON HTF 14 system compared to that of EOPO 3900 systems as the former is comparatively less hydrophobic though having the same EO:PO ratio.

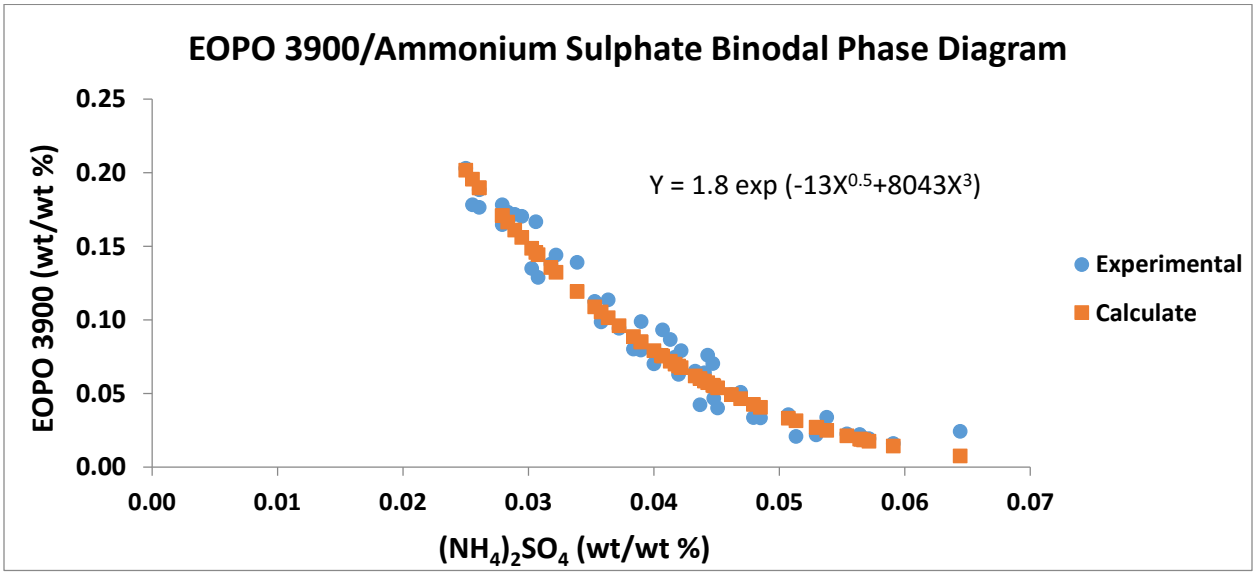


Figure 4.5: Binodal phase diagram of EOPO 3900/Ammonium sulfate

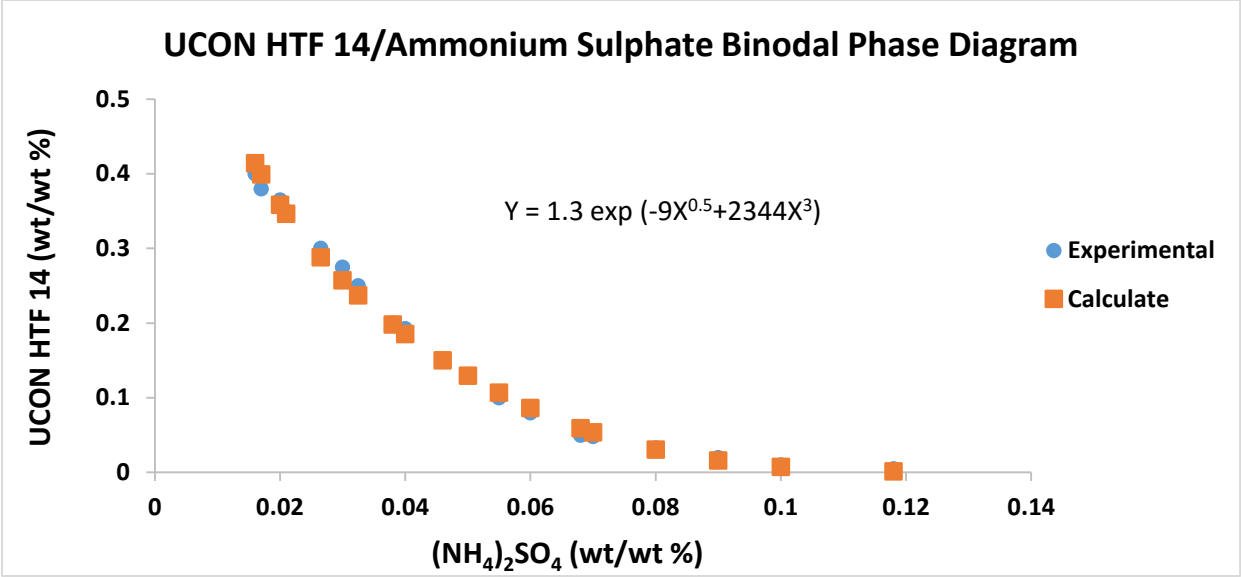


Figure 4.6: Binodal phase diagram of UCON HTF 14/Ammonium sulfate

4.3.2.2 Effect of polymer type and salt concentration

Tie-line length (TLL) of ATPS can be changed by adjusting either phase-forming component concentration or both simultaneously with TLL increase following the phase-forming component concentration. In this part, TLL was first altered by only manipulating the salt concentration at fixed polymer concentration (i.e. 10 wt/wt %). 10 wt/wt % of TSP concentration was chosen to enable the study of a wider range of salt concentration (i.e. from 8 to 22 wt/wt %). Having a higher molecular weight, EOPO 3900 is considerably more hydrophobic as well as require less concentration to form ATPS with ammonium sulfate as shown in Figure 4.5.

Figure 4.7a and Figure 4.7b present the effect of salt concentration on PHAs partitioning for EOPO 3900 and UCON HTF 14 systems respectively. For UCON system, PHAs yield increased from 8 wt/wt % to 14 wt/wt % salt concentration with a maximum of 29 % reached at 14 wt/wt %, then decreased and remained constant afterward. Kpa does not follow a specific trend and has the highest value of 1.63 at 14 wt/wt % of salt concentration. In this

system, purification factor, PF demonstrated a decreasing trend following the increasing salt concentration and has a sudden drop after 14 wt/wt %. This may due to the biopolymer molecules precipitate and accumulate at the interface caused by strong "salting-out" effect as a result of an increase in salt concentration (Jiang et al., 2015). It is also noteworthy that Vr remains constant at a value of approximately 0.25 in the range of 10 to 22 wt/wt % of salt concentration for UCON system.

For EOPO 3900 system, biopolymer yield shared a similar trend as UCON system with a maximum value of 37.9 % obtained at 14 wt/wt % of salt concentration. Similarly, PF decreased with the rise in salt concentration and achieved a fix value of approximately 1.0 after 14 wt/wt % of salt concentration which is caused by the Vr reached constant at a value of approximately 0.22. This can be explained by the increasingly dominating "salting-out" effect of ammonium sulfate which forces more contaminants partitioned to the top copolymer-rich phase (Barbosa et al., 2011). However, the "free-volume" of the top phase reached a minimum and can no longer accommodate more biomolecules after 14 wt/wt % where "volume-exclusion" effect which is stronger than "salting-out" effect started to take place.

The results revealed that the P(3HB) partitioning can be influenced by the types of TSP. Comparing between the two TSPs, a higher Kpa and yield is achieved by EOPO 3900 system over a range of salt concentration which may contributed by the stronger hydrophobic interaction between copolymer-rich phase and PHA biomolecules. EOPO 3900 system generally presented Kpa higher than 1 (range from 1.228 to 2.405) with the highest value (2.405) achieved at 14 wt/wt % of salt concentration, showing that PHAs partitioned preferably into polymer-rich top phase, but this is not the case in UCON system. PHAs partitioning by UCON system mostly gives a Kpa value of unity or less than unity which means that the bioproduct partition equally to both phase or preferably to the bottom phase.

On the other hand, both systems have reached an agreement that the purity of PHAs obtained dropped significantly after 14 wt/wt % of salt concentration which indicates

electrochemical force indeed plays an important role in PHAs partitioning. EOPO 3900 system has a higher purity at the lower range of salt concentration (8 to 14 wt/wt %), but the opposite case happened at the higher range of salt concentration (16 to 22 wt/wt %). Moreover, EOPO 3900 system generally has a much higher PF than UCON system as the latter generally showed a poorer degree of purification. It can be due to the stronger hydrophobicity of the TSP-rich top phase. This is in accordance with the literature as a higher concentration of copolymer is required to form biphasic for UCON HTF 14 and this may cause volume exclusion effect as well as precipitation at the interphase (Ng et al., 2012). These concluded that EOPO 3900 is a more suitable phase-forming TSP for PHAs partitioning and recovery. Therefore, 14 wt/wt % of salt coupled with EOPO 3900 ATPE was selected for further optimization.

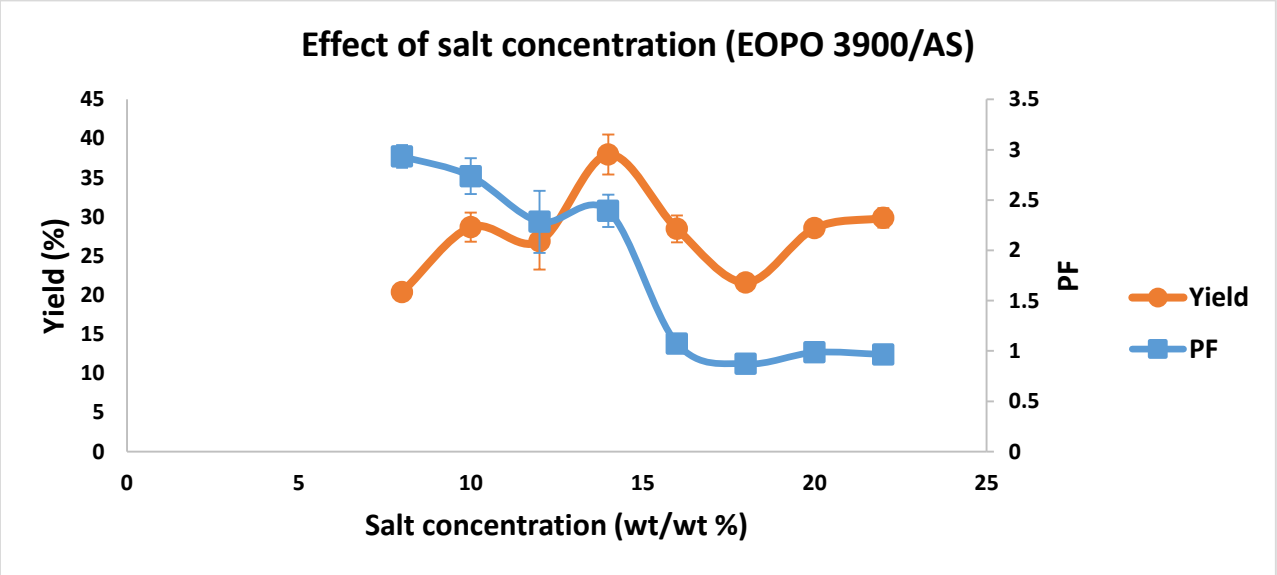


Figure 4.7a. Effect of salt concentration on PHA partitioning (EOPO 3900/AS)

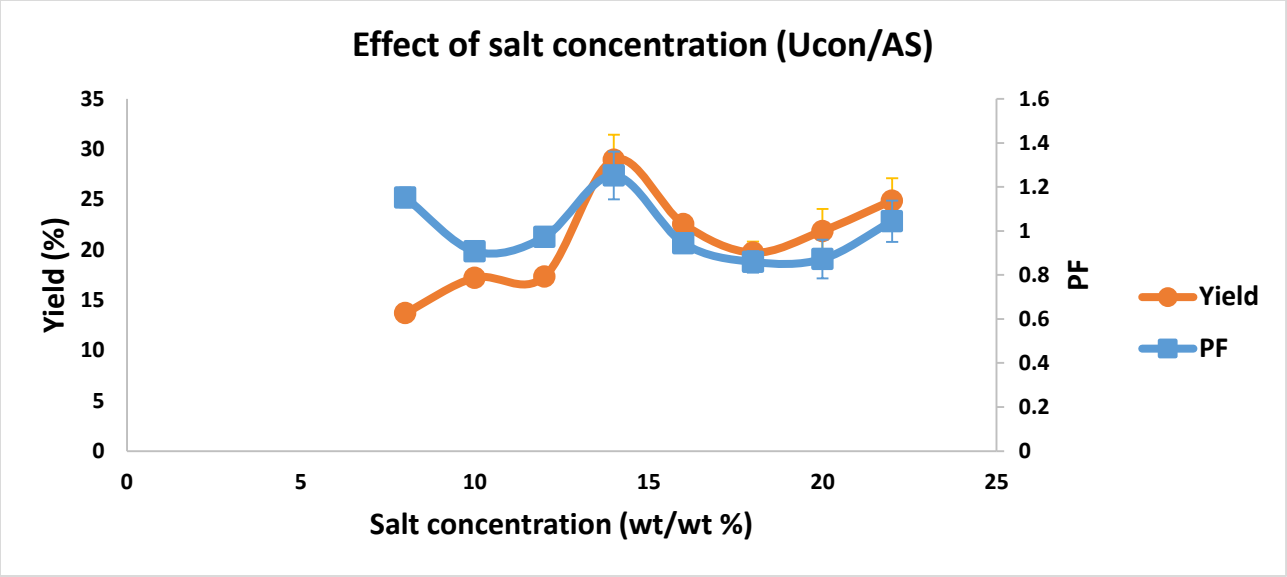


Figure 4.7b. Effect of salt concentration on PHA partitioning (Ucon/AS)

4.3.2.3 Effect of polymer concentration

Following the study on the influence of salt concentration, the effect of TSP concentration was investigated by varying polymer concentration from 4 to 18 wt/wt % at constant 14 wt/wt % salt concentration as illustrated in Figure 4.8. From Figure 4.8, it can be observed that the yield increase following polymer concentration until Vr reaches constant at approximately 0.4 where the biomolecules reached their solubility limits and the "volume-exclusion" effect started to take place. In a system with high concentration of TSPs, the polymer's entropic effect is dominating (Kepka et al., 2004), thus the biomoleculess partition favorably to the salt-rich bottom phase. Therefore, a maximum yield of 40.3 % was achieved at 14 wt/wt % polymer concentration. On the other hand, PF presented a similar trend as yield with highest PF obtained at 14 wt/wt % (2.09 fold). Therefore, ATPS with 14 wt/wt % of EOPO 3900 and 14 wt/wt % of ammonium sulfate was chosen for further optimization.

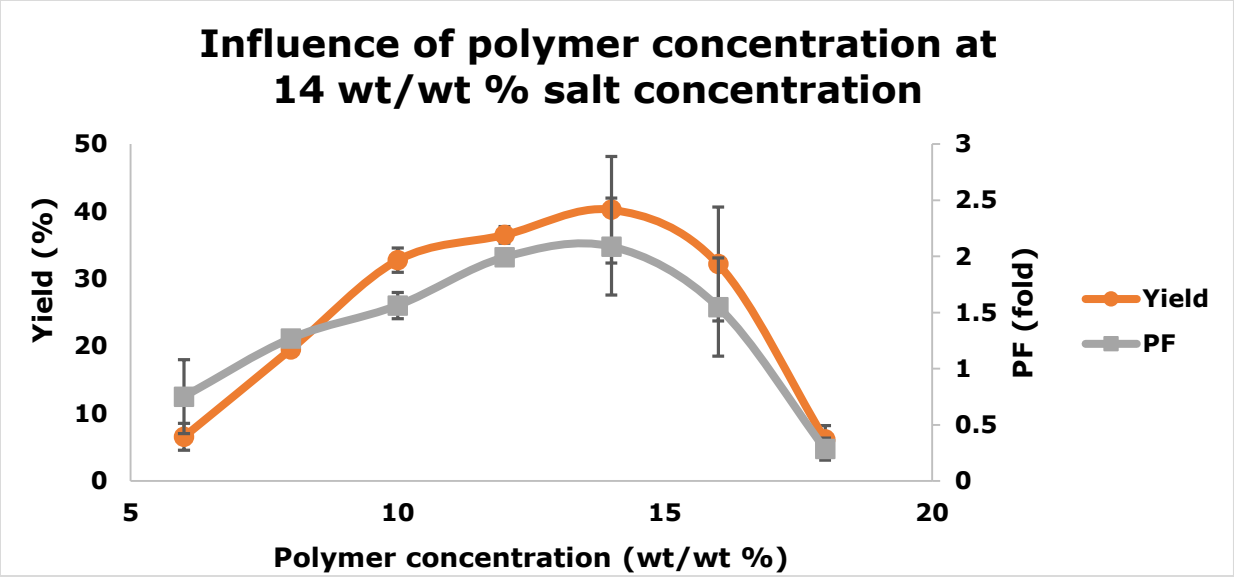


Figure 4.8. Influence of polymer concentration on PHAs partitioning for UCON/AS

4.3.2.4 Effect of system pH on partitioning of PHAs

Serving an important role in the partitioning of PHAs by thermoseparating ATPE, system pH in this study was adjusted to the range of pH 4 to 8.8 by the addition of suitable amounts of H_2SO_4 and NH_3 (aq) solution. Besides than altering bioproduct partitioning, system pH also modifies the phase-forming polymer concentration in both phases (Pandey and Banik, 2011) as polymer either gains or loses charge (Show et al., 2012b). For conventional PEG-based ATPE, literature had reported that there was zero recovery of P(3HB-co-3HV) in top phase for pH less than 7, while better separation and recovery were achieved at pH 8 and above (Divyashree et al., 2009). However, this was not the case in thermoseparating-based ATPS as presented in Figure 4.9. From pH 4 to 8, PHAs partitioning showed a steady yield of 60 to 63.5 % with highest value of 63.5 % achieved at pH 6 (except for the sudden drop of yield at pH 8.8 with value 46.4 %), demonstrating the robustness of thermoseparating-based ATPE in PHAs purification and recovery. On the other hand, partitioning of PHAs presented a slight increase in PF following the pH, with the maximum obtained at pH 8 with the value of

1.35. Therefore, it can be concluded that the optimum separation and extraction of PHAs can be done around pH 6 to 8 with a minimum trade-off between PF and yield (less than 6.3 % for yield and less than 4 % for PF). Also, thermoseparating-based ATPE at pH 6 with the highest Kpa of 3.67 and yield of 63.5 % was selected for further study.

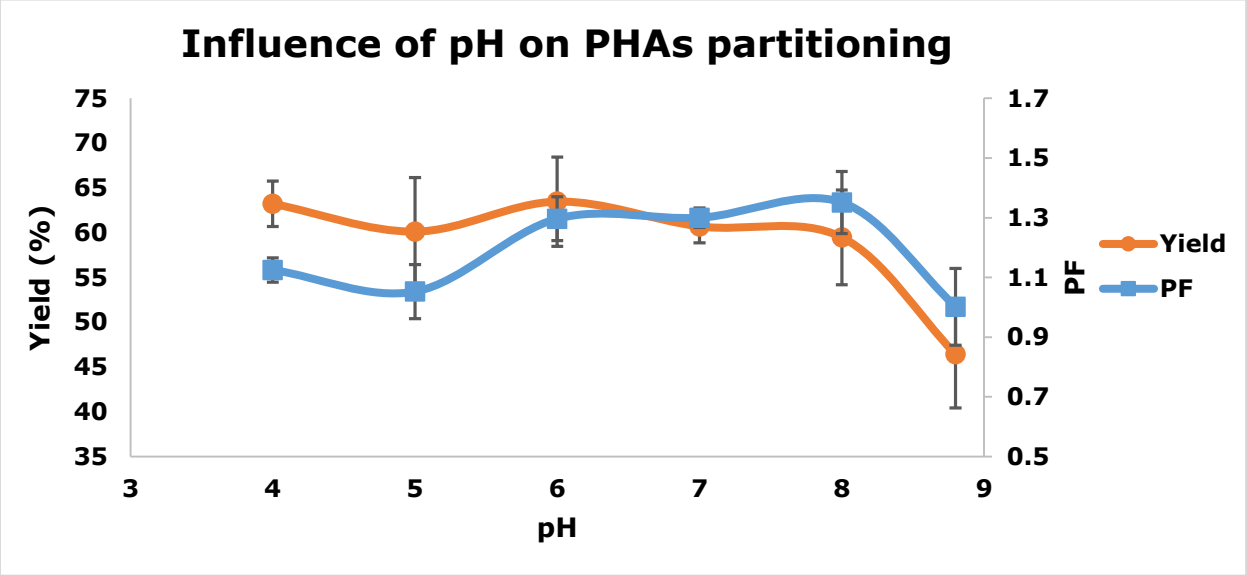


Figure 4.9. Influence of pH on PHAs partitioning

4.3.2.5 Effect of centrifugation speed

As reported in the literature, centrifugation might be able to reduce the time needed for phase separation (Li et al., 2015) and enhance the partitioning of desired product (Tou et al., 2014). Following that, this study was conducted to investigate the effectiveness of centrifugation in the purification and recovery of biopolymers by thermoseparating ATPS. Table 4.5 presents the influence of centrifugal speed on PHAs partitioning where the control is the system which was left to stand still until complete phase separation. Come to an agreement with the literature(Dembczyński et al., 2013), thermoseparating ATPS has a relatively short phase separation time (less than half an hour). From the table, it can be clearly observed that the control system has a much higher purity (69.6 %), thus a higher

value of PF (1.88 fold) was obtained compared to those that have been centrifuged. However, it has a lower yield (31.5 %) than the system with centrifugation carried out at 150 g which has a 41.2 % yield.

The mechanism behind the partitioning of PHAs can be understood through the Lamm equation (Behlke and Ristau, 1998) which is given by $\frac{\partial C}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left[rD\frac{\partial C}{\partial r} - s\omega^2 rC\right]$ where C, t, r, ω are concentration, time, radial position and angular velocity respectively, and s and D are the sedimentation and diffusion coefficients respectively. By assuming the initial condition where $C = C_0$ and $r_0 < r < r_b$ where r_0 and r_b are the meniscus and bottom position respectively, the equation is Laplace transformed and solved to give

$$C = \frac{C_0}{2}e^{-\tau}\left\{2 - erfc\left(\frac{(1-z)+\alpha\tau}{2\sqrt{ae\tau}}\right) - \frac{\alpha}{e}exp\left(\frac{-\alpha(1-z)}{ae}\right)erfc\left(\frac{(1-z)-\alpha\tau}{2\sqrt{ae\tau}}\right)\right.$$

$$\left. + \frac{\gamma}{e}exp\left(\frac{(\gamma^2-\alpha^2)\tau - 2(\alpha+\gamma)(1-z)}{4ae}\right) \times erfc\left(\frac{(1-z)-\gamma\tau}{2\sqrt{ae\tau}}\right)\right\}$$

where

$\alpha = \frac{z(x_b-1)+1}{(x_b-1)} - \frac{\varepsilon}{x_b-1}$, $= \frac{z(x_b-1)+1}{(x_b-1)} + \frac{\varepsilon}{x_b-1}$, $x_b = \left(\frac{r_b}{r_0}\right)^2$, $z = \frac{x-1}{x_b-1}$ and $0 < z < 1$.

If $x_b \to \infty$, we can see that the first and the third terms disappear and we have

$$C \sim \frac{1}{\omega^2}e^{-\frac{1}{\omega^4}}. \qquad \text{Eqn 4a}$$

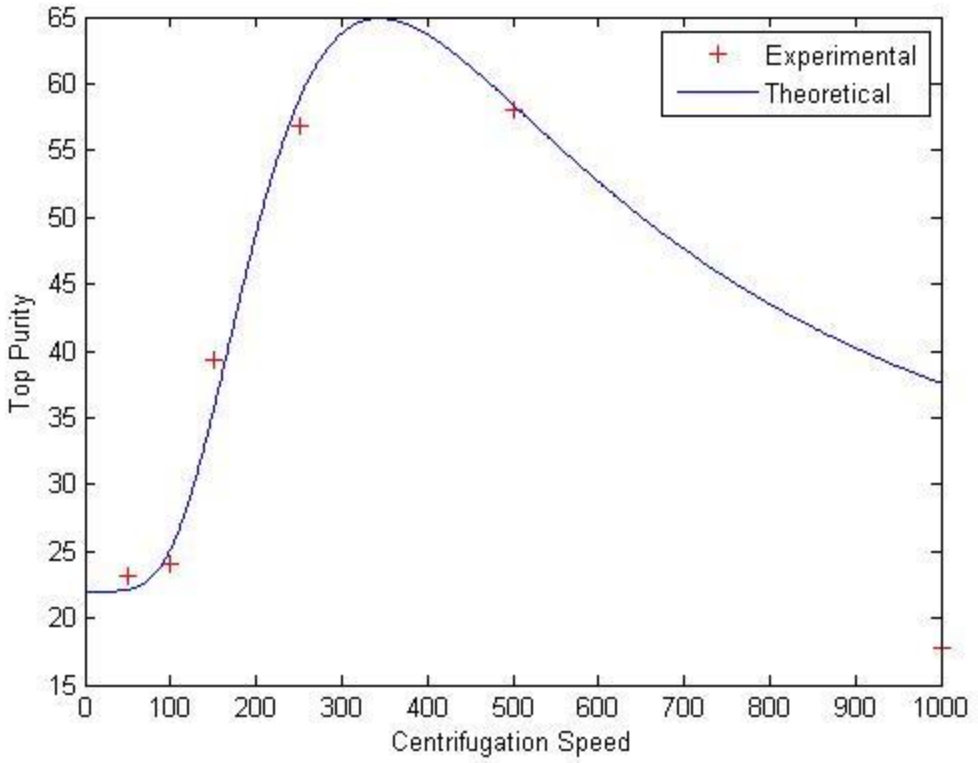
Using equation 4a, the theoretical value of purity in top phase can be predicted and calculated. Figure 4.10 shows the comparison between experimental data and the theoretical prediction. The experimental data is in agreement with the theoretical prediction. It can be clearly seen that the maximum PHAs purity (approximately 65 %) can be obtained at around 350 – 400 g centrifugal force. Therefore, it can be concluded that thermoseparating ATPE does not require additional centrifugation step to facilitate phase separation and promote biomolecule partitioning, thus, reducing overall purification cost.

1598

1599 Table 4.5：influence of centrifugal speed on PHAs partitioning

Centrifugal force (g)	Yield (%)	Purity (%)	PF
50	28.7	23.2	0.63
100	26.9	24.0	0.65
150	41.2	39.3	1.06
250	27.7	56.9	1.54
500	26.7	58.1	1.57
1000	15.2	17.8	0.48
control	31.5	69.6	1.88

1600



1601

1602 Figure 4.10. Influence of centrifugation speed on PHAs partitioning

4.3.2.6 Recycling and reutilization of EOPO copolymer in PHAs partitioning

Other than having the advantage of being more hydrophobic than conventional PEGs, TSPs such as EOPO copolymers have the distinct characteristic of reduced solubility in its aqueous solution as temperature increase which leads to phase separation when exceed certain temperature limits. This enables efficient recovery of desired product exclusively in the waterphase while the copolymers can be recycled and reutilized in a new cycle of bioproduct extraction which is greener and more cost-competitive (Persson et al., 2000b).

Following the in depth study done by Persson and coworkers(Persson et al., 2000b), temperature-induced phase separation was performed at 65 °C for 15 minutes as complete phase separation can only be achieved at temperature few degree Celsius above LCST (around 50 to 55°C) and 15 minutes is the threshold where there is no longer increment for copolymer recovery. The presence of sulfate salts in the top phase enhanced the recovery of the copolymer as literature reported there is an increase from 69 % to 95 % of polymer recovery with the addition of only100 mM sulfate salts (Persson et al., 2000b). Other than that, the EOPO copolymers were recycled and reutilized in a new stage of extraction without the addition of fresh bottom phase. Observation indicated that the Vr reduced after each cycle, due to a slight loss of polymer during recovery, leading to decreasing top phase volume.

The recycling and reutilization of EOPO copolymer in 3 cycles are presented in Table 4.6. In first extraction stage, PHAs purity of 60 % and yield of 72% was achieved, following with a slight fall in both PHAs purity and yield for the second stage of extraction. However, the yield dropped significantly after the third cycle, due to growing amounts of impurities in bottom phase and weaker "salting-out" ability of ammonium sulfate after every cycle. Therefore, it is recommended that the EOPO copolymers can be recycled and reused for two times in PHAs partitioning, in congruent with many bioproducts as reported in the literature,

such as cyclodextrin glycosyltransferase (Ng et al., 2012), ciprofloxacin (Chen et al., 2014) and others.

Table 4.6: Recycling and reutilization of EOPO copolymer

Cycles	Purity (%)	PF	Yield (%)
1	59.6	1.61	72.2
2	54.4	1.47	63.1
3	47.0	1.27	59.7

4.4. CONCLUDING REMARK

Aiming to develop a cost-effective and environmental-friendly extraction strategy for isolation and recovery of PHAs from *C. necator* H16, the partitioning behavior of biopolymers was investigated utilizing the thermoseparation-based ATPE technique called cloud point extraction (CPE) strategy. Cloud-point diagram has shown that both TSPs, which are EOPO 3900 and UCON HTF 14 have a similar cloud point beyond 7.5 wt/wt % concentration. Comparing between two TSPs, EOPO 3900 was preferred as UCON-based system did not show a promising result.

The purification of PHAs utilizing CPE was satisfactorily achieved in the system of 20 wt/wt % EOPO 3900 and 10 mM NaCl addition at thermoseparating temperature of 60 °C with a limit of 37.5 wt/wt % of crude feedstock. With a recovery yield of 96.7 % and PF of 1.45 fold, PHAs were partitioned preferably to the water-rich top phase, which makes the subsequent downstream processing simpler and cheaper. The recycling and reutilization of EOPO 3900 can be done for at least 2 times without significant loss of yield and purity. The results proved that CPE is a powerful and potential technique for PHAs purification and

recovery as the technique showed a much higher PHAs recovery yield (approximately 2 times) in comparison with literature.

To further improve the final quality of PHAs recovered, thermoseparating polymers have been coupled with ammonium sulfate to isolate and purify biopolymers from a crude feedstock of *C. necator*. Thermoseparating-based ATPE of PHAs could be effectively done under the condition of 14 wt/wt % of EOPO 3900 concentration, 14 wt/wt % of ammonium sulfate concentration and pH 6 without the needs of extra centrifugation steps. Yield and purity up to 72 % and 60 % can be achieved with the copolymers can be recycled and reused for two times.

Chapter 5

THERMOSEPARATING AQUEOUS TWO-PHASE EXTRACTION OF POLYHYDROXYALKANOATES (PHAS) FROM *CUPRIAVIDUS NECATOR* FEEDSTOCK USING STATISTICAL DESIGN OF EXPERIMENT (DOE)

5.1 INTRODUCTION

Despite numerous advantages offered, the main downside of bioproduct purification using ATPE is the complex mechanism behind the partition of target product which influenced by a long range (hydrophobic and electrostatic) and short range (van der Waals) molecular interaction between the bioparticle and the system. In addition, there is a shortage of suitable mathematical laws and models to explain it (Dembczyński et al., 2013). As there are many parameters play in parts for bioproduct partitioning by ATPE, this resulting in a highly complex task for optimization. The conventional method of change one-factor-at-a-time (OFAT) (also known as single factor optimization) which optimize one parameter at a time while keeping the other variables constant using a trial and error approach (Dembczyński et al., 2013)as in the previous chapter requires a huge amount of time and labor consumption with a large number of experiments needed to be conducted (Kammoun et al., 2009). Not only that, it also does not guarantee the identification of the global optimum and cannot give insights on interactions between the independent parameters (Wiendahl et al., 2012, Aydog˘an et al., 2010).

In order to overcome this, statistical design of experiment (DOE) was widely utilized for optimization, modeling and identification of main influencing parameters as well as the significant interactions between them by utilizing minimum amount of experiments (Santos et al., 2011). Examples of literature that employed DOE strategy for purification of bioproducts are such as plasmid DNA (Wiendahl et al., 2012), human immunoglobulin G (Ferreira et al., 2008), lysozyme (Lu et al., 2013, Dembczyński et al., 2013), 1,3-propandiol (Aydog˘an et al., 2010), fibrinolytic enzyme (Ashipala and He, 2008), human antibodies (Azevedo et al., 2007), lectin (Porto et al., 2011), β-glucuronidase (Ross and Zhang, 2010), collagenase (Lima et al., 2013), ciprofloxacin (Mokhtarani et al., 2008), human antibodies (Rosa et al., 2007), amylase (Kammoun et al., 2009) and other bioproducts.

Nevertheless, thorough literature review had shown that the application of DOE on PHAs purification has yet to be described. Henceforth, in this chapter, the experimental design methodology have been conducted on the purification and recovery of crude PHAs extract from *Cupriavidus necator,* aiming to identify the optimal ATPE conditions for primary purification of thetarget product. In order to screen the important ATPE-influencing parameters, a two-level full factorial design was utilized to study the influence of and interaction between the parameters, such as the concentration of potassium phosphate, $EO_{50}PO_{50}$, NaCl addition as well as pH on partition behavior of PHAs. Followed by the determination of global optimum region using the method of the path of steep ascent, Box-Wilson central composite design (CCD) was utilized to achieve a global optimum of PHAs purification and recovery.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design and statistical analysis

A two-level full factorial design (2^4) was carried out to investigate the effects and interactions among important parameters in thermoseparating-based ATPE of PHAs. Four independent parameters, including potassium phosphate concentration (X_1), EOPO concentration (X_2), pH (X_3) and addition of sodium chloride (X_4) were taken into account and studied at two widely spaced levels. The low (-) and high (+) levels of the factors were 8 wt/wt % and 15 wt/wt % for potassium phosphate concentration, 10 wt/wt % and 18 wt/wt % for EOPO concentration, 8 and 10 for system pH and 0 mM and 100 mM for sodium chloride addition. The factor combination is presented in Table 5.1. The reason behind the choice of the levels for EOPO concentration and phosphate concentration is to make sure that the formation of two-phase has a high enough volume ratio so that the "volume-exclusion" effect does not happen. Volume-exclusion occurs when the free space available for target products in either one of the phase is reduced, causing partitioning to the opposite phase (de Medeiros

e Silva et al., 2013). System pH levels were chosen based on literature review of PHAs partitioning (Divyashree et al., 2009). The levels of sodium chloride addition were selected following the results of the previous chapter. The partition coefficient (Kpa), recovery yield and purification factor (PF) were selected as response variables.

After the screening of important parameters using full factorial design, the global optimum region is determined using the method of the path of steepest ascent. Following that, the influence of key parameters on partitioning behavior of PHAs was evaluated utilizing a Box-Wilson central composite design (CCD). A total of 13 experiments were conducted, with 5 repetitions of a central point for the evaluation of the experimental error variance. The experiments were conducted in a random manner, obeying the condition for observations to be distributed randomly and independently which helps, as well as, to eliminate the influence of unknown factors. The "Design Expert" software (Version 7.0.0, Stat-Ease) was used for regression and graphical analysis of the data obtained. Three replicates were conducted for each experimental run.

5.2.2 Partitioning of PHAs in thermoseparating ATPE

A 40 g two-phase system was prepared by mixing the phase-forming components which are EOPO and salt (in wt/wt %) with the crude feedstock. The mixture was then allowed to settle for phase separation. After the stages of equilibration and phase separation, the top phase was carefully sampled using a pipette, while the bottom phase was then sampled through the interface. The samples from both phases and disrupted biomass are centrifuged and washed with deionized water for three times before left in the oven for overnight at 70 °C to be dried. The dried samples from both phases and disrupted biomass were analyzed for PHAs content using gas chromatography (GC).

5.3. RESULTS AND DISCUSSION

5.3.1 Screening of important ATPE-influencing parameters

In ATPE, biomolecules have complex partitioning behavior which influenced by charge, molecular size, electrochemical properties and hydrophobicity of the proteins. The partitioning of biomolecules can be manipulated by adjusting factors like theconcentration of phase-forming components, pH and salt additions. A two-level full factorial design (2^4) were conducted to investigate the influence and interaction among 4 ATPE-influencing variables, which are potassium phosphate concentration (X_1), EOPO concentration (X_2), pH (X_3) and NaCl addition (X_4) in PHAs partitioning by thermoseparating-based ATPE.

The full factorial designs and the responses are presented in Table 5.1. It can be observed that there is a wide variation of Kpa (0.402 – 16.547) and recovery yield (16.8 – 93.9 %) which is due to the intended variation in the factor combinations and this revealed the significance of optimization in achieving better recovery yield and purity (Kammoun et al., 2009, Ross and Zhang, 2010). From the results in Table 5.1, it can be seen that run 6 achieved the highest Kpa (16.5) and yield (93.9 %), while run 10 obtained the highest PF with the value of 1.54. Utilizing Design Expert software, the analysis of variance (ANOVA) was performed to verify the validity of the models, evaluate the statistical significance of all factors and determine the influence of these factors on the response variables. These models consisted of four main effects, six two-parameter interactions, and four three-parameter interactions, with the last one that is a four-parameter interaction which was given the assumption of being negligible due to hierarchical reasons (Lima et al., 2013).

Table 5.1: Experimental design and results of response variables where X_1 = potassium phosphate concentration, X_2 = EOPO concentration, X_3 = pH and X_4 = NaCl addition

Run	X_1 (wt/wt %)	X_2 (wt/wt %)	X_3	X_4 (mM)	Kpa	Yield (%)	PF (fold)
1	8	10	10	0	0.814	24.6	0.93
2	8	10	8	0	0.646	21.4	0.93
3	15	10	10	0	3.371	57.4	1.35
4	8	18	8	0	0.431	25.5	1.07
5	8	18	8	100	0.402	24.8	1.04
6	15	18	10	0	16.547	93.9	1.46
7	15	18	10	100	5.585	85.5	1.48
8	15	10	8	0	1.554	38.3	0.95
9	8	10	8	100	0.525	16.8	0.67
10	15	18	8	100	12.291	92.5	1.54
11	15	18	8	0	8.047	86.8	1.52
12	15	10	8	100	1.314	32.9	1.22
13	8	18	10	0	0.512	30.1	0.96
14	15	10	10	100	2.322	45.5	1.04
15	8	18	10	100	0.369	22.7	1.04
16	8	10	10	100	0.817	24.6	1.21

1769

1770

5.3.1.1 Effect on the "Yield"

From a practical point of view, an efficient purification and extraction of ATPE requires the maximum recovery of target product with the purity as high as possible. From Table 5.1, the results demonstrated that the maximum yield was achieved in the run 6 with the value of 93.9 %, while the minimum was obtained in the run 9 with the value of 16.8 %. The full statistical results for the response variable "Yield" are shown in Table 5.2. On the basis of the result, the f-value of 1007.69 with a low probability value (Prob > F = 0.0247) indicates that the overall regression model was significant with 95 % of confidence. The value of the coefficient of determination, R^2 can be utilized to assess the ratio of total variation ascribed to each fit. With value always between 0 and 1, R^2 larger than 0.75 shows a good fit of the model to the response variable (Kammoun et al., 2009), while a value larger than 0.9 are greatly satisfying in the DOE for bioprocess (Santos et al., 2011). For recovery yield, the high value of R^2, which is 0.9999 demonstrates a good response between the model and the experimental results. This also indicates that the interrelationship among the independent variable can be satisfactorily represented by the model with only less than 0.01 % of total variations were not able to be explained by the model. The adjusted coefficient of determination, R^2_{adj} can be used to measure the accuracy of a model for the response variable (Dembczyński et al., 2013). The R^2_{adj} of 0.999 was in a good agreement with the predicted R^2, R^2_{pred} of 0.982, which shows that the predicted values are compatible with the experimental results.

Values of "Prob > F" less than 0.05 reveal that the model terms are statistically significant model terms, while values larger than 0.10 show that those are insignificant model terms (Aydog˘an et al., 2010). For recovery yield, the regression analysis of the experimental design has indicated that the linear model terms (X_1 and X_2) and interactive model term (X_1X_2) were the significant model terms. Nevertheless, the linear model terms (X_3 and X_4) and interactive model terms (X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, $X_1X_2X_3$, $X_1X_2X_4$ and $X_2X_3X_4$) were not

significant at 5 % level. By discarding and pooling all statistically insignificant model terms (Prob > F > 0.05) into the error term and using only significant model terms, the new reduced model was attained for response variable "Yield". Using ANOVA, the statistical analysis demonstrated that the reduced model was significant at a confidence level of 95 % with the p-value much lower than 0.05 (Prob > F < 0.0001). By taking into account the significant linear model terms and interactions, the regression analysis of Yield data provided the following first-order model:

$$Y = 45.21 + 21.39\ X_1 + 12.52\ X_2 + 10.56\ X_1X_2$$

Generally, the contribution of the model terms to the response variables can be evaluated utilizing the degree of the corresponding coefficients of the linear regression equations (Lu et al., 2013). From the regression equation, it can be observed that potassium phosphate concentration (X_1) had the strongest positive effect on recovery yield. As shown in the experimental design results, the recovery yield is generally higher at high (+) level of X_1 where the yield can achieve as high as 93.9 % for run 6. At system of higher phosphate concentrations, the strengthened "salting-out" effect reduces the solubility of PHA in the salt-rich bottom phase, by promoting aggregation and hydrophobic interaction. This, in turn, directs PHA partition to the polymer-rich top phase which has lower salt concentration, facilitating the extraction of PHAs to the EOPO-rich phase (Porto et al., 2011). This is in accordance with other works in thepartitioning of others biomolecules by ATPE, such as recovery of papain (Jiang et al., 2015), lectin from *Carnavaliagrandiflora* seed (Porto et al., 2011) and collagenase from *Penicillium aurantiogriseum*(Lima et al., 2013) as well. More importantly, it is worth mentioning that the highest yield obtained in thecurrent study is significantly higher than that of literature which utilized PEG/phosphate system (40 to 50 %) (Divyashree et al., 2009).

In addition to X_1, the regression equation also suggested that EOPO concentration (X_2) is a significant positive parameter for recovery yield as well. There are two main forces

dominating in the polymer-rich phase, which is "volume-exclusion" effect and hydrophobic interaction. As the "volume-exclusion" effect is no longer in the picture as mentioned above, the increasingly stronger hydrophobic interaction between polymer-rich phase and PHAs molecules due to increasing concentration of thermoseparating polymers causes PHAs partition preferably to top phase (Lu et al., 2013). Several studies on ATPE also showed the similar results, such as lysozyme (Dembczyński et al., 2013, Dembczyński et al., 2010), lectin (Porto et al., 2011) and papain (Jiang et al., 2015). Another essential point is the recovery yield was also positively affected by the synergistic positive interaction between phosphate and EOPO concentration (X_1X_2). The simultaneous rise in the level of both parameters has a stronger impact on the increment of recovery yield than the expected add up of those of the individual parameter. The combined effect can be observed in run 6, 7, 10 and 11 where yield can achieve a high value of at least 85 % when both phosphate and EOPO concentration were at high (+) levels.

Supported by the literature (Divyashree et al., 2009), the recovery yield remains constant in the selected range for pH, thus, it is not a significant model term with 95 % of confidence. Though, with Prob > F of 0.0519, pH still bring positive impacts on recovery yield, agreeing with the results of Table 5.1 (with the exception of run 5 and 15 as well as run 7 and 10). In this respect, it should not forget that system pH serves an important role in thermoseparating ATPE as it influence partitioning of the bioproduct by modifying the solute charge (Lima et al., 2013). Therefore, PHA partitioning is preferable to be performed at a more basic pH (Divyashree et al., 2009), still, it is worth mentioning that extreme pH will cause degradation to PHA granule morphology.

Because of the diverse affinity of ions for the different phases where anions give a stronger effect compared to cations, the addition of co-solutes like salts into two-phase system serve as "counter-ions" which promote the partition of biomolecules to the desired phase (Berggren et al., 1995). The reason of salt addition parameter is not significant at 5 %

level might be due to the high content of multiple salts in the PHAs crude extract used and thus, the NaCl addition does not have a dominating effect (Kepka et al., 2004). Nevertheless, although very slight, salt addition with Prob > F of 0.0717 gives a negative influence on recovery yield, as illustrated in the result of Table 5.1 (except for run 10). Previous ATPE works on clavulanic acid from thefermentation broth of *Streptomyces clavuligerus*(Santos et al., 2011) and bacteriocin-like inhibitory substance from *Pediococcus acidilactici* Kp10 (Abbasiliasi et al., 2014) have also illustrated the similar results. Thus, according to the full factorial design, theaddition of NaCl as co-solute should be avoided for maximum recovery of PHAs. To sum up, a high yield of PHAs can be obtained at ahigh concentration of EOPO 3900 and phosphate salt as well as thecondition of basic pH without any addition of NaCl.

Table 5.2: Full statistical result of ANOVA for response variable "Yield" where X_1 = potassium phosphate concentration, X_2 = EOPO concentration, X_3 = pH and X_4 = NaCl addition

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	12070.8	14	862.2	1007.687	0.0247
X_1*	7323.1	1	7323.1	8558.750	0.0069
X_2*	2507.5	1	2507.5	2930.613	0.0118
X_3	128.3	1	128.3	149.897	0.0519
X_4	66.8	1	66.8	78.107	0.0717
X_1X_2*	1783.0	1	1783.0	2083.799	0.0139
X_1X_3	20.9	1	20.9	24.462	0.1270
X_1X_4	3.3	1	3.3	3.893	0.2986
X_2X_3	100.5	1	100.5	117.459	0.0586
X_2X_4	7.7	1	7.7	9.000	0.2048
X_3X_4	32.2	1	32.2	37.640	0.1029
$X_1X_2X_3$	33.4	1	33.4	38.978	0.1011
$X_1X_2X_4$	20.5	1	20.5	23.931	0.1284
$X_1X_3X_4$	21.4	1	21.4	25.000	0.1257
$X_2X_3X_4$	22.3	1	22.3	26.093	0.1231
Residual	0.9	1	0.9		
Cor Total	12071.7	15			
R^2	0.9999				
R^2_{adj}	0.999				
R^2_{pred}	0.982				

5.3.1.2 Effect on the "Partition Coefficient"

Generally employed to assess the effectiveness of biomolecule separation by ATPE, the extreme value of partition coefficient (Kpa) signifies the effective extraction of target biomolecules from the systems, while the value close to unity (1) indicates the almost equal partition between both phases which is undesirable in ATPE. For thermoseparating ATPE, the partitioning of target products to polymer-rich top phase (Kpa> 1) is highly desirable due to theexclusive recovery of bioparticles in the water-rich top phase during the secondary ATPE. As shown in Table 5.1, the maximum Kpa (16.547) was achieved in run number 6, while run number 15 gave the minimum Kpa (0.369). The highest partition coefficient achieved in this study is comparable to that of literature which utilizes PEG/phosphate system (Kpa = 4 to 15) [8]. The statistical results for reduced model of response variable "Kpa" are presented in Table 5.3. The statistical analysis of Kpa indicated that some parameters were statistically significant with 95 % of confidence. Three out of the four linear model terms, which are X_1, X_3 and X_4 have asignificant influence on Kpa. For this response variable, EOPO concentration only gives a slight but positive effect as the intended selection of X_2 levels to prevent the "volume-exclusion" effect. Not only that, some of the interactive model terms (X_1X_2, X_2X_3 and $X_2X_3X_4$) were significant at 95 % of confidence level as well. The reduced model can be described by the following regression equation:

$$1/Kpa^{0.5} = 0.95 - 0.43\ X_1 - 0.057\ X_3 + 0.046\ X_4 - 0.18\ X_1X_2 + 0.054\ X_2X_3 + 0.033\ X_2X_3X_4$$

It is worth mentioning that the empirical relationship between the Kpa and the test variable is better characterized using an inverse square root function instead of normal linear function due to the significant deviation from normality demonstrated.

The f-value of 221.5 and p-value at a level below 0.0001 demonstrated a very high significance for the regression model and confirms the adequacy of the reduced model. With the high value of R^2 (0.997), it shows that the real relationship between the response variable

and independent parameters is adequately represented by the model. This indicates that 99.7 % of the variability in response could be explained by the model. The close values of R^2_{adj} and R^2_{pred} which are 0.992 and 0.979 respectively demonstrate a good degree of correlation between the theoretical values predicted by the reduced model equation and experimental responses.

Similar to the response variable "yield", the most significant and positive influence on the response variable "Kpa" at a confidence level of 95 % was that exerted by the potassium phosphate concentration (X_1). As shown in Table 5.1, only Kpa for high level (i.e. 15 wt/wt %) of X_1gives value larger than 1 (ranging from lowest of 1.3 to highest of 16.5). The same result is also reported by Dong and his coworkers in extraction of phenylethanoid glycosides from *Cistanche deserticola* stems as the partition coefficient of echinoside increased following the rising sulfate concentration (Dong et al., 2015). The same applies to the partitioning of collagenase (Lima et al., 2013), lysozyme (Dembczyński et al., 2013), and lectin (Porto et al., 2011). Also, a positive interaction effect between X_1 and X_2 was observed, revealing a synergism between the two variables. Therefore, higher Kpa will be achieved with high level of both potassium phosphate and EOPO concentration, which especially can be seen in run 6 and 10 (with Kpa higher than 12).

For salt addition, it is in agreement with "Yield" response that it should be completely avoided to achieve high Kpa. Agreeing with Divyashree and his co-workers which reported that significant increase in Kpa can be observed after pH 8 (Divyashree et al., 2009), the results of Table 5.1 demonstrated that high Kpa can be obtained at a higher pH value. This is also observed in partitioning of fibrinolytic proteases from *Streptomyces* sp. DPUA1576 in PEG/phosphate system (de Medeiros e Silva et al., 2013). However, the simultaneous increase in the level of EOPO concentration and pH lowers the value of Kpa unless there is dominating positive influence from X_1 as in run 6.

For this response variable, there were some significant two- and three- parameters interactions involving every parameter which can be observed. The overall influence of a given parameter on this response variable is affected by the levels of the other parameters. Thus, this makes the estimation of these effects on Kpa very complicated due to the strong influence of one of the parameters in the interaction to the others. Still, it can be concluded that both high salt and EOPO concentration with basic pH and no addition of cosolute will contribute to high Kpa in PHAs partitioning.

Table 5.3: Statistical result of ANOVA for the reduced model of response variable "Kpa" where X_1 = potassium phosphate concentration, X_2 = EOPO concentration, X_3 = pH and X_4 = NaCl addition

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	3.568	9	0.396	221.5	< 0.0001
X_1	2.891	1	2.891	1615.4	< 0.0001
X_3	0.052	1	0.0516	28.8	0.0015
X_4	0.033	1	0.0332	18.5	0.0057
X_1X_2	0.516	1	0.516	288.5	< 0.0001
X_2X_3	0.046	1	0.0460	25.7	0.0022
$X_2X_3X_4$	0.018	1	0.0179	10.0	0.0265
Residual	0.011	6	0.0018		
Cor Total	3.579	15			
R^2	0.997				
R^2_{adj}	0.992				
R^2_{pred}	0.979				

5.3.1.3 Effect on the "Purification Factor"

Serves as the primary purification tool of PHAs, purification factor (PF) is used to define the purification efficiency of thermoseparating ATPE. Contradicted to the wide variation of Kpa and yield, the PF obtained in the factorial design were in a smaller range where an average value of 1.235 with the fluctuation of 0.3 was achieved for most run (with the exception of run 9 which has a value of 0.67). Based on the statistical results for reduced model of response variable of "PF" illustrated in Table 5.4, the reduced model was significant at a confidence level of 95 % with f-value of 42.6 and probability value much lower than 0.05 (Prob > F < 0.0001). With a value of 0.966 for R^2, this indicated that 96.6 % of the experimental data was compatible with the predicted data from the model. The value of R^2_{adj} was calculated to be 0.943, close to that R^2_{pred} (0.893), which demonstrate a high degree of correlation between the predicted and observed values. The model can be adequately utilized to predict the data within the range of variables studied. The reduced model can be described by the following first-order model:

$$PF = 1.15 + 0.17\ X_1 + 0.11\ X_2 - 0.067\ X_1X_2 - 0.062\ X_2X_3 - 0.077\ X_1X_3X_4$$

The regression analysis of PF indicated that the X_1 and X_2 were significant with 95 % of confidence among the investigated independent parameters as shown in the equation. Other than that, the interactions between independent parameters such as X_1X_2, X_2X_3 and $X_1X_3X_4$ also play significant roles in thepurification of PHAs by thermoseparating ATPE. As expected of the positive main effect of phosphate concentration, a dramatic increase of PF values can be observed at the elevated levels of this parameter, especially shown in run 10 and 11 with PF greater than 1.5. This was also demonstrated in thepartition of collagenase from *Penicillium aurantiogriseum* by Lima and his colleagues with improving purification when utilizing higher phosphate concentration (Lima et al., 2013). Similarly, PF was found to be positively correlated with the EOPO concentration (X_2) as well. This trend is congruent with the purification of other bioproducts utilizing ATPE as reported in theliterature. For example,

the purification factor of α-amylase from *Aspergillus oryzae* was increased by threefold at thehighest level of PEG concentration (20 wt/wt %) (Kammoun et al., 2009). Study on isolation of lysozyme from crude hen egg white has reported a high PF value was achieved at high polymer concentration as well (Lu et al., 2013). Despite that, the simultaneous increase of X_1 and X_2, as well as X_2 and X_3, has a negative impact on PF. To summarize, PF is the most complicated response variable to be optimized due to the complicated positive contributions from the linear model terms combined with negative contributions from the interactions between them.

Table 5.4: Statistical result of ANOVA for reduced model of response variable "PF" where X_1 = potassium phosphate concentration, X_2 = EOPO concentration, X_3 = pH and X_4 = NaCl addition

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Model	0.955	6	0.159	42.6	< 0.0001
X_1	0.459	1	0.459	122.9	< 0.0001
X_2	0.205	1	0.205	54.8	< 0.0001
X_1X_2	0.072	1	0.071556	19.2	0.0018
X_2X_3	0.061	1	0.061256	16.4	0.0029
$X_1X_3X_4$	0.095	1	0.094556	25.3	0.0007
$X_1X_2X_3X_4$	0.064	1	0.063756	17.1	0.0026
Residual	0.034	9	0.003734		
Cor Total	0.988	15			
R^2	0.966				
R^2_{adj}	0.943				
R^2_{pred}	0.893				

5.3.2 Method of path of steepest ascent

Following the preliminary screening using full factorial design, it can be clearly observed that the concentration of both phase-forming components, which are phosphate salts and EOPO together with the synergistic interaction between them play a significant role in PHAs partitioning by thermoseparating ATPE. Therefore, these two parameters were chosen to be further optimized utilizing response surface method (RSM) design to obtain a better partitioning results for PHAs from *C. necator* feedstock.

Based on the first-order regression equation from the full-factorial model, the method of the path of steepest ascent was employed to determine the global optimal region for PHAs partitioning conditions. Details of the experiments and results are illustrated in Table 5.5. It clearly demonstrated that ATPS 2 is the system that falls in the global optimal region with highest Kpa, Yield, and PF of 15.45, 93 % and 1.29 respectively. Henceforth, a system with conditions of 17.54 wt/wt % phosphate salts, 18.04 wt/wt % EOPO and pH 9 was selected as the center point for further experiments.

Table 5.5: Result of method of path of steepest ascent

	Phosphate concentration (wt/wt %)	EOPO concentration (wt/wt %)	Kpa	Yield (%)	PF (fold)
-1	8.48	11.98	1.39	51.0	0.97
0	11.50	14.00	2.78	67.6	1.04
1	14.52	16.02	5.65	82.2	0.92
2	17.54	18.04	15.45	93.0	1.29
3	20.56	20.06	14.62	93.0	1.05

5.3.3 Response surface method (RSM) experiments

RSM experiment design was performed to optimize the isolation and purification of PHAs using the minimum amount of experiments, thus reducing the time consumption and overall cost. For all the RSM experiments, the response variables which is the recovery yield and PF, have been experimentally investigated and optimized as a function of two independent parameters, phosphate concentration (X_1) and EOPO concentration (X_2). A Box-Wilson central composite design (CCD) was chosen for the optimization of the PHAs purification process. With a face centered star points design, the CCD is made up of 13 experimental points, including a complete 2^2 factorial matrix, augmented with 2 "star" (axial) points on each design parameter axis at a distance of 1.414 from the center point together with 5 center points (Azevedo et al., 2009, Aydog˘an et al., 2010). Quintuple run of center point are performed to ensure the uniform precision of the experiments. The inconsistency of the experiment results which represents pure experiment error caused by measurement of the dependent variable can be estimated from the inconsistency of the repeated runs (Rosa et al., 2007).

The design matrix of the CCD and the experimental results of PHAs isolation and purification by thermoseparating ATPE are demonstrated in Table 5.6. From Table 5.6, it can be seen that the yield of PHAs achieved a minimum of 80.8 % at run 1, while 8 out of the 13 runs achieved yield as high as 99.9 %. On the other hand, the PF of PHAs purification fell in the range of 1.10 to 1.54 with the maximum was achieved in the run 11 (1.54 fold), while the minimum was obtained in the run 8 (1.10 fold). Yields close to 100 % are repeatedly reported for purification of bioproducts using ATPE (de Medeiros e Silva et al., 2013). Henceforth, we chose to model PF and not the recovery yield as most of the run has reached almost 100 % yield, thus, does not require further optimization through modeling.

Utilizing Design Expert software, the analysis of variance (ANOVA) was done to confirm the validity of the models, assess the statistical significance of all factors (main parameters and all their possible linear and quadratic interactions) and investigate the impacts of these

factors on the response variables (Rosa et al., 2007). The results of ANOVA are illustrated in Table 5.7. Based on the result, the f-value of 12.88 with probability value much lower than 0.05 (Prob > F = 0.002) indicates that the overall regression model was significant at 95 % level of confidence. Combining this with insignificant lack of fit (Prob > F = 0.784), this demonstrates that the response surface of the experimental design can be adequately described by the quadratic model with interaction.

With the high value of R^2 (0.902), it indicates that the real relationship between the independent parameters and response variables can be adequately represented by the model. This shows that 90.2 % of the variability in response can be explained by the model. The R^2_{adj} of 0.8319 was in a reasonable agreement with theR^2_{pred} of 0.7303, which shows a good degree of correlation between the predicted values with the experimental results.The regression analysis of the experimental design has indicated that both the quadratic model terms (X_1^2 and X_2^2) were the significant model terms. However, the linear model terms (X_1 and X_2) and interactive model term (X_1X_2) were found to be not significant at 95 % of confidence level.

Applying multiple regression analysis, a second-order polynomial equation was used to describe the empirical relationship between the purification factor and the independent variables. Although there were some factors which were insignificant statistically at a confidence level of 95 %, none of the model terms were discarded and pooled into the error terms due to hierarchical reason.

$$PF = 1.42 - 0.03\ X_1 + 0.041\ X_2 - 0.016\ X_1X_2 - 0.15\ X_1^2 - 0.091\ X_2^2$$

Generally, the impact of the model terms to the response variables can be determined by the degree of the corresponding coefficients of the regression equations (Lu et al., 2013). Although EOPO concentration (X_2) is not a significant model term at a confidence level of 95 % with the Prob > F of 0.0786, the independent parameter still brings positive impacts on the PF. This was also demonstrated in the purification of lysozyme from crude hen egg white at

high concentration of polymer (Lu et al., 2013). Similarly, a threefold increase in purification factor has been observed at high level of PEG concentration for isolation of α-amylase from *Aspergillus oryzae*(Lima et al., 2013).

Table 5.6: Experimental design and results of response variables

Run	Phosphate concentration (wt/wt %)	EOPO concentration (wt/wt %)	Yield (%)	PF (fold)
1	13.28	18.05	80.8	1.14
2	14.53	20.05	98.8	1.30
3	14.53	16.03	86.2	1.17
4	17.55	15.18	99.9	1.20
5	17.55	18.05	99.9	1.42
6	17.55	20.90	99.9	1.29
7	20.55	20.05	90.5	1.17
8	20.55	16.03	99.9	1.10
9	21.80	18.05	91.6	1.12
10	17.55	18.05	99.9	1.40
11	17.55	18.05	99.9	1.54
12	17.55	18.05	99.9	1.37
13	17.55	18.05	99.9	1.39

Table 5.7: Full statistical result of ANOVA for response variable "PF"

Source	Sum of Squares	DF	Mean Square	F-Value	Prob > F
Model	0.21	5	0.042	12.88	0.002
X_1	7.30E-03	1	7.30E-03	2.26	0.1762
X_2	0.014	1	0.014	4.24	0.0786
$X_1 X_2$	1.02E-03	1	1.02E-03	0.32	0.5908
X_1^2	0.15	1	0.15	46.25	0.0003
X_2^2	0.058	1	0.058	17.84	0.0039
Residual	0.023	7	3.23E-03		
Lack of Fit	4.84E-03	3	1.61E-03	0.36	0.784
Pure Error	0.018	4	4.44E-03		
Cor Total	0.23	12			
R^2	0.9020				
R^2_{adj}	0.8319				
R^2_{pred}	0.7303				

5.3.4 Model analysis

The quadratic model obtained was utilized to estimate the response surface for response variable "PF". Three-dimensional graph was constructed to visualize the relationship between independent parameters and response variable. The interaction between phosphate salt concentration and thermoseparating polymer concentration as well as their optimum range can be well defined via the response surface plots. According to the response surface as in Figure 5.1, PF does not have a linear correlation with either phosphate concentration or EOPO 3900 concentration. In addition, it reached an optimum of 1.431 fold at 17.12 wt/wt % of phosphate concentration and 18.52 wt/wt % EOPO 3900 concentration. The purification factor achieved in this study is in congruent with isolation and purification of other bioproducts as reported in the literature. Examples are such as extraction of recombinant cutinase from *E. coli* cell homogenate (2.5 fold) (Kepka et al., 2003), purification of papain (3.6 fold) (Jiang et al., 2015), and partitioning of α-amylase from *Aspergillus oryzae* (3.2 fold) (Kammoun et al., 2009).

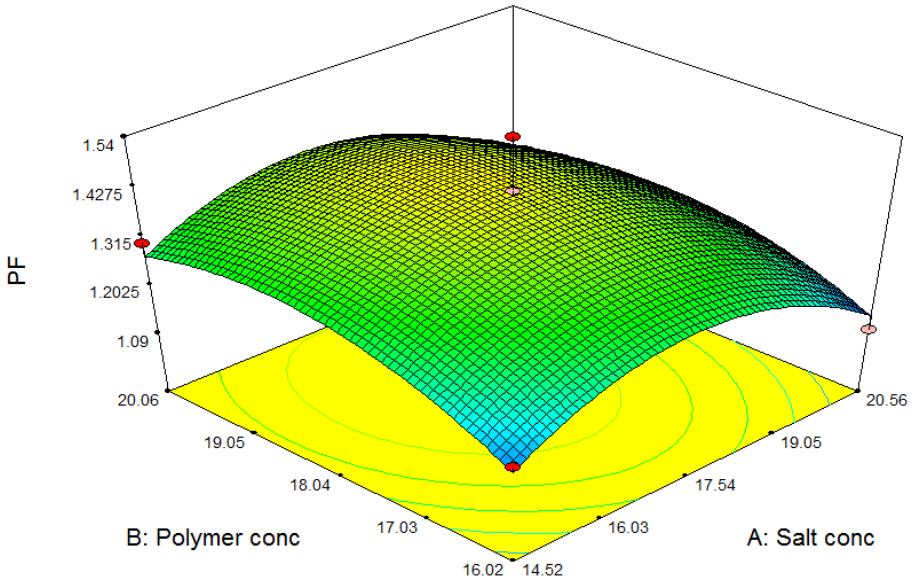


Figure 5.1: Three-dimensional contour plots showing interactive effect of salt and polymer concentration on PF

5.3.5 Recycling and reutilization of phase-forming components

Employing the technique of change one-factor-at-a-time in previous work, we studied the isolation and recovery of PHAs in an EOPO/phosphate system by replacing conventional PEG with thermoseparating copolymer which is thermoresponsive and recyclable. Recovery and reutilization of thermoseparating polymers has been successfully performed in previous study as well as extraction of other biomolecules as shown in literature. However, phosphate salts used in ATPE might pose environmental threat, thus, there will be significant advantages for recycling phosphate salts as well. Therefore, the recycling and reutilization of bottom phase-forming components was also introduced and described to lower the purification cost and minimize the environmental impact.

During the first extraction of ATPE, the EOPO-rich phase was removed from the primary system and placed into another centrifuge tube. Following that, the EOPO-rich phase was diluted with distilled water of 1:1 ratio and heated at 65 °C in water bath for 15 mins which allow for temperature-induced phase separation. With top phase contains of almost 100 % of water and bottom phase with EOPO concentration of 40-60%, a secondary two-phase system for PHA extraction and polymer recycling was formed.

To maintain the optimal conditions for PHAs isolation and recovery, fresh EOPO and phosphate salts were topped up at the next cycle of PHA extraction. This is shown in Table 5.8, where the primary two-phase systems have only small variation in volume ratio (< 0.22). This is due to the fact that the composition of all the systems were similar to each other. The similar trend is observed for PF where the purification efficiency of ATPE stayed in the range of 1.78 to 1.90. On the other hand, the yield of PHAs extraction via ATPE presented a decreasing trend throughout the 4 cycles, which may due to the increasing amount of contaminants in the systems. In the last cycle of extraction (i.e., 4^{th} cycle), the yield has

dropped approximately 10 % compared to the first extraction, though still remained as an acceptable yield (87.2 %).

Therefore, a total recovery yield greater than 85 % was obtained for two-step ATPE separation for four cycles. This indicated that most of the PHAs were exclusively partitioned to the water-phase in the secondary two-phase system. Following the models of Flory-Huggins solution theory, "excluded-volume" effect plays an important role in the separation of PHAs from the polymer phase, as the water-rich phase is entropically more favorable for PHAs compared to the polymer-rich phase.

Table 5.8: Results of PHA extraction in recycling EOPO/phosphate ATPE

PHA extraction	Overall Yield (%)	PF (fold)	Vr
First extraction	97.4	1.80	1.06
I recycling	90.5	1.78	0.98
II recycling	96.2	1.90	0.85
III recycling	87.2	1.80	0.91

5.3.6 Comparison between DOE and single factor optimization strategies

As mentioned previously, the conventional method of single factor optimization (also OFAT) is often used in finding the optimal condition for partitioning of bioproducts using ATPE. Therefore, this section compares the results obtained using OFAT and DOE strategies in the partitioning of PHAs utilizing thermoseparating ATPE. Table 5.9 compared the optimum result obtained from two different strategies, while table 5.10 and 5.11 tabulates the statistical result of ANOVA for response variable "Yield" and "PF" from the experimental data using the OFAT strategy. The ANOVA analysis has shown similar result as the previous section, where both salt and polymer concentration are key influencing parameters for both yield and PF at a confidence level of 95 % with probability value much lower than 0.05 (Prob > F < 0.0001),

while pH does not cause a significant impact. Therefore, statistically speaking, OFAT and DOE deliver the same result, although OFAT does not provide insight on the interaction between different parameters.

On the other hand, the yield optimized using OFAT strategy is not as high as its counterpart from DOE, this might due to the global optimum was not achieved during optimization. Though, the PF obtained for both methods are comparable. In conclusion, DOE plays an important role in pinpointing the key influencing parameters and demonstrating the interaction between parameters with minimum runs of experiments, while OFAT can use to demonstrate the trend of increasing effect of the specific parameter with the input from experimental data enable detailed analysis on the cause of the trend.

Table 5.9: Comparison between optimum result for one factor at a time (OFAT) and design of experiment (DOE)

Strategy	Yield (%)	PF (fold)
OFAT	72.2	1.61
DOE	97.4	1.80

Table 5.10: Statistical result of ANOVA for response variable "Yield" using OFAT

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
X_1	607.979	7	86.854	24.342	< 0.0001
X_2	3604.489	6	600.748	29.333	< 0.0001
X_3	426.2207	5	85.244	1.990	0.152612

Table 5.11: Statistical result of ANOVA for response variable "PF" using OFAT

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
X_1	16.46975	7	2.353	109.442	< 0.0001
X_2	7.624743	6	1.271	21.709	< 0.0001
X_3	0.168009	5	0.034	0.778	0.584365

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5.4. CONCLUDING REMARKS

Aiming to obtain the global optimal condition of PHAspurification as well as study the interactions between the independent parameters, the chapter researched on the purification and recovery of biopolymers from fermentation broth of *C. necator* via thermoseparating polymer/salt ATPE utilizing statistical design strategy. The screening of the main influencing parameters which consider four different factors which are concentration of phosphate salts and EOPO, addition of salts and pH on partitioning of PHAs was performed utilizing two-level full factorial design with the responses selected are recovery yield, Kpa and PF.

The full factorial design allowed the definition of appropriate models for the estimation of the most crucial process parameters, such as yield, Kpa and PF. For all the responses, the most influencing factors were the concentration of phosphate salts and EOPO as not only they provided significant positive impacts on most of the responses, while the synergistic positive interaction between the two factors demonstrated greater influences as well. PF is the most complicated response variable to be optimized due to the complicated positive contributions from the linear model terms combined with negative contributions from the interactions between them. Utilizing the full factorial design, the highest partition coefficient (16.6) and yield (93.9 %) was obtained at the conditions of 15 wt/wt % phosphate concentration, 18 wt/wt % EOPO concentration and pH 10 without the addition of NaCl. These results are more promising than the recovery of PHAs by PEG/phosphate system previously reported by Divyashree and his coworkers (Divyashree and Shamala, 2010). On the other hand, the highest purification factor (1.54) can be achieved at the same concentration of phosphate and EOPO, but with pH 8 and 100 mM addition of salt as cosolutes.

Using both the influential factors screened, the method of path of steepest ascent was employed to determine the global optimal region for PHAs partitioning conditions. The recovery yield of PHAs from *C. necator* fermented broth achieved as high as 99.9 % utilizing DOE strategy. On the other hand, Box-Wilson central-composite design showed that

PFreached an optimum of 1.43 fold at 17.12 wt/wt % of phosphate concentration and 18.52 wt/wt % EOPO 3900 concentration.

The recycling and reutilization of both phase-forming components has also shown that thermoseparating ATPE is a more environmental-friendly and economical favorable technique for isolation and purification of PHAs. A total of 4 successive purification cycles with satisfying yield and purification factor were demonstrated. In order to improve the partition yield, some further improvements can be considered to be applied such as the complete removal of PHAs and contaminants in phosphate phase using regeneration technique (for example, membrane diafiltration technique) after several extraction cycles. In conclusion, this opens promising standpoints for utilizing thermoseparating ATPE as the primary step in the isolation and purification of PHAs from fermented broth.

Chapter 6

EXTRACTIVE BIOCONVERSION OF POLYHYDROXYALKANOATES (PHAs) FROM *CUPRIAVIDUS NECATOR*

6.1 INTRODUCTION

Extractive bioconversion, also known as in situ product recovery is a technique that integrate upstream fermentation and downstream purification. This integrated approach offered several benefits, which includes lower product degradation, preservation of biological activity, minimize inhibition by end-product, steps reduction, as well as energy and time savings. Extractive bioconversion can be done via aqueous two-phase system (ATPS) due to several advantages offered by ATPS. Other than providing a mild environment for cell growth and bioseparation due to high water content, ATPS has the capability to handle large volume and scale-up can be reliably predicted from laboratory data. Examples of extractive fermentation via ATPS including alkaline phosphatase (Pandey and Banik, 2011), cyanobacterial products (Chavez-Santoscoy et al., 2010), cyclodextrin glycolsyltransferase (Ng et al., 2013), lactic acid (Kwon et al., 1995), lipase (Ooi et al., 2011, Show et al., 2012b), nisin (Li et al., 2000), xylanase (Kulkarni et al., 1999) and other bioproducts.

Prior proceeding to extractive bioconversion of polyhydroxyalkanoates (PHAs) via thermoseparating ATPS, there is a need to select a suitable carbon source(Koller et al., 2011). There are some important elements that needed to be considered when choosing the ideal microorganism and carbon source for industrial scale biosynthesis of PHAs, such as substrate cost, maximum polymer yield, growth rate, PHAs productivity, quality and quality of biopolymer accumulated, and downstream processing cost (Choi and Lee, 1997, Choi and Lee, 1999b). It is noteworthy that carbon source has been reported as the most influencing factor for PHAs price (Chanprateep, 2010).

How green are green plastics?The "environmental friendliness" of PHAs has been questioned as a product which is produced from renewable or natural source or is biodegradable is automatically equalled to environmental-friendly material (Akiyama et al., 2003). As the "green" substitute of petrochemical-based plastics, the overall process of PHAs biosynthesis must be environmental-friendly enough and causes less negative impacts on the

environment (Belboom and Léonard, 2016). Being a well-established and systematic concept that implements environmental sustainability (Kylili et al., 2016), Life Cycle Assessment (LCA) has been applied by several researchers (Kim and Dale, 2005, Kim and Dale, 2008, Gurieff and Lant, 2007b, Koller et al., 2013, Pawelzik et al., 2013, Harding et al., 2007a, Khoo and Tan, 2010, Akiyama et al., 2003, Hottle et al., 2013, Heimersson et al., 2014, Álvarez-Chávez et al., 2012, Pietrini et al., 2007, Heyde, 1998, Gerngross, 1999) on "cradle-to-grave" or "cradle-to-gate" environmental analysis of PHAs biosynthesis, comparison with conventional plastics and other bioplastic, and from many different points of view. Most works demonstrates that PHAs are more environmental-friendly compared to conventional plastics, while some of the earlier works give different conclusions (Gerngross, 1999, Heyde, 1998).

Literature have always reported environmental impacts in terms of carbon dioxide equivalent emissions (global warming potential) and/or energy consumptions (Akiyama et al., 2003, Kim and Dale, 2005, Kim and Dale, 2008, Gurieff and Lant, 2007b). However, these are not comprehensive enough and hardly cover every aspect, includes smog formation, ozone depletion, acid rain formation, water pollution, and other pollutions (Harding et al., 2007a). Developed based on sustainability concept of LCA, WAR algorithm is an environmental index that can covers a wide range of environmental concerns (Young et al., 2000). Also, R&D stage is a crucial stage to integrate environmental concern inherently into the process design as there are plenty of opportunities and the modification cost is cheap (Koller et al., 2013). Therefore, focusing on R&D stage, this chapter aimed to develop a preliminary integrated analysis of economic and environmental performances to screen suitable carbon source in a simple and rapid way for extractive bioconversion of PHAs by coupling material cost evaluation and WAR algorithm.

Following that, no literature sources to date have reported the extractive bioconversion of PHBs from *Cupriavidus necator* using thermoseparating ATPS. Focusing on important parameters such as polymer concentration, cultivation temperature and pH, this chapter

studied the influence of these parameters on biomass growth and PHB accumulation. Finally, scaling up to 2 L fermenter was performed to investigate the reliability and predictability of scaling-up ATPS from laboratory data.

6.2 MATERIALS AND METHODS

6.2.1 Materials

In the preliminary screening of carbon source, the substrates used are soybean oil, waste cooking oil, crude and refined glycerol. *C. necator* were cultivated for 72 hours at 30 °C and pH 7 in 5 L bioreactor containing 3.5 L working volume of defined medium with 2 g/L yeast extract and optimized carbon source of 5 % wt/v for soybean oil and waste cooking oil, while 3 % wt/v for crude and refined glycerol. The stirring speed was controlled by dissolved O_2 (DO) concentration which maintained at 50 % and air flow rate at 1vvm. The process flow diagram for production of PHAs from *C. necator* was presented in Figure 6.1.

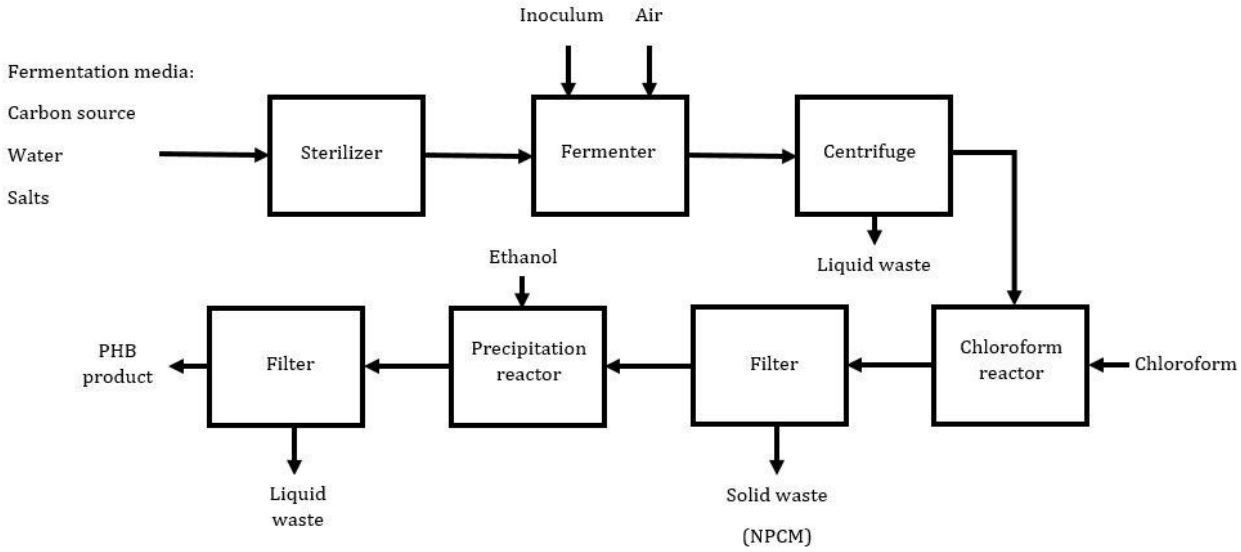


Figure 6.1. Process flow diagram for PHAs production

6.2.2 Integrated economic and environmental assessment

For all assessment, the system boundary only covered the "gate-to-gate" analysis of upstream PHA biosynthesis as the main objective of this work is to screen the suitable carbon source that satisfies both economic and environmental criteria. Cost and environmental impacts of utility is assumed to be same for each carbon source due to the same fermentation condition.

6.2.2.1 Economic assessment of PHA biosynthesis

The economic performances of PHA biosynthesis are evaluated by calculating the raw material cost which dominates the major shares of PHA's production cost. Below shows the material cost calculation,

$$Material\ cost = \sum(Cost\ of\ chemical\ components\ per\ kg\ \times R^{cp}) \quad (6.1)$$

Where cp = chemical components, R = chemical components used per kg PHA produced (kg cp/kg PHA).

6.2.2.2 Environmental assessment of PHA biosynthesis

Based on the potential environmental impact (PEI) balance concept introduced by Hilaly and Sikdar (Hilaly and Sikdar, 1995), Young and Cabezas (Young and Cabezas, 1999) have introduced WAR algorithm that focuses on waste minimization across the process boundary. With an extensive chemicals database, WAR algorithm covers four local toxicological impact categories which are human toxicity potential by ingestion (HTPI), terrestrial toxicity potential (TTP), human toxicity potential by either inhalation or dermal

exposure (HTPE), and aquatic toxicity potential (ATP) and four global atmospheric impacts which are global warming potential (GWP), ozone depletion potential (ODP), photochemical oxidation or smog formation potential (POP), and acidification or acid-rain potential (AP). The equations used are as shown below

$$Impact_i^{total} = \sum_{cp}^{n}(Impact_i^{cp} \times R^{cp}) \quad (6.2)$$

$$PEI = \sum_i^m Impact_i^{total} \quad (6.3)$$

$$EB = \sum PEI\ introduced\ - \sum PEI\ deducted \quad (6.4)$$

Where EB = environmental burdens

PEI categories are combined into a single PEI index utilizing weighing factors which show the relative or site-specific considerations of user. Following the literature (Young et al., 2000), with no specific site in mind, the weighting factor for all categories in this case study will be given equivalent values of unity.

6.2.3 Multiple-objective optimization

In this case study, there are two objectives, which are economic and environmental concerns that needed to be taken in account for alternative selection. As these objectives have the possibility to be conflicting in nature, this becomes a multiple-objective optimization problem. Thus, there is a need for utilizing "fuzzy optimization algorithm" as a decision making tool on the trade-off between the different objectives of the process optimization. In fuzzy objective optimization, a degree of satisfaction, λ_o has to be introduce to each objective which can be expressed as a linear function bounded by lower and upper limits of the target objective as shown in equations (6.5) and (6.6). Equation (6.5) is used for a target objective to be maximized, while equation (6.6) is used for a target objective to be minimized.

$$\lambda_o = \begin{cases} 0 & V_o \leq v_o^L \\ \frac{V_o - v_o^L}{v_o^U - v_o^L} & v_o^L \leq V_o \leq v_o^U \\ 1 & V_o \geq v_o^U \end{cases} \quad (6.5)$$

$$\lambda_0 = \begin{cases} 0 & V_o \geq v_o^U \\ \frac{v_o^U - V_o}{v_o^U - v_o^L} & v_o^L \leq V_o \leq v_o^U \\ 1 & V_o \leq v_o^L \end{cases} \quad (6.6)$$

$$0 \leq \lambda_0 \leq 1 \quad (6.7)$$

Where V_o is the score of the carbon source (eg. Material cost for economic, Total PEI and EB for environmental), v_o^L and v_o^U is the lower and upper limit respectively.

All λ_0 have to be maximized in order for all λ_0 to achieve high level of satisfaction. Developed by Zimmermann (Zimmermann, 1978), max-min operator tool can be utilized to maximize the least satisfied degree of satisfaction by guaranteeing all λ_0 will be satisfied to at least the value of λ. Therefore, the overall objective now is to maximize λ as shown in equation below.

$$\lambda_0 \geq \lambda \quad (6.8)$$

6.2.4 Sensitivity analysis

Sensitivity analysis was conducted to determine the robustness and sensitivity of the integrated assessment. For this purpose, 5 different cases were considered where the most influencing factors were manipulated. In the first 4 cases, yield and price of both refined and crude glycerol were manipulated, while in the last case, the weighting factor was utilized for multi-objective optimization instead of fuzzy optimization algorithm using the equations below.

PEI weightage = 1 – Cost weightage (6.9)

EB weightage = 1 – Cost weightage (6.10)

6.2.5 Extractive bioconversion of PHB using thermoseparating ATPE

Using the carbon source selected, the two-phase medium was prepared by autoclaving stock solution of EOPO 3900 along with some distilled water in the flask and then mixing with sterilized stock culture medium to give final desired concentration of all components in 100 mL. The respective media were inoculated with 5 % v/v of *C. necator* inoculum. Samples of 5 mL which are withdrawn periodically during fermentation were centrifuged and dried in the oven for overnight at 70 °C for cell mass and PHB content determinations.

A 40 g two-phase system was extracted from the fermentation medium and incubated in water bath at 65 °C for thermoseparation. After the stages of equilibration and phase separation, the top phase was carefully removed using a pipette, while the bottom phase was then sampled through the interface. The samples from both phases and disrupted biomass are centrifuged and washed with deionized water for three times before left in the oven for overnight at 70 °C to be dried. The dried samples from both phases and disrupted biomass were analyzed for PHAs content using gas chromatography (GC).

6.2.6 Scaled-up bioreactor fermentation

The scaling-up of extractive bioconversion was performed in a 2 L fermentor with a working volume of 1.5 L. The respective media were inoculated with 5 % v/v of C. necator inoculum. The cultivation was carried out at 30 °C and pH 6 for 72 hours with air flowrate of 1vvm and dissolved oxygen (DO) of 50 %.

6.3 RESULTS AND DISCUSSION

6.3.1 Preliminary screening of carbon source

6.3.1.1 Basis for assessment of PHA biosynthesis

Showing the raw material cost at year 2016 in USD $/kg and the PEI score for each chemical components in each categories respectively, the foundation for the economic and environment assessment of PHA production is presented in Table 6.1. The inoculum cells were deemed negligible to be considered as raw material cost due to rapid dividing rate of bacteria cells (Raynolds et al., 2000). The PHA biosynthesis from *C. necator* H16 using four different substrates, which are soybean oil, waste cooking oil (WCO), refined and crude glycerol has been studied and optimized with the yield obtained are 20.73, 11.05, 31.07 and 25.01 g/L of PHAs respectively. Following that, the relative mass of carbon sources of soybean oil, WCO, refined and crude glycerol are 2.412, 4.524, 0.966 and 1.2 kg/kg PHA. The material and energy flows used in the study are provided in Table 6.2.

2347 Table 6.1: Data of raw material cost and PEI

Chemicals[a]	Price (USD $/kg)[a]	HTPI (kg^{-1})[b]	TTP (kg^{-1})[b]	HTPE (kg^{-1})[b]	ATP (kg^{-1})[b]	GWP (kg^{-1})[b]	ODP (kg^{-1})[b]	PCOP (kg^{-1})[b]	AP (kg^{-1})[b]
Na_2HPO_4	0.25	0.045	0.045	0	0.002	0	0	0	0
KH_2PO_4	0.3	0.156	0.156	0	0.005	0	0	0	0
$(NH_4)_2SO_4$	0.1	0.177	0.177	0	0.025	0	0	0	0
$MgSO_4$	0.05	0.070	0.070	0	0	0	0	0	0
$CaCl_2$	0.08	0.191	0.191	0	0	0	0	0	0
Na_2EDTA	1.55	0.156	0.156	0	0.007	0	0	0	0
$FeCl_3$	0.2	0.835	0.835	0.082	0.011	0	0	0	0
H_3BO_3	0.4	0.141	0.141	0.119	0.028	0	0	0	0
$MnCl_2$	0.3	0.488	0.488	0.021	0.014	0	0	0	0
$ZnCl_2$	0.18	1.070	1.070	0.238	0.065	0	0	0	0
$CoCl_2$	0.8	0.490	0.490	0.595	0.033	0	0	0	0
$CuSO_4$	1.15	1.250	1.250	0.095	1.380	0	0	0	0
Yeast Extract	0.3	0	0	0	0	0	0	0	0
Soybean Oil	0.615	0.059	0.059	0	0.489	0	0	0	0
Refined Glycerol	0.4	0.030	0.030	0.048	0	0	0	0.993	0
Crude Glycerol	0.2	0.030	0.030	0.048	0	0	0	0.993	0

Waste Cooking Oil[c]	0.2	0.098	0.098	0	0.792	0	0	0	0

[a] All the chemical prices were obtained from Alibaba.com [32], with exception of soybean oil [33].

[b] The PEI score can be obtained from the database of WAR Algorithm Graphical User Interface (Environmental Protection Agency, U.S.)

[c] PEI for waste cooking oil are of palmitic acid as most of the waste cooking oil are palm oil-based.

Table 6.2. Material and energy values for PHAs production

Feed			
Electricity (MJ)[a]	648	Salts:	
Air (kg)	19.4	Na_2HPO_4 (kg)	23.45
Water (kg)	3.5	KH_2PO_4 (kg)	5.25
Carbon source:		$(NH_4)_2SO_4$ (kg)	8.75
Crude glycerol (kg)	105	$MgSO_4$ (kg)	0.7
Pure glycerol (kg)	105	$CaCl_2$ (kg)	0.035
Soybean Oil (kg)	175	Trace mineral salts (kg)	0.25
Waste Cooking Oil (kg)	175		
PHAs Products		Solid waste (biomass)	
Crude glycerol (kg)	87.54	Crude glycerol (kg)	40.29
Pure glycerol (kg)	108.75	Pure glycerol (kg)	38.01
Soybean Oil (kg)	72.56	Soybean Oil (kg)	56.88
Waste Cooking Oil (kg)	38.68	Waste Cooking Oil (kg)	83.44

[a]Energy requirement by fermenter is assumed as 0.5 kW/m^3 (Harding et al. 2007)

6.3.1.2 Economic assessment of PHA biosynthesis

The results for both economic and environmental assessment for PHAs biosynthesis are displayed in Table 6.3. From the result, it is clearly shown that crude glycerol (0.362 USD$/kg PHA) is the most economic competitive substrate, followed by refined glycerol (0.484 USD$/kg PHA) as they gave a better biopolymer yield compared to others. The raw material cost calculated is comparable with literature value of 0.118 and 0.149 USD$/kg for crude and refined glycerol respectively (Posada et al., 2011). As the by-product in biodiesel synthesis, glycerol's price fall drastically following the rapid growth of biodiesel industry and has huge potential as carbon source for PHA biosynthesis (Posada et al., 2011). Being the unwanted waste from food industry, WCO also demonstrates its value as substrate for PHA production (1.180 USD$/kg PHA). Agreeing with literature (Akaraonye et al., 2012), further analysis has shown that carbon source has dominated the share of raw material cost which contribute at least 66.3 % of the total cost. Internal rate of return (IRR) and total annual cost (TAC) has always been used by literature (Gurieff and Lant, 2007b, Van Wegen et al., 1998, Choi et al., 2010) to evaluate economic performance of PHAs biosynthesis process. However, these assessment techniques are not suitable for rapid screening at early stage as tedious and rigorous calculations are required.

6.3.1.3 Environmental assessment of PHA biosynthesis

During environmental assessment for different process alternatives, WAR algorithm considers the "worst case scenario" which is the accidental release and disperse of all chemicals into the environment, achieves dangerous levels of concentration, causing short or long term impacts to human, terrestrial, aquatic and global atmospheric. Therefore, from Table 6.3, the production of PHA from refined glycerol gives the least environmental impact

(Total PEI = 1.130), where WCO scores the worst (Total PEI = 4.657) as total loss of containment for this process will give the most negative impact on the environment.

Table 6.3. Economic and environmental evaluation between different carbon sources for *C. necator*

Carbon Sources	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Material Cost (USD$/kg PHA)	1.631	1.180	0.484	0.362
HTPI (kg PHA^{-1})	0.192	0.533	0.061	1.000
TTP (kg PHA^{-1})	0.192	0.533	0.061	1.000
HTPE (kg PHA^{-1})	0.0002	0.0004	0.046	0.095
ATP (kg PHA^{-1})	1.184	3.590	0.003	0.003
GWP (kg PHA^{-1})	0.000	0.000	0.000	0.000
ODP (kg PHA^{-1})	0.000	0.000	0.000	0.000
POP (kg PHA^{-1})	0.000	0.000	0.959	1.958
AP (kg PHA^{-1})	0.000	0.000	0.000	0.000
Total PEI (kg PHA^{-1})	1.568	4.657	1.130	1.404
Environmental Burdens (kg PHA^{-1})	+1.568	-4.275	-1.062	-1.320

Detailed environmental analysis showed that biosynthesis of PHA does not cause much burden on global atmospheric except when utilizing glycerol as carbon source. Release of crude and refined glycerol to the surroundings will cause formation of photochemical smog with POP of 0.959 and 1.404 kg PHA^{-1} respectively. Other than that, comparing between different carbon sources, accidental release of soybean oil and WCO into water body will cause water pollution and endangers aquatic life with ATP of 1.184 and 3.590 kg PHA^{-1} respectively. Thus, these concerns needed to be taken into account when choosing PHA production plant site.

On the other hand, the process of turning unwanted byproducts into value-added products helps in reduction of environmental impacts by eliminating the release of waste to the environment from the point of view of overall sustainability. Therefore, utilizing glycerol and WCO as substrate for PHA biosynthesis cause deduction in environmental burden as presented in Table 6.3. Hence, the environmental burdens for PHAs synthesized from WCO, refined and crude glycerol has the value of -4.275, -1.062 and -1.320 respectively, while environmental burden for soybean oil have the same value as that of Total PEI.

Compared to CML 2 baseline 2000 V2.03 and TRACI model used by Harding and his coworkers (Harding et al., 2007a) and Kim and Dale (Kim and Dale, 2008) respectively, the chemical database of both the environmental assessment techniques are not comprehensive enough to cover the whole range of chemicals consumed in biosynthesis of PHAs from *C. necator*. On the other hand, non-renewable energy use (NREU) or fossil energy consumption and global warming potential (GWP) or greenhouse gases (GHG) emissions used by Akiyama and his colleagues (Akiyama et al., 2003), Gurieff and Lant (Gurieff and Lant, 2007b), Yu and Chen (Yu and Chen, 2008) as well as Miller and his coworkers (Miller et al., 2013) requires massive amount of information to obtain the impact value for every single component. Although these strategies may not be suitable in rapid screening for choice of bacteria and carbon source, they are able to provide detailed insights on cradle-to-gate environmental

performance of PHAs and can be further improved if the assessment could cover a wider range of environmental impacts.

6.3.1.4 Multi-objective optimization using fuzzy approach

Following the two different environmental perspectives, there are two different scenario for multi-objective optimization. First scenario aims to minimize both raw material cost and PEI by considering the "worst case scenario", while the second scenario focusing on overall sustainability which minimize the raw material cost as well as environmental burden. The result of multi-objective optimization using fuzzy approach is displayed in Table 6.4. In the first scenario, crude glycerol obtains the highest λ (λ = 0.922), which may due to its high yield and considerably cheap price among other substrates. Similarly, crude glycerol also achieves the highest λ (λ = 0.494) for second scenario, due to its economic advantages and sustainability using it as carbon source for PHA biosynthesis. This has concluded that the crude glycerol is the most optimal carbon source for PHA biosynthesis.

Table 6.4. Results of multi-objective optimization for two different scenarios

Carbon Source	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Cost λ_o	0	0.355	0.904	1.000
PEI λ_o	0.876	0	1.000	0.922
EB λ_o	0	1.000	0.450	0.494
(Cost +PEI) λ	0	0	0.904	0.922
(Cost + EB) λ	0	0.355	0.450	0.494

6.3.1.5 Sensitivity analysis

Aiming to investigate the consequences of "what if ?" scenarios, a sensitivity analysis was conducted by considering the main parameters that influence both economic and environmental performance of PHA production by different carbon sources. Five different cases were considered as the parameters have huge influence on the results of multi-objective optimization and might have an influence on the result when the value of the parameters fluctuates. The results of 5 cases were displayed in Table 6.5a, b, c and d respectively as well as Figure 6.2a and b.

In case 1, the raw material cost, PEI and EB of PHA production from crude glycerol increase from 0.362 to 0.538 USD$/kg PHA, 1.404 to 1.560 kg PHA^{-1}, and -1.320 to -1.466 kg PHA^{-1} respectively with the yield decrease by 10 %. Following that, for crude glycerol, the (Cost + PEI) λ decrease from 0.922 to 0.878, while (Cost + EB) λ increase slightly from 0.494 to 0.519. Thus, refined glycerol has become the best carbon source from the point of view "worst-case scenario".

In case 2, the raw material cost, PEI and EB of PHA synthesized from refined glycerol decrease from 0.484 to 0.440 USD$/kg PHA, 1.130 to 1.027 kg PHA^{-1} and -1.062 to -0.966 kg PHA^{-1} respectively following 10 % rise in yield. On the basis of that, for refined glycerol, the (Cost + PEI) λ increase to 0.969 from 0.904, while the (Cost + EB) λ reduce from 0.450 to 0.434. Same as previous case, the multi-objective optimization give the same result where refined glycerol became a better substrate than crude glycerol.

In case 3, following 10 % increase in the crude glycerol price (ie. USD$ 0.22), the overall raw material cost of PHA from crude glycerol increase to 0.386 from 0.362 USD$/kg PHA. Following that, the (Cost + PEI) λ of refined glycerol increase from 0.904 to 0.921, become quite close to that of crude glycerol (0.922). Therefore, it can be predicted that the

refined glycerol will replace crude glycerol as the best carbon source as the price of crude glycerol further increase.

In case 4, the total raw material cost of PHA from refined glycerol drop from 0.484 to 0.446 USD$/kg PHA following the reduction of 10 % in refined glycerol price (i.e. USD$ 0.36). With that, the (Cost + PEI) λ of refined glycerol rise to 0.934 from 0.904, making the refined glycerol a more desired substrate for PHA biosynthesis. Therefore, it can be concluded that the integrated assessment is sensitive to the price and PHA yield of the substrate.

In case 5, weighting factor is utilized for multi-objective optimization instead of fuzzy approach. From "worst case scenario" viewpoint, WCO and crude glycerol has the similar increasing trend as the weightage of cost increase, while soybean oil and refined glycerol presents a different trend. Crude and refined glycerol maintains a high score of at least 0.9 throughout the range of weightage with crude glycerol take over the most optimal substrate after cost weightage of 0.5.

On the other hand, based on the concept of overall sustainability, WCO's score falling as the cost weightage increase, while soybean oil's score remains 0 throughout the range of weightage. Both crude and refined glycerol has increasing score following the rise of cost weightage, with crude glycerol's score constantly on top of refined glycerol. Agreeing with fuzzy approach, crude glycerol is the most optimum carbon source for both scenario, followed by refined glycerol.

2473 Table 6.5a. Result of case 1 where crude glycerol yield decrease by 10%

Carbon Source	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Cost (USD$/kg PHA)	1.631	1.180	0.484	0.402
PEI (kg PHA^{-1})	1.568	4.657	1.130	1.560
EB (kg PHA^{-1})	1.568	-4.275	-1.062	-1.466
Cost λ_o	0	0.367	0.933	1
PEI λ_o	0.876	0	1	0.878
EB λ_o	0	1	0.450	0.519
(Cost +PEI) λ	0	0	0.933	0.878
(Cost + EB) λ	0	0.367	0.450	0.519

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2476 Table 6.5b. Result of case 2 where refined glycerol yield increase by 10%

Carbon Source	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Cost (USD$/kg PHA)	1.631	1.180	0.440	0.362
PEI (kg PHA^{-1})	1.568	4.657	1.027	1.560
EB (kg PHA^{-1})	1.568	-4.275	-0.966	-1.466
Cost λ_o	0	0.367	0.969	1
PEI λ_o	0.851	0	1	0.853
EB λ_o	0	1	0.434	0.519
(Cost +PEI) λ	0	0	0.969	0.853
(Cost + EB) λ	0	0.367	0.434	0.519

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2479 Table 6.5c. Result of case 3 where crude glycerol price increase by 10%

Carbon Source	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Cost (USD$/kg PHA)	1.631	1.180	0.484	0.386
PEI (kg PHA^{-1})	1.568	4.657	1.130	1.404
EB (kg PHA^{-1})	1.568	-4.275	-1.062	-1.32
Cost λ_o	0	0.367	0.921	1
PEI λ_o	0.876	0	1	0.922
EB λ_o	0	1	0.450	0.494
(Cost +PEI) λ	0	0	0.921	0.922
(Cost + EB) λ	0	0.367	0.450	0.494

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2482 Table 6.5d. Result of case 4 where refined glycerol price decrease by 10%

Carbon Source	Soybean oil	WCO	Refined Glycerol	Crude Glycerol
Cost (USD$/kg PHA)	1.631	1.180	0.446	0.362
PEI (kg PHA^{-1})	1.568	4.657	1.130	1.404
EB (kg PHA^{-1})	1.568	-4.275	-1.062	-1.320
Cost λ_o	0	0.355	0.934	1
PEI λ_o	0.876	0	1	0.922
EB λ_o	0	1	0.450	0.494
(Cost +PEI) λ	0	0	0.934	0.922
(Cost + EB) λ	0	0.355	0.450	0.494

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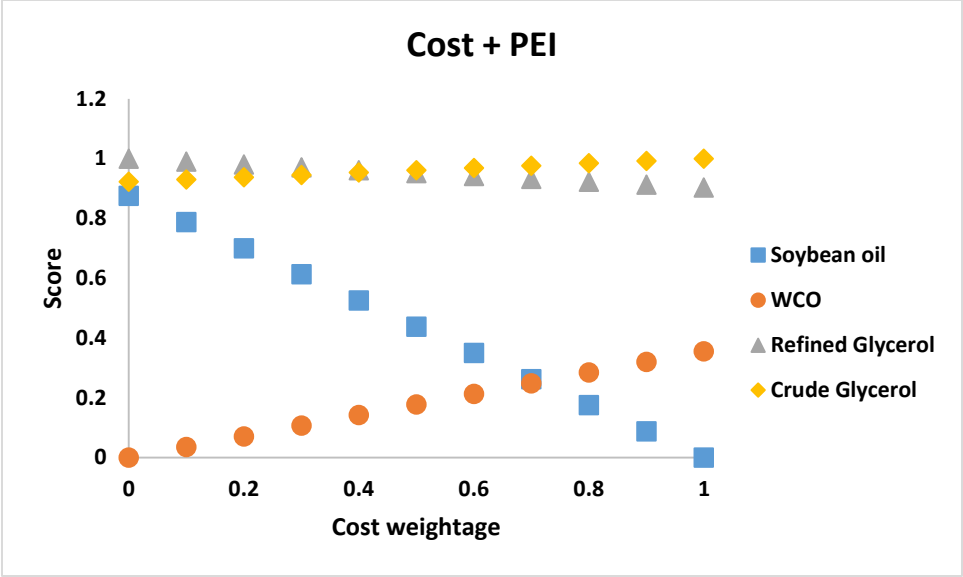


Figure 6.2a. Multi-objective optimization for Cost + PEI using weighting factor

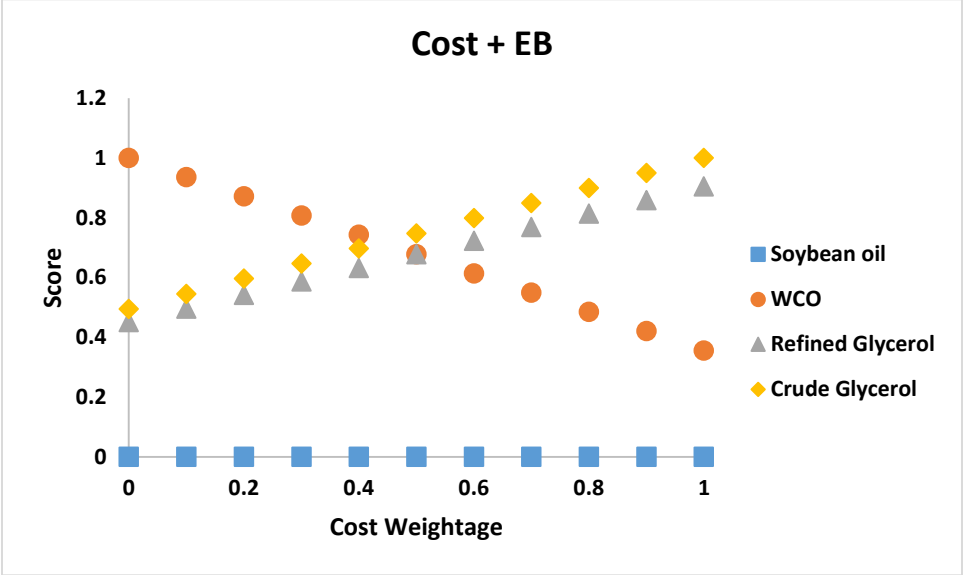


Figure 6.2b. Multi-objective optimization for Cost + EB using weighting factor

6.3.2 Extractive Bioconversion of PHAs via ATPS

6.3.2.1 PHB partitioning in thermoseparating ATPS

The aim of the extractive fermentation of PHAs in a thermoseparating polymer-based aqueous two-phase system is to integrate cultivation, centrifugation, washing, purification and recovery steps into fewer steps, thus minimizing processing steps, time and energy. The examples of extractive fermentation of intracellular products include glucose-6-phosphate dehydrogenase from *Bacillus subtilis* using poly(ethylene glycol)/dextran system (Tanaka et al., 1993), β-galactosidase from *Escherichia coli* using poly(ethylene glycol)/phosphate system (Kuboi et al., 1995) and heat shock proteins from *E. coli* using poly(ethylene glycol)/dextran system (Umakoshi et al., 1996).

6.3.2.2 Growth behavior of *C. necator* at different polymer concentration

When performing the extractive bioconversion of PHB from *C. necator*, it is paramount to determine the polymer concentration that can sustain stable cultivation of *C. necator* and support PHB accumulation. Therefore, *C. necator* cells were grown in fermentation media containing different concentration of thermoseparating polymers. Figure 6.3 displays the biomass growth and PHB accumulation curve at different thermoseparating polymer concentration. At all polymer concentrations, biomass growth does not display a growth lag phase, which means EOPO 3900 does not inhibit cell growth. Also, all the concentrations demonstrate a similar initial growth rate, ranging from 0.05 to 0.056 g/L.hr. This especially observed in 5 wt/wt % which has exponential growth until 24th hour. However, the cell growth slowed down after 12^{th} hour for polymer concentration more than 10 wt/wt %. It can be seen that *C. necator* at 5 wt/wt % of thermoseparating polymer concentration has the highest cell growth with maximum of 1.4 g/L achieved at 72th hour as well as PHB accumulation with maximum of 0.224 g/L obtained at 72th hour. Another observation that can be made is that similar cell growth and PHB accumulation curve were achieved at 10 and 15 wt/wt % of

polymer concentration, with 15 wt/wt % generally has slightly higher cell growth. It has been reported that low concentration of polymer is preferred in extractive fermentation, so that the two-phase system which is also the fermentation system is less viscous to ensure efficient oxygen mass transfer for cell growth and PHB accumulation (Kuboi et al., 1995, Kwon et al., 1995). Furthermore, literature stated that high concentration of polymer might cause precipitant forming at the interphase (Ng et al., 2013). Therefore, thermoseparating polymer concentration of 5 wt/wt % was chosen for subsequent experiments.

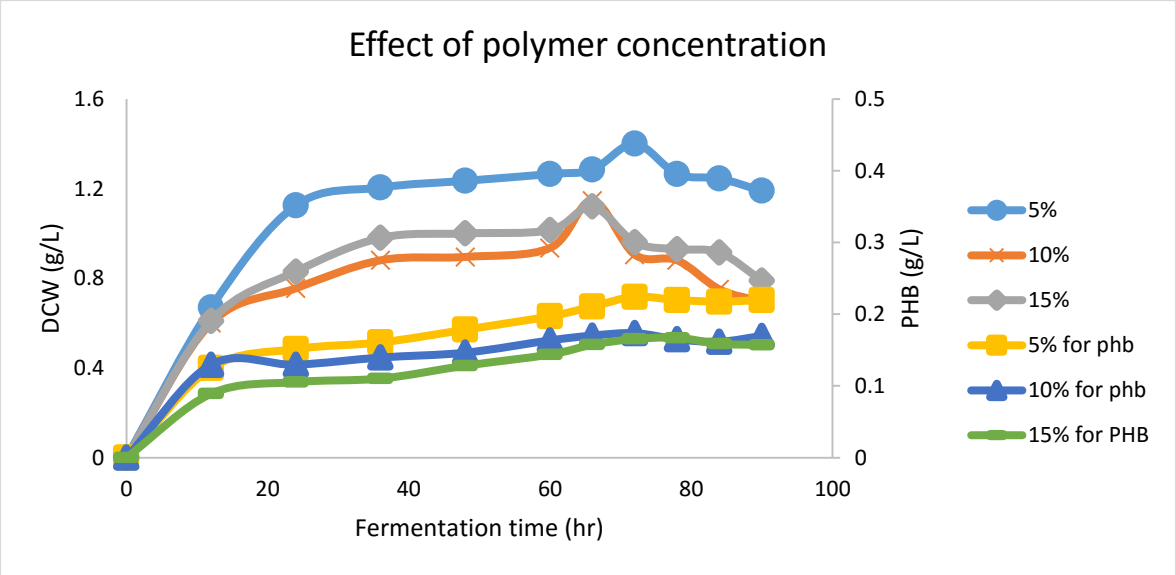


Figure 6.3: Effect of polymer concentration on biomass growth and PHB accumulation

6.3.3.3 Growth behavior of *C. necator* at different cultivation temperature

Temperature plays an important role in extractive bioconversion of PHB from *C. necator* as most microorganisms have the best growth at an optimal temperature. Low temperature will inhibit the bacteria growth, while high temperature may result in denaturation of protein. Figure 6.4 presents the influence of temperature on biomass growth and PHB accumulation. As observed, *C. necator* cultivated at 30 °C has the highest biomass growth of 1.8 g/L at 60th hour as well as highest PHB accumulation of 0.249 g/L at 72th hour.

Although with different initial growth rate, DCW of *C. necator* reached the same at 1.4 g/L after 72th hour for both 25 °C and 35 °C. However, PHB accumulation reached the highest at 0.224 g/L at 35 °C cultivation temperature, while only reached 0.18 g/L for 25 °C.

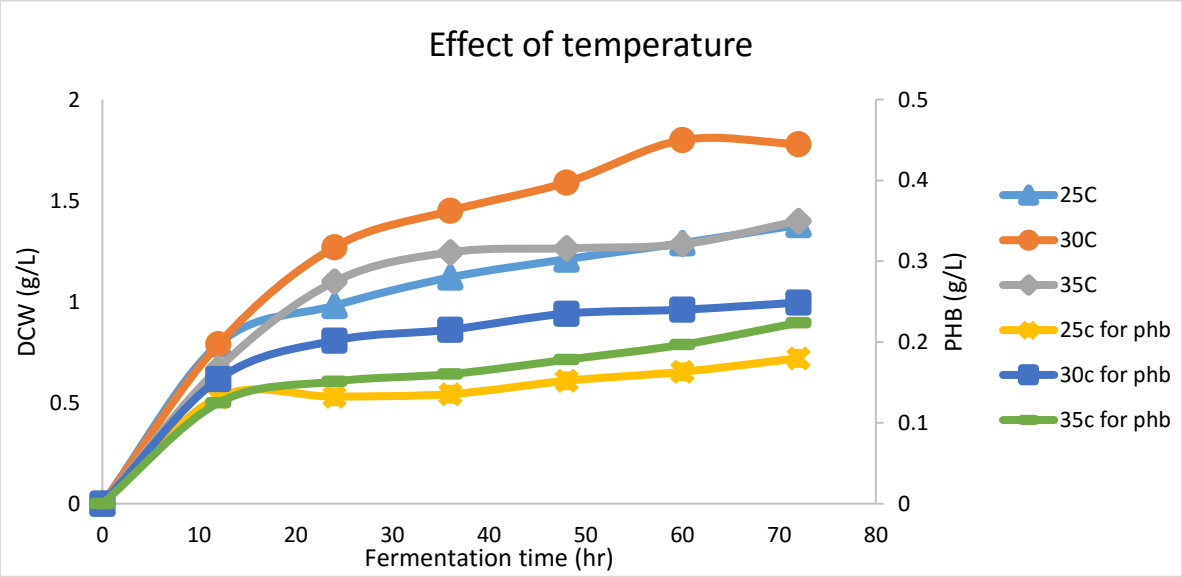


Figure 6.4: Effect of temperature on biomass growth and PHB accumulation

6.3.3.4 Effect of pH

System pH is a key factor in extractive bioconversion of PHB from *C. necator* as extreme pH may inhibit cell growth which cause long lag phase and lead to low PHB yield. Furthermore, pH also influence the partitioning of PHB as it alter the concentration of polymer in each phase (Pandey and Banik, 2011). To investigate the influence of pH on extractive bioconversion of PHB in a 5 wt/wt % solution of EOPO 3900, the study was conducted by manipulating pH of the fermentation medium in the range of 5 to 9, which was done by adding appropriate amounts of sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) solution. Table 6.6 summarizes the result of biomass growth as well as PHB accumulation and partitioning due to influence of pH. It can clearly observed that the growth of *C. necator* is strongly inhibited at pH 5, an acidic condition. This may due to disruption of cell membrane at acidic

condition and a lower cultivation pH might stop the biomass growth at all. However, a slight acidic condition, pH 6 support the biomass growth and PHB accumulation as it achieved both highest DCW and PHB content at 2.02 g/L and 21.8 % respectively. It also seems that pH does not really have a significant influence on PHB accumulation. For partitioning of PHB, the highest yield of 99.1 % is obtained at pH 8, which followed by pH 6 at 97.6 %. For purification factor, pH 8 achieved the highest value with 1.42 fold. Screening for a cultivation pH which provides high biomass growth and PHB accumulation with acceptable yield and PF, pH 6 was chosen for subsequent experiment as it has a significantly higher biomass growth and PHB accumulation compared to pH 8.

Table 6.6: Effect of pH on biomass growth as well as PHB accumulation and partitioning

pH	DCW (g/L)	PHB content (%)	Yield (%)	PF (fold)
5	0.59	17.8	61.6	1.16
6	2.02	21.8	97.6	1.36
7	1.39	17.6	85.1	1.37
8	1.48	16.9	99.1	1.42
9	1.65	19.7	58.5	1.05

6.3.3.5 Scale-up to bioreactor

In order to investigate the scale-up capability of extractive bioconversion via thermoseparating ATPS, the two-phase system with 5 wt/wt % polymer concentration was scaled-up to 2 L bioreactor with operating volume 1.5 L. The fermentation was done at 30 °C and pH 6 for 72 hours with air flowrate of 1vvm and dissolved oxygen (DO) of 50 %. To reduce energy required and chemical cost, the fermentation was done without pH control.

The biomass growth and PHB accumulation in 2 L bioreactor is demonstrated in Figure 6.5. The 2 L fermenter showed initial exponential growth rate until 12th hour and has an initial growth rate of *C. necator* in 2 L bioreactor is 0.363 g/L.hr, which is much higher compared to that of laboratory flask scale (0.056 g/L.hr). The biomass reached a maximum of 5.77 g/L at 48th hour, almost three times compared to the DCW obtained for laboratory flask scale. On the other hands, the PHB content topped at 0.398 g/L at 60th hour. For partitioning behavior of PHB in 2 L bioreactor, the PF obtained is 1.36 fold and the yield achieved is 96.7 %, which is similar to the result demonstrated by laboratory flask scale.

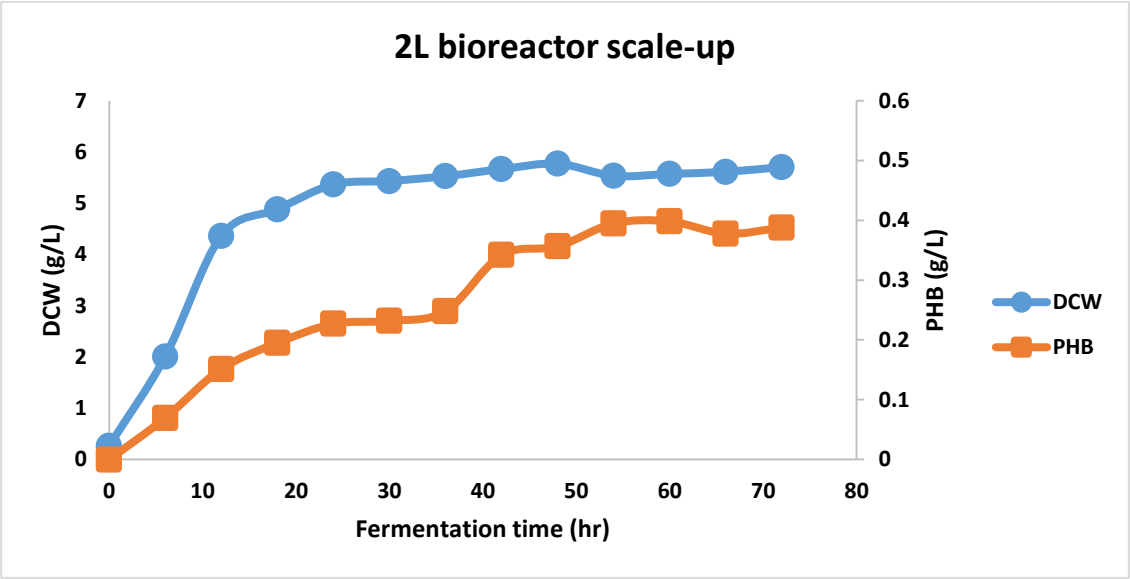


Figure 6.5. Biomass growth and PHB accumulation in 2 L fermenter

6.4 CONCLUDING REMARKS

Aiming to screen suitable carbon source for extractive bioconversion of PHAsin a simple and rapid way, a preliminary integrated analysis of economic and environmental performances have been developed by coupling material cost evaluation and WAR algorithm. The integrated economic and environmental assessment has successfully compared and

analysed PHAs production from 4 different carbon sources, which are soybean oil, waste cooking oil (WCO), refined and crude glycerol. In congruent with fuzzy optimization, it can be concluded that the crude glycerol is the optimal carbon source for PHAs biosynthesis from the viewpoint of both economic and environmental with the material cost of 0.362 USD$/kg PHA and total PEI of 1.404. Agreeing with literature, carbon sources have shown to dominate the major share of raw material cost. For potential environmental impacts, crude and refined glycerol may cause formation of photochemical smog, while soybean oil and WCO might endanger aquatic life if leaked into water body. Insight from the sensitivity analysis has showed that this integrated assessment is sensitive to PHAs yield and price of the substrate, while giving similar results when using different multi-objective optimization tools. This integrated economic and environmental assessment has successfully proved to aid in providing a rapid preliminary analysis of carbon sources for PHAs biosynthesis.

Aiming to integrate upstream fermentation and downstream purification into fewer steps, extractive bioconversion of PHAs via thermoseparation-based ATPE was developed. The results proved that the thermoseparating ATPS demonstrated a huge potential as it is able to sustain stable cultivation of *C. necator* and support PHB accumulation. Under the condition of 5 wt/wt % EOPO 3900 concentration, 30 °C fermentation temperature and pH 6, the strategy achieved highest DCW and PHB content of 2.02 g/L and 21.8 % respectively with a yield and PF of 97.6 % and 1.36 fold respectively. Scaling up to 2L bioreactor, the biomass and PHB content reached a maximum of 5.77 g/L and 0.389 g/L respectively with a yield and PF of 96.7 % and 1.36 fold respectively. Thus, it can be concluded that that the scaling-up can be easily and reliably to be predicted from experimental data.

Chapter 7

ECONOMIC AND ENVIRONMENTAL ANALYSIS OF PHAs PRODUCTION PROCESS

7.1 INTRODUCTION

As mentioned in previous chapters, ATPE serves as a primary integrated recovery step of isolation, purification and concentration which partially removing the impurities from the target product and reducing the subsequent downstream processing volume. Other than providing a mild environment for bioseparation, ATPE also utilizes safe and relatively environmental-friendly phase-forming components, consumes little time and energy as well as the scale-up can be predicted reliably from experimental data. However, there are doubts that does utilization of thermoseparating ATPE as primary recovery step really provide an economic competitive edge as well as arguments that it being not environmental-friendly enough. Henceforth, there is a need to perform a detailed economic analysis to evaluate the economic performance of PHAs purification and recovery via thermoseparating ATPE.

For environmental performance evaluation, life cycle analysis (LCA) has been widely utilized for the environmental performance assessment of products and services. However, LCA can be very tedious or not suitable to be applied at some process design stages due to insufficient data and the needs of rigorous calculation. Therefore, some researchers focus on certain part of the life cycle by applying the concept of LCA and developed sets of indicators and indexes based on the sustainability concept, such as Sustainable Process Index (SPI) (Krotscheck and Nardolawsky, 1996), Waste Reduction (WAR) algorithm (Young and Cabezas, 1999), Environmental Fate and Risk Assessment Tool (Shonnard and Hiew, 2000), Eco-indicator 99 (Goedkoop and Spriensma, 2001), GreenPro (Khan et al., 2001), IChemE sustainability metrics (IChemE, 2002) and others. The researchers have also applied LCA on PHA's biosynthesis, comparison with conventional plastics and other bioplastic, and from many different points of view (Hyde, 1998, Grengross and Slater, 2000, Akiyama et al., 2003, Harding et al., 2007a, Kim and Dale, 2005, Kim and Dale, 2008, Pietrini et al., 2007, Gurieff and Lant, 2007a, Heimersson et al., 2014, Khoo and Tan, 2010, Zhong et al., 2009). Nevertheless, agree with literature (Heimersson et al., 2014), researchers have always

reported environmental impacts in terms of carbon dioxide equivalent emissions (global warming potential) and/or energy consumptions, especially the early LCA studies of PHAs (Gurieff and Lant, 2007; Miller et al., 2013; Fernandez-Dacosta et al., 2015). These are not comprehensive enough and hardly cover every aspect, include smog formation, ozone depletion, acid rain formation, water pollution, and other pollutions.

Henceforth, in the final section of the research, it is essential to design the PHAs production process using thermoseparation ATPE as primary recovery strategy and evaluate its performance as well as compare with other isolation and purification strategies as there has not been any study reported on this to date. Furthermore, it is important to pinpoint the processing steps that can be further improved. Economic and environmental analysis of the production and recovery of PHAs by thermoseparating ATPE will allow for the industrial-scale PHA production price estimation and for the evaluation of the economic and environmental feasibility of PHA production. In this chapter, both economic and environmental performance of PHAs production process with thermoseparating ATPE as primary recovery step was evaluated and compared with other purification and recovery strategy. Computer-aided process simulation has been utilized as planning, design, optimization and evaluation tool of chemical process. Full-scale process flowsheets of PHA production processes were drawn designed with concrete simulation basis and detailed explanation on the process flow provided. Detailed cost analysis was utilized to calculate equipment, raw material and utility cost, PHAs selling price, and profitability analysis as well as identify economy "hot-spots". WAR algorithm which was selected as the environmental indicator not only requires minimum amount of data, it also covers a wide range of environmental aspects.

7.2. MATERIALS AND METHODS

7.2.1 Process analysis tool

The conceptual process design follows a hierarchical design strategy based on heuristics and experience, to develop the processing flowsheet. Process analysis and

economic evaluation for PHAs biosynthesis and recovery were carried out using SuperPro Designer v6.0 from Intelligen Inc. (Scotch Palins, NJ, USA). Microbial biomass was modeled as dextrose (Choi et al., 2010), while PHA was modeled as repeating unit of PHA ($C_4H_6O_2$) and the analysis did not account for polymerization energy. Two different process flowsheets for the biosynthesis and recovery of PHAs are shown in Figure 7.1a and b. The first one is extractive bioconversion of PHAs via thermoseparating polymer-based ATPS coupled with recovery using surfactant and sodium hypochlorite digestion, while the latter is a conventional PHAs production with recovery using surfactant and sodium hypochlorite digestion. Some equipment and other secondary pieces such as valves and piping are omitted for simplicity in the flowsheet but are taken into consideration in the economic analysis. Pumps are provided wherever necessary. For all assessment, the system boundary only covered the "gate-to-gate" analysis from upstream PHAs biosynthesis to the final product of PHAs powder formation.

7.2.2 Raw materials

The media of PHAs biosynthesis consist of glycerol as a carbon source and various inorganic salts. The cost of inorganic salts was excluded due to the insignificant share in the final production cost of PHAs (Choi and Lee, 1997). *Cupriavidus necator* H16 was the bacterial strain selected for this analysis.

7.2.3 Fermentation

Fermentation medium was prepared in a blending tank (P-12) was sterilized in a continuous heat sterilizer (P-13). Then, the medium and air were supplied to fermenter (P-2) for 42 hours of fermentation period at 30 ℃. After the cultivation, fermentation broth was moved to the holding tank (P-14).

7.2.4 Recovery of PHAs by thermoseparation coupled with surfactant-hypochlorite digestion (Figure 7.1a)

Culture broth collected in the holding tank was transferred to the thermoseparation tank (P-3 and P-4) for thermoseparation at 60 ℃ for 15 minutes residence time. The PHAs after thermoseparation achieved a purification factor of 1.42 and recovery yield of 94.8 % based on results in the previous chapter. 95 % of thermoseparating polymer was assumed to thermoseparate to the bottom phase which makes up approximately 60 wt/wt % of bottom phase with remainder is mostly water. Afterward, the bottom phase was sent for wastewater treatment, while the top phase consisted of mostly water and the PHAs product was transferred to a centrifuge (P-4) to separate PHAs from aqueous solution. Centrifugation also removes soluble cellular materials (especially nucleic acids) to improve the efficiency of subsequent downstream processing and reduce the viscosity of process fluid (Van Wegen et al., 1998).

Following that, surfactant solution (10 % w/v) was added to the outflow of aqueous solution from centrifuge with surfactant to biomass ratio 1:3 and mixed at 55 ℃ for 15 minutes of mean residence time to obtain recovery yield of 86.6 % and purity of 98 % (Dong and Sun, 2000). PHAs were then separated from the aqueous solution containing dissolved NPCM by centrifugation (P-7). The PHAs was then cleaned with 30 % wt/v sodium hypochlorite solution to achieve purity of 99 % with a yield of 95 % (Fernandez-Dacosta et al., 2015a, Ramsay et al., 1990). After centrifugation (P-8), the PHAs granules were then washed with an equal volume of water (P-9 and P-10) (Yu and Chen, 2006) and were finally spray dried (P-11) using continuous spray drying system to obtain PHAs products of 99.9 wt/wt % purity. This process will be referred as process 1 throughout the chapter.

7.2.5 Recovery of PHAs by surfactant-hypochlorite digestion (Figure 7.1b)

The process is similar as above except the culture broth was directly transferred to continuous centrifugation (P-4) after the holding tank skipping the thermoseparation step. The PHAs recovery strategy of surfactant-hypochlorite was employed in this study as it was demonstrated to be more economical and environmental-friendly than the others (Choi and Lee, 1997, Jacquel et al., 2008, Posada et al., 2011, Fernandez-Dacosta et al., 2015b). This process will be referred as process 2 throughout the chapter.

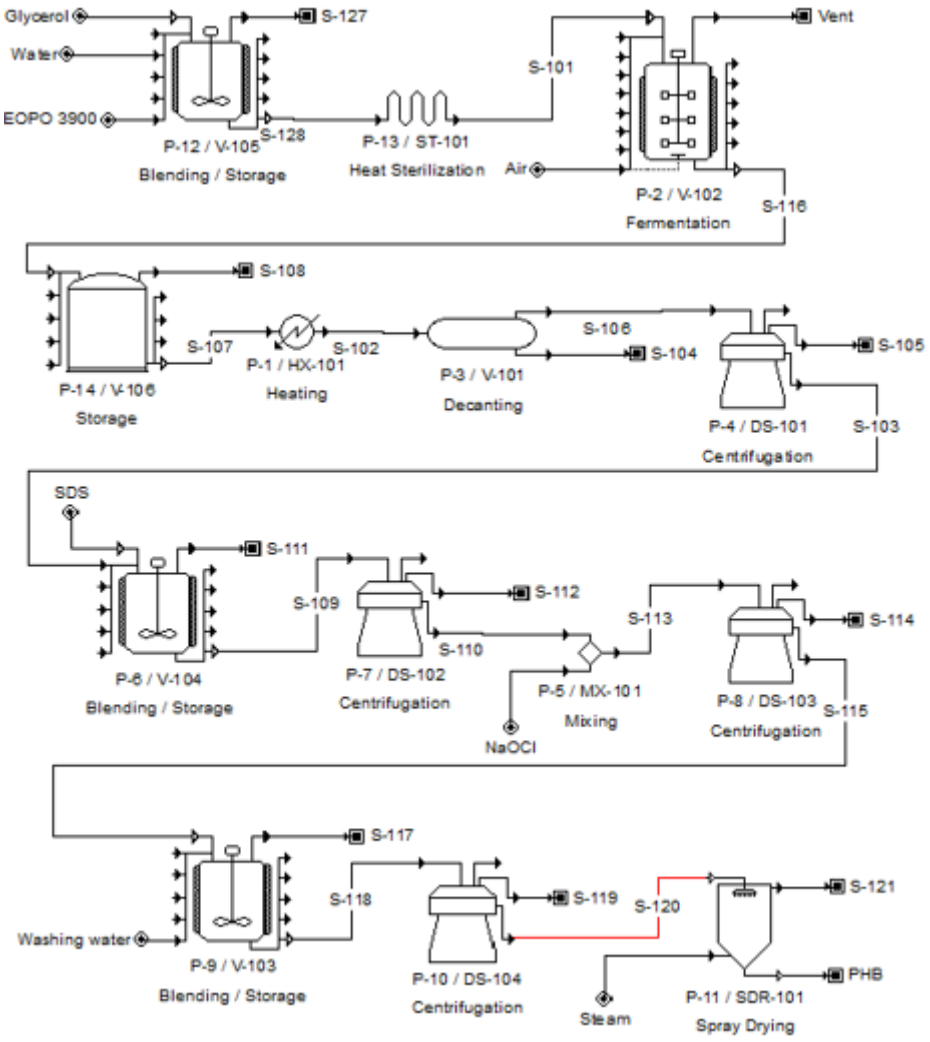
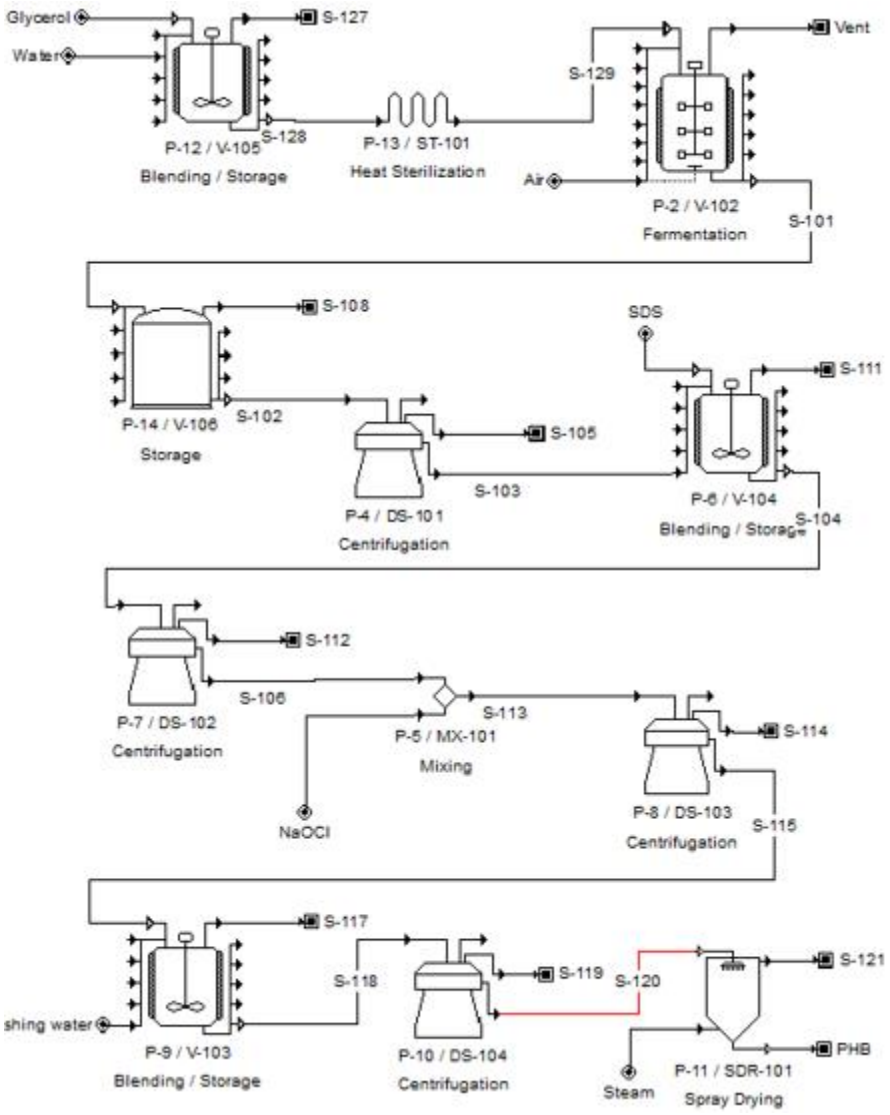


Figure 7.1a. Process flowsheet of PHAs production using recovery by thermoseparation coupled with surfactant-hypochlorite digestion (Process 1)



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2731 Figure 7.1b. Process flowsheet of PHAs production using recovery by surfactant-hypochlorite

2732 digestion (Process 2)

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7.2.6 Economic Analysis

All costs in this chapter are given in 2016 US$. This analysis was estimated for a 10-year period at an annual interest rate of 6 %, considering the straight line depreciation method and a 40 % income tax. The price of carbon source and other major raw materials were as following in US$/kg: glycerol, 0.5; EOPO 3900, 1.0; surfactant (SDS), 0.8; hypochlorite solution, 0.2; water, 0.0012. Also, the prices used for electricity, water and low-pressure vapor were 0.03044 US$/kWh, 0.0004 US$/kg and 0.0042 US$/kg, respectively. The labor cost used for operatives was assumed to be 25US$/man-hour. The above-mentioned software has been used to estimate the capital costs of process units and the operating cost as well as perform the profitability analysis.

7.2.7 Environmental Assessment

Based on the potential environmental impact (PEI) balance concept introduced by Hilaly and Sikdar (Hilaly and Sikdar, 1995), Young and Cabezas (Young and Cabezas, 1999) have introduced WAR algorithm that focuses on waste minimization across the process boundary. With an extensive chemicals database, WAR algorithm covers four local toxicological impact categories which are human toxicity potential by ingestion (HTPI), terrestrial toxicity potential (TTP), human toxicity potential by either inhalation or dermal exposure (HTPE), and aquatic toxicity potential (ATP) and four global atmospheric impacts which are global warming potential (GWP), ozone depletion potential (ODP), photochemical oxidation or smog formation potential (POP), and acidification or acid-rain potential (AP). Using WAR algorithm, the environmental performances for both process were evaluated with the most potential hazard equipment was identified.

7.2.8 Sensitivity Analysis

Sensitivity analysis was conducted to investigate the influence of different operating costs on the PHAs price, and to identify the opportunity for further improvement. For these purposes, 4 different cases were considered where the most critical and influencing factors were manipulated using spreadsheet software, Excel 2013 (Microsoft, Redmond, WA, USA).

7.3. RESULTS AND DISCUSSION

7.3.1 Material and Energy Flows

The targeted amount of PHAs production in the simulations was 9,000 tons per year. The total operating hour was assumed to be 7,920 hours per year. Considering 42 hours of fermentation period added with the 12 hours of turnaround time which required for cultivation tank cleaning and recharging, 146 fermentation runs can be carried out per year. These values were used to determine the material flow as well as estimate the sizes of the processing equipment. Table 7.1 presents the summary of material and energy flows for both processes, while Table 7.2 a and b tabulate the summary of material flow in each stream. Based on the process flowsheet, 190.3 tons of glycerol are required as carbon source per batch to achieve 9,000 tons of annual PHAs production. It is clearly shown that process 2 requires almost 10 folds of surfactant and hypochlorite solution compared to process 1. For utility usage, both processes consume slightly over 25,000 MWh of electricity, 129 kilotons of chilled water and approximately 50 kilotons of steam per year. For material flow, the two processes have similar mass flowrate at the fermentations stage with process 1 has additional EOPO 3900, while process 2 has a higher mass flowrate at the downstream processing due to a large amount of surfactant and hypochlorite solution utilized.

Table 7.1. Material and energy flows per batch for 9,000 tons PHAs production per annum using two different recovery strategies

Input (to/batch)	Process 1	Process 2	Outputs (ton/batch)	Process 1	Process 2
			Carbon		
Glycerol	190.3	190.3	dioxide	29.5	29.5
Oxygen	43.3	43.3	Biomass	70.3	70.4
Surfactant (SDS)	3.1	46.3	Oxygen	1.5	1.5
Hypochlorite					
solution	0.9	9.7	PHAs	68.4	68.5
Water	576.4	1,031.70	Water	639.9	1,095.30
EOPO 3900	38.2	0.0			
Energy (per year)					
			Electricity		
Chilled water (ton)	129,302	129,267	(MWh)	25,602	27,290
Steam (ton)	52,630	47,914			

Table 7.2a. Material flows for each stream for process 1 (in tons/batch)

Chemicals	Glycerol	Water	EOPO 3900	S-128	S-101	Air	Vent	S-116	S-107
PHAs								68.4	68.4
Biomass								70.3	70.3
Water		535.6		535.6	535.6		30	569.2	569.2
Glycerol	190.3			190.3	190.3				
Oxygen						43.3	1.5		
CO2							29.5		
EOPO 3900			38.2	38.2	38.2			38.2	38.2

Chemicals	S-102	S-106	S-104	S-105	S-103	SDS	S-109	S-112	S-110
PHAs	68.4	64.8	3.6		64.8		64.8	8.7	56.2
Biomass	70.3	27.7	42.6		27.7		27.7	26.6	1.2
Water	569.2	514.7	54.5	514.7	0.05	27.8	27.8	27.9	
SDS						3.1	3.1	3.1	
EOPO 3900	38.2	1.9	36.3	1.9					

Chemicals	NaOCl	S-113	S-114	S-115	Washing Water	S-118	S-119	S-120	PHB
PHAs		56.2	2.8	53.4		53.4		53.4	53.4
Biomass		1.2	0.6	0.6		0.6		0.6	0.6
Water	2.1	2.1	2.1		54.8	54.8	54.8		
NaOCl	0.9	0.9	0.9						

2792 Table 7.2b. Material flows for each stream for process 2 (in tons/batch)

Chemicals	Glycerol	Water	S-128	S-129	Air	S-101	Vent	S-102
PHAs						68.5		68.5
Biomass						70.4		70.4
H_2O		535.6	535.6	535.6		569.3	30	569.3
Glycerol	190.3		190.3	190.3				
O_2					43.3		1.5	
CO_2							29.5	
NaOCl								

Chemicals	S-105	S-103	SDS	S-104	S-112	S-106	NaOCl	S-113
PHAs		68.5		68.5	9.2	59.3		59.3
Biomass		70.4		70.4	69.2	1.2		1.2
H_2O	559.2		416.6	416.6	416.6	0.04	22.6	22.6
SDS			46.3	46.3	46.3			
NaOCl							9.7	9.7

Chemicals	S-114	S-115	Washing Water	S-118	S-119	S-120	PHB	
PHAs	3	56.3		56.3		56.3	56.3	
Biomass	0.6	0.6		0.6		0.6	0.6	
H_2O	22.6		56.9	56.9	56.9			
NaOCl	9.7							

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7.3.2 Economic analysis

By integrating a holding tank preceding the recovery process, fermentation and recovery process could be decoupled. Thus, the entire purification process can be carried out continuously during the fermentation plus its turnaround period. Increasing the production capacity has shown to be able to reduce total capital and operating cost of PHAs production process (results not shown). The capital cost included: cost of rotary and static equipment, piping, wastewater treatment system, etc. On the other hands, the operation and maintenance (O&M) cost included: cost of chemicals, manpower, utilities, wastewater treatment, maintenance and insurance. Table 7.3a and b summarize the specification and purchase costs of the major equipment for both process. To reduce the equipment cost and optimize the operating cost, the sizes of the process unit in the isolation and recovery operations were adjusted so that the entire recovery process could be operated in 54 hours. As shown from the tables, much of the capital cost is due to the fermenter and the centrifuges. Based on these results, the fixed capital cost, the annual operating cost, and profitability analysis were estimated and summarized in Table 7.4a and b respectively. For the direct cost, total (f.o.b) equipment costs were calculated to be USD$ 22.5 million and USD$ 27.5 million for process 1 and 2 respectively. The capital cost of process 1 and 2 are USD$ 130.6 million and USD$ 160.1 million respectively, while the annual operating cost for process 1 and 2 are USD$ 51.7 million and USD$ 57.9 million respectively.

The profitability analysis was done based on the assumption of selling price of PHAs at 10.0 US$/kg. The production cost of PHAs for process 1 and 2 is 5.77 US$/kg and 6.12 US$/kg, respectively at this production scale. The price obtained was comparable to those reported in literature, with examples of 5.35 US$/kg at production scale of 4,300 tons for PHA production from *E. coli* using dairy whey (Van Wegen et al., 1998), 6.14 US$/kg at production scale of 2,850 tons (Choi and Lee, 1997), 8.32 US$/kg at production scale of 100,000 tons (Lee and Choi, 1998), , 5-10 €/kg for PHA production from whey (Koller et al., 2007), and 5.38

USD$/kg at fermentation capacity of 1,000 m^3/day (Mudliar et al., 2007). Due to the high amount of surfactant and sodium hypochlorite used in process 2, the wastewater treatment cost also doubled compared to process 1. Therefore, it proved that utilizing thermoseparation-based ATPE as primary recovery step not only helps to reduce chemical cost, it also minimizes downstream equipment cost and wastewater treatment cost. At the selling price assumed, it was shown that PHAs production using thermoseparation recovery strategy have a payback period of fewer than 4 years and ROI of 25.2 %, while the surfactant-hypochlorite recovery strategy requires more than 4 and half years with ROI of 21.8 %. This is due to the higher facility-dependent cost which includes depreciation, equipment maintenance, insurance, local taxes and factory expenses.

Table 7.3a. Major equipment specification and purchase cost (based on 2016 year) to achieve 9,000 tons PHAs production per annum using the recovery strategy of thermoseparation coupled with surfactant-hypochlorite digestion.

Equipment (Quantity)	Description	Cost ($)
Fermenter P-2 (1)	Volume = 321.3 m^3	9,774,000
	Power = 2189 kW	
Blending Tank P-12 (1)	Volume = 80.9 m^3	506,000
Heat Sterilizer P-13 (1)	Diameter = 1.2 m	758,000
	Length = 12.8 m	
Disk-Stack Centrifuge P-4 (6)	Sigma factor = 188335 m^2	3,498,000
	Power = 648 kW	
Flat Bottom Tank P-14 (2)	Volume = 117.6 m^3	166,000
Spray Dryer P-11 (1)	Dryer diameter = 0.127 m	115,000
	Dryer height = 0.382 m	
Thermoseparation Tank P-1 & P-3 (1)	Volume = 46.2 m^3	278,000
Blending Tank P-6 (2)	Volume = 67.7 m^3	948,000
	Power = 12kW	
Disk-Stack Centrifuge P-7 (3)	Sigma factor = 154435 m^2	1,569,000
	Power = 121 kW	
Disk-Stack Centrifuge P-8 (2)	Sigma factor = 196405 m^2	1,192,000
	Power = 76 kW	
Blending Tank P-9 (1)	Volume = 36.8 m^3	390,000
	Power = 6 kW	
Disk-Stack Centrifuge P-10 (2)	Sigma factor = 145513 m^2	1,014,000
	Power = 65 kW	

Table 7.3b. Major equipment specification and purchase cost (based on 2016 year) to achieve 9,000 tons PHAs production per annum using the recovery strategy of surfactant-hypochlorite digestion.

Equipment (Quantity)	Description	Cost ($)
Fermenter P-2 (3)	Volume = 305.3 m^3	9,519,000
	Power = 2073 kW	
Blending Tank P-12(1)	Volume = 76.6 m^3	496,000
Heat Sterilizer P-13 (1)	Diameter = 0.8 m	747,000
	Length = 15.6 m	
Disk-Stack CentrifugeP-4 (6)	Sigma factor = 214650 m^2	3,750,000
	Power =365 kW	
Flat bottom tank P-14 (1)	Volume = 111.5 m^3	159,000
Spray dryer P-11(1)	Dryer diameter = 0.3 m	115,000
	Dryer height = 0.9 m	
Blending TankP-6 (9)	Volume =73.5 m^3	4,392,000
	Power = 60 kW	
Disk-Stack CentrifugeP-7 (5)	Sigma factor = 218230 m^2	3,155,000
	Power = 285 kW	
Disk-Stack Centrifuge P-8(2)	Sigma factor =132394 m^2	964,000
	Power = 65 kW	
Blending Tank P-9(1)	Volume = 63.8 m^3	465,000
	Power = 6 kW	
Disk-Stack Centrifuge P-10 (2)	Sigma factor = 144942 m^2	1,012,000
	Power = 67kW	

Table 7.4a. Economic analysis of PHAs production (9,000 tons per annum) using the recovery strategy of thermoseparation coupled with surfactant-hypochlorite digestion.

FIXED CAPITAL ESTIMATE SUMMARY (2016 prices)	
A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)	Cost ($)
a. Equipment Purchase Cost (PC)	22,454,000
b. Installation (summed over all units)	6,287,000
c. Process Piping (0.35 X PC)	7,859,000
d. Instrumentation (0.40 X PC)	8,982,000
e. Insulation (0.03 X PC)	674,000
f. Electrical (0.10 X PC)	2,245,000
g. Buildings (0.45 X PC)	10,104,000
h. Yard Improvement (0.15 X PC)	3,368,000
i. Auxiliary Facilities (0.40 X PC)	8,982,000
	70,955,000
B. TOTAL PLANT INDIRECT COST (TPIC)	
a. Engineering (0.25 X TPDC)	17,739,000
b. Construction (0.35 X TPDC)	24,834,000
	42,573,000
C. OTHER COSTS (OTC)	
a. Contractor's fee (0.05 X (TPDC + TPIC))	5,676,000
b. Contingency (0.10 X (TPDC + TPIC))	11,353,000
	17,029,000
D. DIRECT FIXED CAPITAL (DFC)	
TPDC + TPIC + OTC	130,557,000

ANNUAL OPERATING COST (2016 prices)	Cost ($)

A. RAW MATERIALS	
a. Glycerol	15,982,000
b. Water	116,000
c. Surfactant (SDS)	415,000
d. Hypochlorite solution	31,000
e. EOPO 3900	6,418,000
	22,962,000
B. LABOR-DEPENDENT ITEMS	
a. Lumped operating labor (57,370 h X 57.5 $/h)	3,299,000
B. Laboratory (0.15 X a)	494,850
	3,793,850
C. FACILITY-DEPENDENT ITEMS	23,858,000
D. OTHER CONSUMABLES	0
E. UTILITIES	1,052,081
F. WASTE TREATMENT/DISPOSAL	61,000
Total annual operating cost	51,726,931

PPROFITABILITY ANALYSIS (2016 prices)	Cost ($)
A. Direct Fixed Capital	130,557,000
B. Working Capital	2,490,000
C. Startup Cost	6,528,000
D. Total Investment	139,575,000
	Cost ($/year)
E. Revenues	
PHAs	89,619,000
F. Annual Operating Cost	51,727,000

G. Gross Profit (E-F)	37,892,000
H. Taxes (40 %)	15,156,800
I. Net Profit (G-H + Depreciation)	35,138,000
J. Return On Investment (ROI)	25.18 %
K. Payback Period	3.97 years
L. IRR (After Taxes)	21.48 %
M. NPV (at 7.0 % interest)	130,369,000 $

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2843 Table 7.4b. Economic analysis of PHAs production (9,000 tons per annum) using the
2844 recovery strategy of surfactant-hypochlorite digestion.

FIXED CAPITAL ESTIMATE SUMMARY (2016 prices)	
A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)	Cost ($)
a. Equipment Purchase Cost (PC)	27,535,000
b. Installation (summed over all units)	7,710,000
c. Process Piping (0.35 X PC)	9,637,000
d. Instrumentation (0.40 X PC)	11,014,000
e. Insulation (0.03 X PC)	826,000
f. Electrical (0.10 X PC)	2,753,000
g. Buildings (0.45 X PC)	12,391,000
h. Yard Improvement (0.15 X PC)	4,130,000
i. Auxiliary Facilities (0.40 X PC)	11,014,000
	87,010,000
B. TOTAL PLANT INDIRECT COST (TPIC)	
a. Engineering (0.25 X TPDC)	21,752,000
b. Construction (0.35 X TPDC)	30,453,000
	52,205,000
C. OTHER COSTS (OTC)	
a. Contractor's fee (0.05 X (TPDC + TPIC))	6,961,000
b. Contingency (0.10 X (TPDC + TPIC))	13,922,000
	20,883,000
D. DIRECT FIXED CAPITAL (DFC)	
TPDC + TPIC + OTC	160,098,000

ANNUAL OPERATING COST (2016 prices)	Cost ($)

A. RAW MATERIALS	
a. Glycerol	15,982,000
b. Water	208,000
c. Surfactant (SDS)	6,221,000
d. Hypochlorite solution	325,000
	22,736,000
B. LABOR-DEPENDENT ITEMS	
a. Lumped operating labor (70,861 h X 57.5 $/h)	4,075,000
c. Laboratory (0.15 X a)	611,250
	4,686,250
C. FACILITY-DEPENDENT ITEMS	29,256,000
D. OTHER CONSUMABLES	0
E. UTILITIES	1,083,663
F. WASTE TREATMENT/DISPOSAL	122,000
Total annual operating cost	57,883,913

PPROFITABILITY ANALYSIS (2016 prices)	Cost ($)
A. Direct Fixed Capital	160,098,000
B. Working Capital	2,555,000
C. Startup Cost	8,005,000
Total Investment	170,658,000
	Cost ($/year)
E. Revenues	
PHAs	94,656,000
F. Annual Operating Cost	57,883,000
G. Gross Profit (E-F)	36,772,000

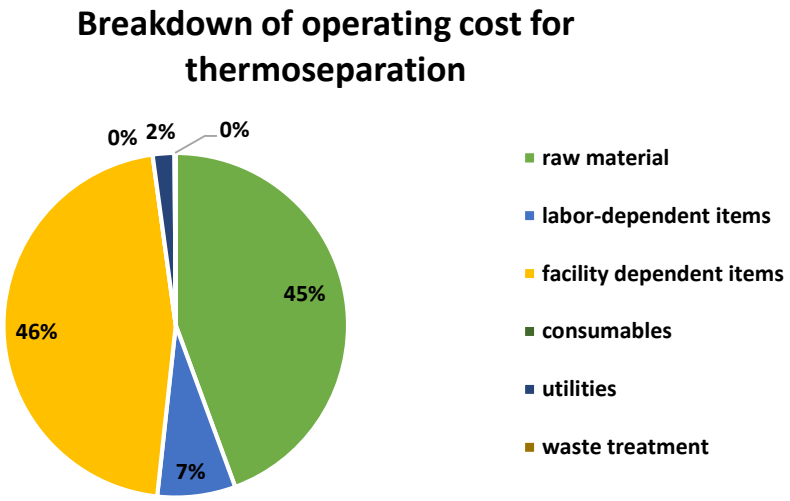
H. Taxes (40 %)	14,708,800
I. Net Profit (G-H + Depreciation)	37,273,000
J. Return On Investment (ROI)	21.84 %
K. Payback Period	4.58 years
L. IRR (After Taxes)	18.05 %
M. NPV (at 7.0 % interest)	117,213,000 $

Table 7.4c: Summary of comparison between Process 1 and Process 2

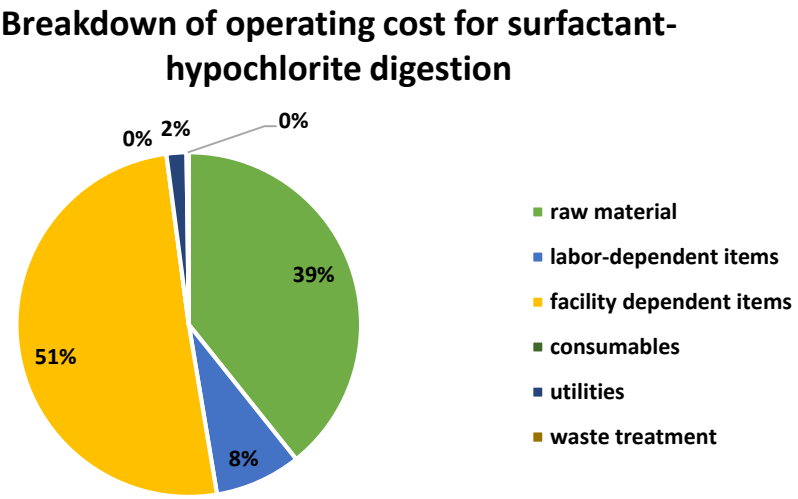
	Process 1 (with thermoseparation)	Process 2 (without thermoseparation)
Total (f.o.b) equipment cost	USD$ 22.5 million	USD$ 27.5 million
Capital cost	USD$ 130.6 million	USD$ 160.1 million
Annual operating cost	USD$ 51.7 million	USD$ 57.9 million
Production cost	5.77 USD$/kg	6.12 USD$/kg
Payback period	> 4 years	< 4.5 years
ROI	25.2 %	21.8 %

Table 7.4c has summarized the comparison between economic feasibility of process 1 and 2. Process 1 has shown to be much cost efficient compared to Process 2. Figure 7.2 a and b depict the breakdown of operating cost for PHAs production using the two different recovery strategies. For process 1, contribution of both raw material cost and facility-dependent cost are almost the same (45 % and 46 % respectively). The high raw material cost is due to the high cost of EOPO 3900 which can be further reduced if it is recycled as EOPO 3900 can be recycled up to twice as reported in previous chapter. On the other hands, facility-dependent cost dominated operating cost for process 2 with 51 % share, while raw material cost contributed 39 %. The cost of the carbon source also contributes significantly

as glycerol alone was as high as 30.9 % and 27.6 % of total operating cost for process 1 and 2 respectively. This was comparable to those as reported in literature, such as 30.7 % for PHAs production by recombinant E. coli utilizing glucose as carbon source (Choi and Lee, 1997) and up to 45 % as mentioned by Posada and coworkers (Posada et al., 2011). Henceforth, utilizing a cheaper carbon source such as cheese whey, waste cooking oil, cane and beet molasses, sludge waste, POME and others has the potential to reduce the final PHAs production price significantly. Nevertheless, it must be keep in mind that PHAs content, productivity, yield and downstream recovery process should be considered as well when using cheaper carbon source.



A.



B.

Figure 7.2. The breakdown of operating cost for PHAs production (9,000 tons per annum) using the recovery strategy: (A) thermoseparation coupled with surfactant-hypochlorite digestion and (B) surfactant-hypochlorite digestion.

7.3.3 Environmental analysis

Following the rising concern of community and government on the environment, minimizing the environmental impact of PHA production process is a necessity other than achieving economic competitiveness. Among range of indicators stated in "Introduction" section, SPI (Koller et al., 2013), Tools for the Reduction and Assessment of Chemical and Other Environmental Impacts model (Kim and Dale, 2004), CML 2 Baseline 2000 v2.03 assessment method (Harding et al., 2007b) and Eco-indicator 99 (Zhong et al., 2009) have been used to evaluate the environmental performance of PHAs production aside from those that utilized complete LCA study. In this chapter, environmental analysis was done to evaluate and compare the environmental performance of both process using WAR algorithm due to its wide-covering environmental impacts and comprehensive chemical database. There are 7 main chemicals which involved in the processes, including glycerol, water, oxygen, carbon

dioxide, SDS, sodium hypochlorite solution and EOPO 3900. However, it should be noted that the environmental data on SDS and EOPO 3900 is not available. Being toxic to human (through inhalation) as well as terrestrial and aquatic life, sodium hypochlorite solution is the most hazardous chemical presents in the PHA production process. Since the consumption for both processes are almost similar for most chemical except for SDS, hypochlorite solution and EOPO 3900, it is obviously that process 2 has a worse environmental performance as it utilizes almost 10 fold hypochlorite solution compared to process 1. Therefore, it is advised that the recycling of hypochlorite solution should be done as much and as efficient as possible.

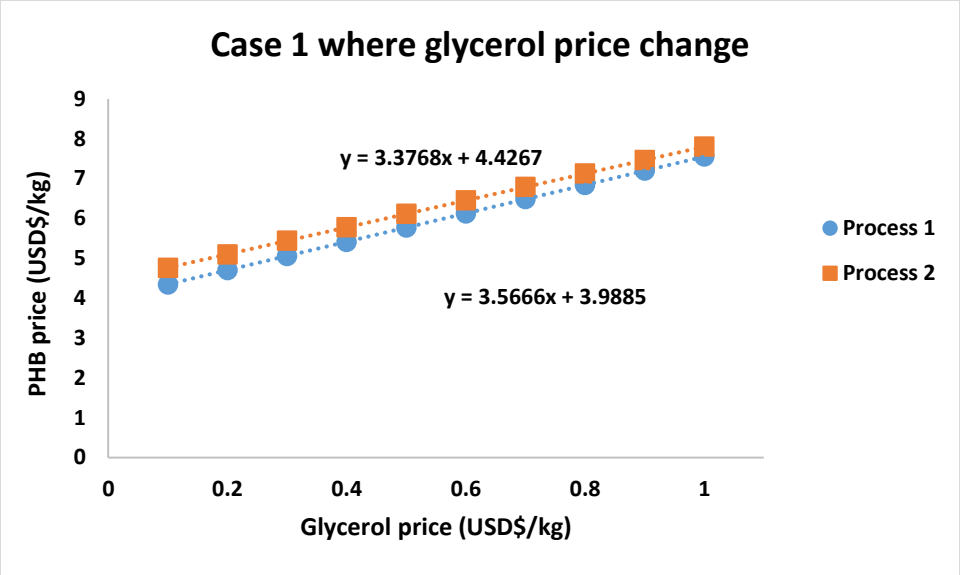
On the other hand, the chemical which brought the most negative impacts on the environment is glycerol due to the amount utilized. Glycerol dominates as much as 99.7 % and 97.2 % of environmental impacts for process 1 and 2 respectively. Other than causing harm to human via inhalation and exposure as well as terrestrial and aquatic life, glycerol also causes the formation of photochemical smog. Careful handling of chemical and excellent ventilation is required to avoid any accident or leakage to the surroundings. Other than that, carbon dioxide produced during fermentation is one type of greenhouse gases and contributes to global warming. Henceforth, the fermenter is the piece of equipment among all the process units that needed most attentions to minimize environmental impacts of the process.

7.3.4 Sensitivity analysis

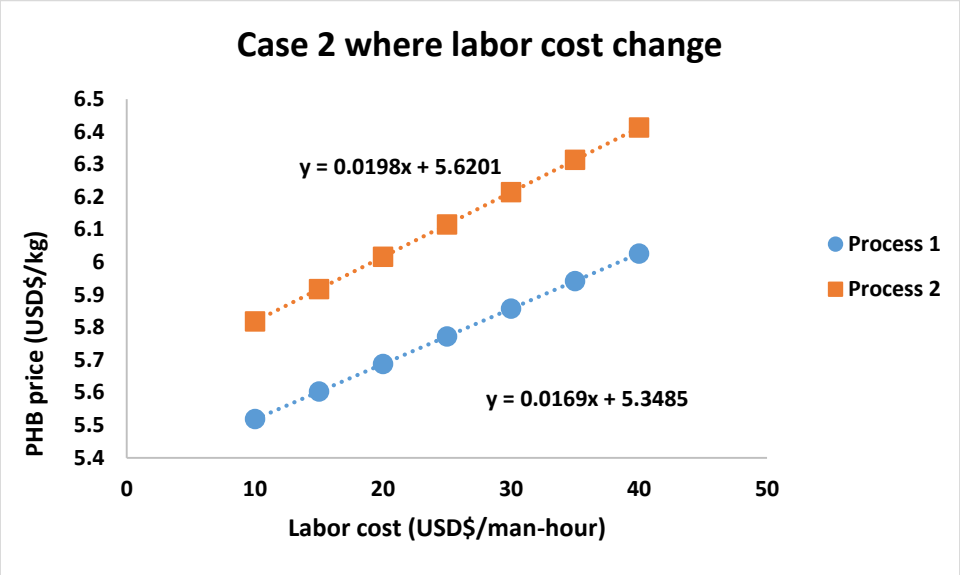
Aiming to investigate the consequences of "what if?" scenarios, a sensitivity analysis was conducted by considering the main parameters that influence economic performance of PHA production by different recovery strategies. Four different cases were considered as the parameters contributes to significant share of operating cost and might have an influence on the result when the value of the parameters. The results of the 4 cases were displayed in Figure 7.3 a, b, c and d.

In case 1, the price of carbon source, which is glycerol was varied from 0.1 to 1.0 USD$/kg due to it significant contribution to operating cost as mentioned in previous section. As predicted, the PHAs price of both processes increase linearly with similar increment (0.34 to 0.36 USD$/kg PHAs increment per 0.1 USD$/kg glycerol price increased) as the glycerol price increase. On the other hand, the labor cost was varied from 10 to 40 USD$/man-hour in case 2 to investigate the effect of fluctuation of labor cost on PHAs production price. Though lesser than previous case, manipulating labor cost still has a significant influence on PHAs price for both processes. Similarly, the price of electricity was manipulated from 0.01 to 0.055 USD$/kWh in case 3 to study the sensitivity of PHAs price towards fluctuation of electricity price. Compared to previous 2 cases, PHAs price is much less sensitive to change in electricity price in both processes as insignificant increment in PHAs price is observed over a range of electricity price.

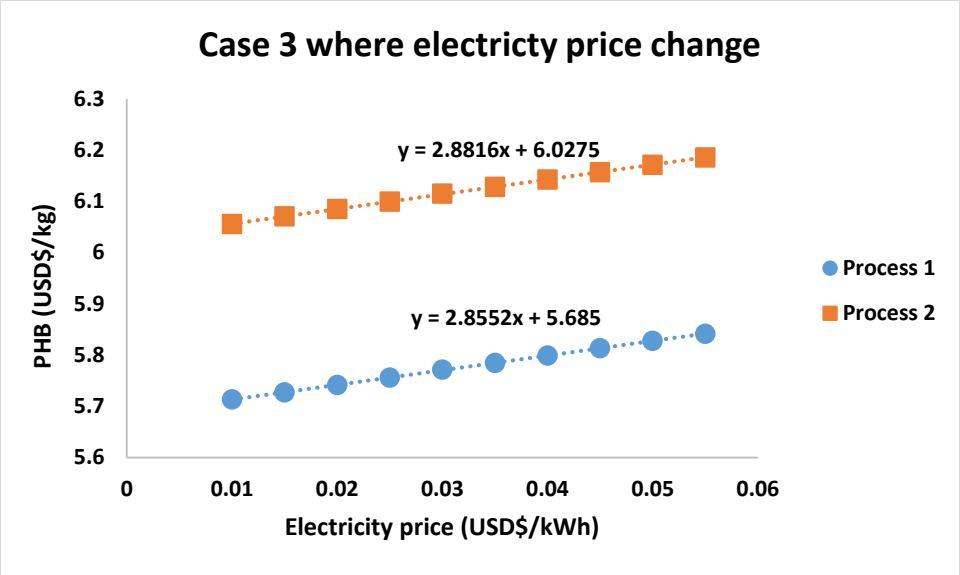
Case 4 is a sensitivity analysis which investigates the impact of change in surfactant price towards PHAs price in both cases. The price of SDS is manipulated from 0.3 to 1.1 USD$/kg. For process 1, The PHAs price is insensitive to change of the SDS price due to small amount of surfactant consumed. However, this is not the case for process 2 as the PHAs price changes drastically as the surfactant price varied. The PHAs prices drops following the fall in surfactant price, even until a point (SDS price ~ 0.35 USD$/kg) where process 2 become more economic favorable than process 1. It should be noted that original price of SDS is 0.8 USD$/kg. Therefore, it is possible for process 2 to outperform process 1 in term of economic performance (though not environmental performance) where a low cost surfactant with similar performance as SDS is present. In conclusion, the cost of carbon source and labor have significant influences on PHAs price for both processes, while the surfactant price is a crucial parameter to evaluate the economic performance of process 2.


Case 1 where glycerol price change
PHB price (USD$/kg)
Glycerol price (USD$/kg)
y = 3.3768x + 4.4267
y = 3.5666x + 3.9885
Process 1
Process 2
0
1
2
3
4
5
6
7
8
9
0
0.2
0.4
0.6
0.8
1

2933


Case 2 where labor cost change
PHB price (USD$/kg)
Labor cost (USD$/man-hour)
y = 0.0198x + 5.6201
y = 0.0169x + 5.3485
Process 1
Process 2
5.4
5.5
5.6
5.7
5.8
5.9
6
6.1
6.2
6.3
6.4
6.5
0
10
20
30
40
50

2934



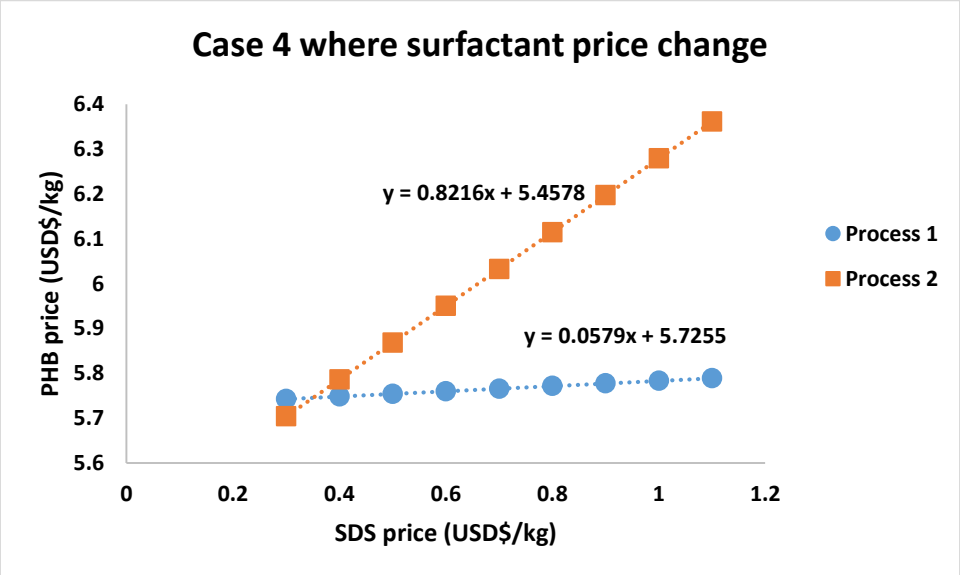


Figure 7.3: Sensitivity analysis where: (A) Case 1 where glycerol price change, (B) Case 2 where labor cost change (C) Case 3 where electricity price change and (D) Case 4 where surfactant price change.

7.4. CONCLUDING REMARKS

In the final part of the research, the PHAs production process using thermoseparation ATPE as primary purification and recovery strategy was design and the performance was evaluated. The evaluations on economic and environmental performance of two PHAs production processes using surfactant-hypochlorite digestion recovery strategy with and without thermoseparation were performed. The results shown that most of O&M cost is contributed by facility-dependent cost and raw material cost, while the main contributor of raw material cost is carbon source.

An integrated approach including process design, economic and environmental assessments identifies PHAs production process with thermoseparating ATPE as primary recovery step outperformed the other in term of both economically and environmentally. PHAs production process which utilize thermoseparation ATPE recovery strategy achieves PHA production cost of 5.77 US$/kg with payback period of less than 4 years and ROI of 25.2 % assuming selling cost of 10 US$/kg.

Insight from the sensitivity analysis has demonstrated that while costs of carbon source and labor have significant influence on PHAs price, the cost of surfactant is still the crucial parameter when comparing performance of both the processes. This proved that utilizing thermoseparation-based ATPE as primary recovery step not only helps to reduce chemical consumption, it also minimizes downstream equipment cost and wastewater treatment cost. It also demonstrated that thermoseparating ATPE is an economical viable and technically feasible PHAs primary purification strategy.

Chapter 8

CONCLUSION AND FUTURE PROSPECTS

8.1 Conclusion

Aiming to develop a cost-effective and environmental-friendly extraction strategy for isolation and recovery of PHAs from *C. necator* H16, the partitioning behavior of biopolymers was first investigated utilizing the thermoseparation-based ATPE technique called cloud point extraction (CPE) strategy. The purification of PHAs utilizing CPE was satisfactorily achieved in the system of 20 wt/wt % EOPO 3900 and 10 mM NaCl addition at thermoseparating temperature of 60 °C with a limit of 37.5 wt/wt % of crude feedstock with a recovery yield of 96.7 % and PF of 1.45 fold. To further improve the final quality of PHAs recovered, thermoseparating polymers have been coupled with ammonium sulfate to isolate and purify biopolymers from a crude feedstock of *C. necator*. Thermoseparating-based ATPE of PHAs could be effectively done under the condition of 14 wt/wt % of EOPO 3900 concentration, 14 wt/wt % of ammonium sulfate concentration and pH 6 without the needs of extra centrifugation steps with yield and purity up to 72 % and 60 %.

Following that, design of experiment (DOE) technique has been used to obtain the optimum PHAs purification condition by thermoseparating ATPE. Using two-level full factorial design, the most influencing factors were the concentration of phosphate salts and EOPO as not only they provided significant positive impacts on most of the responses, while the synergistic positive interaction between the two factors demonstrated greater influences as well. Using Box-Wilson central-composite design, the recovery yield of PHAs from *C. necator* fermented broth achieved as high as 99.9 %, while the PF reached an optimum of 1.43 fold at 17.12 wt/wt % of phosphate concentration and 18.52 wt/wt % EOPO 3900 concentration.

Without adding any fresh phase-forming components, the recycling and reutilization of EOPO 3900 can be done for at least 2 times without significant drop in purification purpose. On the other hands, with the addition of fresh phase-forming components, a total of 4 successive purification cycles with satisfying yield and purification factor were demonstrated.

Thus, it has been shown that thermoseparating ATPE is a more environmental-friendly and economical favorable purification technique for recovery of PHAs.

Using the preliminary integrated economic and environmental assessment developed, the crude glycerol is the optimal carbon source for PHAs biosynthesis from *C. necator* H16. The extractive bioconversion of PHAs via thermoseparation-based ATPE was performed. The results proved that the thermoseparating ATPE demonstrated a huge potential as it is able to sustain stable cultivation of *C. necator* and support PHAs accumulation. Under the condition of 5 wt/wt % EOPO 3900 concentration, 30 °C fermentation temperature and pH 6, biomass and PHB content of 5.77 g/L and 0.389 g/L with a yield and PF of 96.7 % and 1.36 fold was obtained in a 2L bioreactor.

In the final part of the research, the evaluations on the economic and environmental performance of two PHAs production processes with and without thermoseparation as primary purification strategy were performed. The results showed that most of O&M cost is contributed by facility-dependent cost and raw material cost, while the main contributor toraw material cost is carbon source. An integrated approach including process design, economic and environmental assessments identifies PHAs production process with thermoseparating ATPE as primary recovery step outperformed the other in term of both economically and environmentally. PHAs production process which utilizes thermoseparation ATPE recovery strategy achieves PHA production cost of 5.77 US$/kg with a payback period of fewer than 4 years and ROI of 25.2 % assuming selling cost of 10 US$/kg.

The results proved that thermoseparating ATPE is a powerful and potential primary purification step in the total PHA purification train as the technique showed a much higher PHAs recovery yield (approximately 2 times) in comparison with the literature. These results are more promising than the recovery of PHAs by PEG/phosphate system previously reported by Divyashree and his coworkers (Divyashree and Shamala, 2010).

In conclusion, this opens promising standpoints for utilizing thermoseparating ATPE as the primary step in the isolation and purification of PHAs from fermented broth. The economic and environmental evaluation proved that utilizing thermoseparation-based ATPE as primary recovery step not only helps to reduce chemical consumption, it also minimizes downstream equipment cost and wastewater treatment cost. It also demonstrated that thermoseparating ATPE is an economically viable and technically feasible PHAs primary purification strategy.

8.2 Limitation and Future Works

As ATPE is usually not selective enough, it should be remembered that it is not a total solution for isolation, extraction and final separation of bioproducts. Therefore, coupling of thermoseparating ATPE with highly selective yet many expensive techniques such as chromatographic method, enzyme digestion, etc to achieve final desired purity for purification and recovery of PHAs can be studied and optimized.

In this work, mathematical models have been used to fit the ATPS bimodal curves, predict partitioning of PHAs in centrifugation as well as estimate the response variable under the influence of ATPE parameters. However, the work doesn't go into depth on mathematical modeling as it is outside the scope of the research. Henceforth, mathematical models can be developed to understand the mechanism of thermoseparating ATPE and predict partitioning of PHAs.

Moreover, other scope future works for this research includes develop new type of thermoseparating polymer which better properties, such as lower thermoseparating temperature. In order to improve the partition yield, some further improvements can also be considered to be applied such as the complete removal of PHAs and contaminants in phosphate phase using regeneration technique (for example, membrane diafiltration technique) after several extraction cycles. The extractive bioconversion of PHAs can also be studied in a larger scale (e.g. 50L) as well as the design of industrial bioreactor optimal for

extractive bioconversion purpose. With the knowledge and insight obtained, it is sincerely hoped that this research will be able to shed some light on the underlying mechanism of PHA purification by ATPE and build a solid foundation for further study.

Chapter 9

REFERENCES

3063 ABBASILIASI, S., TAN, J. S., IBRAHIM, T. A., KADKHODAEI, S., NG, H. S., VAKHSHITEH, F., AJDARI, Z.,
3064 MUSTAFA, S., LING, T. C., RAHIM, R. A. & ARIFF, A. B. 2014. Primary recovery of a bacteriocin-like
3065 inhibitory substance derived from Pediococcus acidilactici Kp10 by an aqueous two-phase system.
3066 *Food Chem,* 151**,** 93-100.
3067 AGUS, J., KAHAR, P., ABE, H., DOI, Y. & TSUGE, T. 2006a. Altered expression of polyhydroxyalkanoate
3068 synthase gene and its effect on poly[(R)-3-hydroxybutyrate] synthesis in recombinant Escherichia
3069 coli. *Polymer Degradation and Stability,* 91**,** 1645-1650.
3070 AGUS, J., KAHAR, P., ABE, H., DOI, Y. & TSUGE, T. 2006b. Molecular weight characterization of poly[(R)-3-
3071 hydroxybutyrate] synthesized by genetically engineered strains of Escherichia coli. *Polymer*
3072 *Degradation and Stability,* 91**,** 1138-1146.
3073 AGUS, J., KAHAR, P., HYAKUTAKE, M., TOMIZAWA, S., ABE, H., TSUGE, T., SATOH, Y. & TAJIMA, K. 2010.
3074 Unusual change in molecular weight of polyhydroxyalkanoate (PHA) during cultivation of PHA-
3075 accumulating Escherichia coli. *Polymer Degradation and Stability,* 95**,** 2250-2254.
3076 AKARAONYE, E., KESHAVARZ, T. & ROY, I. 2010. Production of polyhydroxyalkanoates: the future green
3077 materials of choice. *Journal of Chemical Technology & Biotechnology,* 85**,** 732-743.
3078 AKARAONYE, E., MORENO, C., KNOWLES, J. C., KESHAVARZ, T. & ROY, I. 2012. Poly(3-hydroxybutyrate)
3079 production by Bacillus cereus SPV using sugarcane molasses as the main carbon source.
3080 *Biotechnol J,* 7**,** 293-303.
3081 AKIYAMA, M., TSUGE, T. & DOI, Y. 2003. Environmental life cycle comparison of polyhydroxyalkanoates
3082 produced from renewable carbon resources by bacterial fermentation. *Polymer Degradation and*
3083 *Stability,* 80**,** 183-194.
3084 ALBERTSSON, P. A. 1986. *Partition of cell particles and macromolecules,* New York, Wiley.

ÁLVAREZ-CHÁVEZ, C. R., EDWARDS, S., MOURE-ERASO, R. & GEISER, K. 2012. Sustainability of bio-based plastics: general comparative analysis and recommendations for improvement. *Journal of Cleaner Production,* 23**,** 47-56.

ANDREESSEN, B., LANGE, A. B., ROBENEK, H. & STEINBUCHEL, A. 2010. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant Escherichia coli. *Appl Environ Microbiol,* 76**,** 622-6.

ASHIPALA, O. K. & HE, Q. 2008. Optimization of fibrinolytic enzyme production by Bacillus subtilis DC-2 in aqueous two-phase system (poly-ethylene glycol 4000 and sodium sulfate). *Bioresour Technol,* 99**,** 4112-9.

AYDOĞAN, Ö., BAYRAKTAR, E., MEHMETOĞLU, Ü., KAEDING, T. & ZENG, A.-P. 2010. Selection and optimization of an aqueous two-phase system for the recovery of 1,3-propandiol from fermentation broth. *Engineering in Life Sciences***,** NA-NA.

AZEVEDO, A. M., ROSA, P. A., FERREIRA, I. F. & AIRES-BARROS, M. R. 2007. Optimisation of aqueous two-phase extraction of human antibodies. *J Biotechnol,* 132**,** 209-17.

AZEVEDO, A. M., ROSA, P. A., FERREIRA, I. F., DE VRIES, J., VISSER, T. J. & AIRES-BARROS, M. R. 2009. Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* 877**,** 50-8.

BANIK, R. M., SANTHIAGU, A., KANARI, B., SABARINATH, C. & UPADHYAY, S. N. 2003. Technological aspects of extractive fermentation using aqueous two-phase systems. *World Journal of Microbiology and Biotechnology,* 2003**,** 227-248.

BARBOSA, J. M., SOUZA, R. L., FRICKS, A. T., ZANIN, G. M., SOARES, C. M. & LIMA, A. S. 2011. Purification of lipase produced by a new source of Bacillus in submerged fermentation using an aqueous two-phase system. *J Chromatogr B Analyt Technol Biomed Life Sci,* 879**,** 3853-8.

BAUER, C. B. & ROGERS, R. D. 1996. Partitioning behavior of group 1 and 2 cations in poly(ethylene glycol)-based aqueous biphasic systems. *Journal of Chromatography B,* 680**,** 237-241.

BEHLKE, J. & RISTAU, O. 1998. An improved approximate solution of the Lamm equation for the simultaneous estimation of sedimentation and diffusion coefficients from sedimentation velocity experiments. *Biophyscial Chemistry,* 70**,** 133-146.

BELBOOM, S. & LÉONARD, A. 2016. Does biobased polymer achieve better environmental impacts than fossil polymer? Comparison of fossil HDPE and biobased HDPE produced from sugar beet and wheat. *Biomass and Bioenergy,* 85**,** 159-167.

BERGER, E., RAMSAY, B. A., RAMSAY, J. A., CHAVARIE, C. & BRAUNEGG, G. 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. *Biotechnology Techniques,* 3**,** 227-232.

BERGGREN, K., JOHANSSON, H.-O. & TJERNELD, F. 1995. Effect of salts and the surface hydrophobicity of proteins on partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide-propylene oxide copolymers. *Journal of Chromatography A,* 718**,** 67-79.

BERGGREN, K., NILSSON, A., JOHANSSON, G., BANDMANN, N., NYGREN, P.-A. & TJERNELD, F. 2000. Partitioning of peptides and recombinant protein-peptides fusions in thermoseparating aqueous two-phase systems: effect of peptide primary structure. *Journal of Chromatography B,* 743**,** 295-306.

BERGGREN, K., VEIDE, A., NYGREN, P.-A. & TJERNELD, F. 1999. Genetic engineering of protein-peptide fusion for control of protein partitioning in thermoseparating aqueous two-phase systems. *Biotechnol Bioeng,* 62**,** 135-144.

BIOPLASTICS, E. 2016. *What are bioplastics* [Online]. http://www.european-bioplastics.org/bioplastics/: European Bioplastics. [Accessed 13 June 2016 2016].

CANALES, M., BALLESTEROS, C., MORENO-CID, J. A., ESPINOSA, A. M., VILLAR, M. & DE LA FUENTE, J. 2009. Extractive bioconversion to produce the Aedes albopictus akirin in an aqueous two-phase system supporting Pichia pastoris growth and protein secretion. *Biochemical Engineering Journal,* 46**,** 105-114.

CASTILHO, L. R., MITCHELL, D. A. & FREIRE, D. M. 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresour Technol,* 100**,** 5996-6009.

CHA, S.-H., SON, J.-H., JAMAL, Y., ZAFAR, M. & PARK, H.-S. 2016. Characterization of polyhydroxyalkanoates extracted from wastewater sludge under different environmental conditions. *Biochemical Engineering Journal*.

CHANPRATEEP, S. 2010. Current trends in biodegradable polyhydroxyalkanoates. *J Biosci Bioeng,* 110**,** 621-32.

CHAVEZ-SANTOSCOY, A., BENAVIDES, J., VERMAAS, W. & RITO-PALOMARES, M. 2010. Application of Aqueous Two-Phase Systems for the Potential Extractive Fermentation of Cyanobacterial Products. *Chemical Engineering & Technology,* 33**,** 177-182.

CHEN, B., HAN, J., WANG, Y., SHENG, C., LIU, Y., ZHANG, G. & YAN, Y. 2014. Separation, enrichment and determination of ciprofloxacin using thermoseparating polymer aqueous two-phase system combined with high performance liquid chromatography in milk, egg, and shrimp samples. *Food Chem,* 148**,** 105-11.

CHEN, J., SPEAR, S. K., HUDDLESTON, J. G. & ROGERS, R. D. 2005. Polyethylene glycol and solutions of polyethylene glycol as green reaction media. *Green Chemistry,* 7**,** 19.

CHEN, Q., WANG, Q., WEI, G., LIANG, Q. & QI, Q. 2011. Production in Escherichia coli of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with differing monomer compositions from unrelated carbon sources. *Appl Environ Microbiol,* 77**,** 4886-93.

CHEN, Y., YANG, H., ZHOU, Q., CHEN, J. & GU, G. 2001. Cleaner recovery of poly(3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus. *Process Biochemistry,* 36**,** 501-506.

CHOI, D., CHIPMAN, D. C., BENTS, S. C. & BROWN, R. C. 2010. A techno-economic analysis of polyhydroxyalkanoate and hydrogen production from syngas fermentation of gasified biomass. *Appl Biochem Biotechnol,* 160**,** 1032-46.

CHOI, J.-I. & LEE, S. Y. 1999a. Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. *Biotechnology and Bioengineering,* 62**,** 546-553.

CHOI, J.-I. & LEE, Y. S. 1997. Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation. *Bioprocess Engineering,* 17**,** 335-342.

CHOI, J. & LEE, Y. S. 1999b. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology,* 51**,** 13-21.

CONOR, M., JOHN, F. & LOUISE, C. 2013. *Bioplastic: Materials, Markets and Management* [Online]. http://www.rx3.ie/MDGUploadedFiles/file/rx3publications/rx3_Bioplastics_Review_of_Materials_Markets_Management.pdf: rx3, Department of the Environment, Community & Local Goverment, Republic of Ireland. [Accessed 8/2/2016 2016].

COZAR, A., ECHEVARRIA, F., GONZALEZ-GORDILLO, J. I., IRIGOIEN, X., UBEDA, B., HERNANDEZ-LEON, S., PALMA, A. T., NAVARRO, S., GARCIA-DE-LOMAS, J., RUIZ, A., FERNANDEZ-DE-PUELLES, M. L. & DUARTE, C. M. 2014. Plastic debris in the open ocean. *Proc Natl Acad Sci U S A,* 111**,** 10239-44.

CUNHA, M. T., TJERNELD, F., CABRAL, J. M. S. & AIRES-BARROS, M. R. 1998. Effect of electrolytes and surfactants on the thermoseparation of an ethylene oxide-propylene oxide random copolymer in aqueous solution. *Journal of Chromatography B,* 711**,** 53-60.

DAVIS, R., ANILKUMAR, P. K., CHANDRASHEKAR, A. & SHAMALA, T. R. 2008. Biosynthesis of polyhydroxyalkanoates co-polymer in E. coli using genes from Pseudomonas and Bacillus. *Antonie Van Leeuwenhoek,* 94**,** 207-16.

DE ALMEIDA, A., GIORDANO, A. M., NIKEL, P. I. & PETTINARI, M. J. 2010. Effects of aeration on the synthesis of poly(3-hydroxybutyrate) from glycerol and glucose in recombinant Escherichia coli. *Appl Environ Microbiol,* 76**,** 2036-40.

DE ALMEIDA, A., NIKEL, P. I., GIORDANO, A. M. & PETTINARI, M. J. 2007. Effects of granule-associated protein PhaP on glycerol-dependent growth and polymer production in poly(3-hydroxybutyrate)-producing Escherichia coli. *Appl Environ Microbiol,* 73**,** 7912-6.

DE MEDEIROS E SILVA, G. M., VIANA MARQUES, D. D. A., PORTO, T. S., FILHO, J. L. L., TEIXEIRA, J. A. C., PESSOA-JÚNIOR, 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.

DEMBCZYŃSKI, R. & BIAŁAS, W. 2013. Pilot-scale separation of lysozyme from hen egg white by integrating aqueous two-phase partitioning and membrane separation processes. *Process Biochemistry,* 48**,** 1992-1998.

DEMBCZYNSKI, R., BIALAS, W. & JANKOWSKI, T. 2010. Recycling of phase components during lysozyme extraction from hen egg white in the EO50PO50/K2HPO4 aqueous two-phase system. *Biochemical Engineering Journal,* 51**,** 24-31.

DEMBCZYŃSKI, R., BIAŁAS, W. & JANKOWSKI, T. 2013. Partitioning of lysozyme in aqueous two-phase systems containing ethylene oxide-propylene oxide copolymer and potassium phosphates. *Food and Bioproducts Processing,* 91**,** 292-302.

DEMBCZYŃSKI, R., BIAŁAS, W., REGULSKI, K. & JANKOWSKI, T. 2010. Lysozyme extraction from hen egg white in an aqueous two-phase system composed of ethylene oxide–propylene oxide thermoseparating copolymer and potassium phosphate. *Process Biochemistry,* 45**,** 369-374.

DIAMOND, A. D. & HSU, J. T. 1992. Aqueous two-phase systems for biomolecule separation. *Advances in Biochemical Engineering,* 47**,** 89-135.

DIVYASHREE, M. S. & SHAMALA, T. R. 2009. Effect of gamma irradiation on cell lysis and polyhydroxyalkanoate produced by Bacillus flexus. *Radiation Physics and Chemistry,* 78**,** 147-152.

DIVYASHREE, M. S. & SHAMALA, T. R. 2010. Extractability of polyhydroxyalkanoate synthesized by Bacillus flexus cultivated in organic and inorganic nutrient media. *Indian Journal of Microbiology,* 50**,** 63-69.

DIVYASHREE, M. S., SHAMALA, T. R. & RASTOGI, N. K. 2009. Isolation of polyhydroxyalkanoate from hydrolyzed cells of Bacillus flexus using aqueous two-phase system containing polyethylene glycol and phosphate. *Biotechnology and Bioprocess Engineering,* 14**,** 482-489.

DONG, B., YUAN, X., ZHAO, Q., FENG, Q., LIU, B., GUO, Y. & ZHAO, B. 2015. Ultrasound-assisted aqueous two-phase extraction of phenylethanoid glycosides from Cistanche deserticola Y. C. Ma stems. *Journal of Separation Science,* 38**,** 1194-1203.

DONG, Z. & SUN, X. 2000. A new method of recovering polyhydroxyalkanoate fromAzotobacter chroococcum. *Chinese Science Bulletin,* 45**,** 252-256.

FERIL JR, L. B., KONDO, T., ZHAO, Q.-L., OGAWA, R., TACHIBANA, K., KUDO, N., FUJIMOTO, S. & NAKAMURA, S. 2003. Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. *Ultrasound in Medicine & Biology,* 29**,** 331-337.

FERNANDEZ-DACOSTA, C., POSADA, J., KLEEREBEZEM, R., CUELLAR, M. & RAMIREZ, A. 2015. Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment. *Bioresource Technology* 185**,** 368-377.

FERREIRA, I. F., AZEVEDO, A. M., ROSA, P. A. & AIRES-BARROS, M. R. 2008. Purification of human immunoglobulin G by thermoseparating aqueous two-phase systems. *J Chromatogr A,* 1195**,** 94-100.

FIORESE, M. N. L., FREITAS, F., PAIS, J., RAMOS, A. M., DE ARAGÃ£O, G. U. M. F. & REIS, M. A. M. 2009. Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1,2-propylene carbonate. *Engineering in Life Sciences,* 9**,** 454-461.

FONSECA, C. & ANTONIO, R. 2006. Use of vegetable oil as substrates for medium-chain-length polyhydroxyalkanoates production by recombinant Escherichia coli. *Biotechnology,* 5**,** 277-279.

FURUKI, T., MAEDA, S., IMAJO, S., HIROI, T., AMAYA, T., HIROKAWA, T., ITO, K. & NOZAWA, H. 2003. Rapid and selective extraction of phycocyanin from Spirulina platensis with ultrasonic cell disruption. *Journal of Applied Phycology,* 15**,** 319-324.

GAO, X., CHEN, J. C., WU, Q. & CHEN, G. Q. 2011. Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels. *Curr Opin Biotechnol,* 22**,** 768-74.

GERNGROSS, T. U. 1999. Can biotechnology move us toward a sustainable society. *Nat Biotechnol,* 17**,** 541-544.

GHATNEKAR, M. S., PAI, J. S. & GANESH, M. 2002. Production and recovery of poly-3-hydroxybutyrate from Methylobacterium sp V49. *Journal of Chemical Technology & Biotechnology,* 77**,** 444-448.

GOEDKOOP, M. & SPRIENSMA, R. 2001. The Eco-indicator 99: A damage oriented method for Life Cycle Impact Assessment. Methodology Report.

GRENGROSS, T. & SLATER, S. 2000. How green are green plastics? *Sci Am,* 283**,** 36-41.

GRIMI, N., DUBOIS, A., MARCHAL, L., JUBEAU, S., LEBOVKA, N. I. & VOROBIEV, E. 2014. Selective extraction from microalgae Nannochloropsis sp. using different methods of cell disruption. *Bioresource Technology,* 153**,** 254-259.

GUERRERO, S., LÓPEZ-MALO, A. & ALZAMORA, S. M. 2001. Effect of ultrasound on the survival of Saccharomyces cerevisiae: influence of temperature, pH and amplitude. *Innovative Food Science & Emerging Technologies,* 2**,** 31-39.

GULDHE, A., SINGH, B., RAWAT, I., RAMLUCKAN, K. & BUX, F. 2014. Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production. *Fuel,* 128**,** 46-52.

GUMEL, A. M., ANNUAR, M. S. M. & CHISTI, Y. 2012. Recent Advances in the Production, Recovery and Applications of Polyhydroxyalkanoates. *Journal of Polymers and the Environment,* 21**,** 580-605.

GURIEFF, N. & LANT, P. 2007. Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. *Bioresource Technology,* 98**,** 3393-3403.

HARDING, K., DENNIS, J., BLOTTNITZ, H. & HARRISON, S. 2007a. Environmental analysis of plastic production process: comparing petroleum based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis. *Journal of Biotechnology,* 130**,** 57-66.

HASMANN, F. A., SANTOS, V. C., GURPILHARES, D. B., PESSOA-JUNIOR, A. & ROBERTO, I. C. 2008. Aqueous two-phase extraction using thermoseparating copolymer: a new system for phenolic compounds removal from hemicelullosic hydrolysate. *Journal of Chemical Technology & Biotechnology,* 83**,** 167-173.

HEIMERSSON, S., MORGAN-SAGASTUME, F., PETERS, G. M., WERKER, A. & SVANSTROM, M. 2014. Methodological issues in life cycle assessment of mixed-culture polyhydroxyalkanoate production utilising waste as feedstock. *N Biotechnol,* 31**,** 383-93.

HEJAZI, P., VASHEGHANI-FARAHANI, E. & YAMINI, Y. 2003. Supercritical Fluid Disruption of Ralstonia eutropha for Poly(β-hydroxybutyrate) Recovery. *Biotechnology Progress,* 19**,** 1519-1523.

HILALY, A. & SIKDAR, S. 1995. Pollution balance method and the demonstration of its application to minimizing waste in a biochemical process. *Ind Eng Chem* 34**,** 2051-2059.

HIROE, A., TSUGE, K., NOMURA, C. T., ITAYA, M. & TSUGE, T. 2012. Rearrangement of gene order in the phaCAB operon leads to effective production of ultrahigh-molecular-weight poly[(R)-3-hydroxybutyrate] in genetically engineered Escherichia coli. *Appl Environ Microbiol,* 78**,** 3177-84.

HIROE, A., USHIMARU, K. & TSUGE, T. 2013. Characterization of polyhydroxyalkanoate (PHA) synthase derived from Delftia acidovorans DS-17 and the influence of PHA production in Escherichia coli. *J Biosci Bioeng,* 115**,** 633-8.

HOTTLE, T. A., BILEC, M. M. & LANDIS, A. E. 2013. Sustainability assessments of bio-based polymers. *Polymer Degradation and Stability,* 98**,** 1898-1907.

HOU, D., LI, Y. & CAO, X. 2014. Synthesis of two thermo-sensitive copolymers forming aqueous two-phase systems. *Separation and Purification Technology,* 122**,** 217-224.

HUDDLESTION, J. G., ROGERS, R. D. & WILLAUER, H. D. 2002. Solute partitioning in aqueous biphasic systems composed of polyethylene glycol and salt: The partitioning of small neutral organic species. *Industrial & Engineering Chemistry Research,* 41**,** 1892-1904.

HYDE, M. 1998. Ecological considerations on the use and production of biosynthetic and synthetic biodegradable polymers. *Polymer Degradation and Stability,* 59**,** 3-6.

IBRAHIM, M. H. A. & STEINBÜCHEL, A. 2009. Poly(3-Hydroxybutyrate) Production from Glycerol by Zobellella denitrificans MW1 via High-Cell-Density Fed-Batch Fermentation and Simplified Solvent Extraction. *Applied and Environmental Microbiology,* 75**,** 6222-6231.

ICHEME. 2002. The sustainability metrics. *The sustainability metrics* [Online].

INSTITUTE, B. P. 2015. *Confused by the terms Biodegradable & Biobased* [Online]. http://www.bpiworld.org/Resources/Documents/Confused%20by%20the%20terms%20Biodegradable%20Jan%2015.pdf: Biodegradable Products Institute. [Accessed 28 July 2016 2016].

JACQUEL, N., LO, C.-W., WEI, Y.-H., WU, H.-S. & WANG, S. S. 2008. Isolation and purification of bacterial poly(3-hydroxyalkanoates). *Biochemical Engineering Journal,* 39**,** 15-27.

JAMBECK, J. R., GEYER, R., WILCOX, C., SIEGLER, T. R., PERRYMAN, M., ANDRADY, A., NARAYAN, R. & LAW, K. L. 2015. Plastic waste inputs from land into the ocean. *Science,* 347**,** 768-771.

JIAN, J., LI, Z. J., YE, H. M., YUAN, M. Q. & CHEN, G. Q. 2010. Metabolic engineering for microbial production of polyhydroxyalkanoates consisting of high 3-hydroxyhexanoate content by recombinant Aeromonas hydrophila. *Bioresour Technol,* 101**,** 6096-102.

JIANG, X., RAMSAY, J. A. & RAMSAY, B. A. 2006. Acetone extraction of mcl-PHA from Pseudomonas putida KT2440. *J Microbiol Methods,* 67**,** 212-9.

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**,** 22-26.

JIANG, Z. G., ZHANG, H. D. & WANG, W. T. 2015. Purification of papain by metal affinity partitioning in aqueous two-phase polyethylene glycol/sodium sulfate systems. *J Sep Sci,* 38**,** 1426-32.

JINDAL, P. & TIWARI, D. P. 2013. Biosynthesis of PHA and it's copolymer - a review. *International Journal of Scientific and Engineering Research,* 4**,** 501-507.

JOHANSSON, H.-O., PERSSON, J. & TJERNELD, F. 1999. Thermoseparating water/polymer system: A novel one-polymer aqueous two-phase system for protein purification. *Biotechnology and Bioengineering,* 66**,** 247-257.

JOHANSSON, H. O., KARLSTROM, G. & TJERNELD, F. 1997. Temperature-induced phase partitioning of peptides in water solution of ethylene oxide and propylene oxide random copolymers. *Biochim Biophys Acta,* 1335**,** 315-325.

JOHANSSON, H. O., KARLSTROM, G., TJERNELD, F. & HAYNES, C. A. 1998. Driving forces for phase separatio and partitioning in aqueous two-phase systems. *Journal of Chromatography B,* 71**,** 3-17.

JONSSON, M. & JOHANSSON, H. O. 2003. protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer. *Journal of Chromatography A,* 983**,** 133-144.

KAHAR, P., AGUS, J., KIKKAWA, Y., TAGUCHI, K., DOI, Y. & TSUGE, T. 2005. Effective production and kinetic characterization of ultra-high-molecular-weight poly[(R)-3-hydroxybutyrate] in recombinant Escherichia coli. *Polymer Degradation and Stability,* 87**,** 161-169.

KAMMOUN, R., CHOUAYEKH, H., ABID, H., NAILI, B. & BEJAR, S. 2009. Purification of CBS 819.72 α-amylase by aqueous two-phase systems: Modelling using Response Surface Methodology. *Biochemical Engineering Journal,* 46**,** 306-312.

KANG, Z., DU, L., KANG, J., WANG, Y., WANG, Q., LIANG, Q. & QI, Q. 2011. Production of succinate and polyhydroxyalkanoate from substrate mixture by metabolically engineered Escherichia coli. *Bioresour Technol,* 102**,** 6600-4.

KAPRITCHKOFF, F. M., VIOTTI, A. P., ALLI, R. C., ZUCCOLO, M., PRADELLA, J. G., MAIORANO, A. E., MIRANDA, E. A. & BONOMI, A. 2006. Enzymatic recovery and purification of polyhydroxybutyrate produced by Ralstonia eutropha. *J Biotechnol,* 122**,** 453-62.

KARKAŞ, T. & ÖNAL, S. 2012. Characteristics of invertase partitioned in poly(ethylene glycol)/magnesium sulfate aqueous two-phase system. *Biochemical Engineering Journal,* 60**,** 142-150.

KATHIRASER, Y., AROUA, M. K., RAMACHANDRAN, K. B. & TAN, I. K. P. 2007. Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. *Journal of Chemical Technology & Biotechnology,* 82**,** 847-855.

KAUL, A. 2000. The Phase Diagram. *In:* HITTI-KAUL, R. (ed.) *Aqueous Two-Phase Systems: Methods and Protocols.* Totowa, New Jersey: Humana Press.

KEPKA, C., COLLET, E., PERSSON, J., STÅHL, Å., LAGERSTEDT, T., TJERNELD, F. & VEIDE, A. 2003. Pilot-scale extraction of an intracellular recombinant cutinase from E. coli cell homogenate using a thermoseparating aqueous two-phase system. *Journal of Biotechnology,* 103**,** 165-181.

KEPKA, C., RHODIN, J., LEMMENS, R., TJERNELD, F. & GUSTAVSSON, P.-E. 2004. Extraction of plasmid DNA from Escherichia coli cell lysate in a thermoseparating aqueous two-phase system. *Journal of Chromatography A,* 1024**,** 95-104.

KESHAVARZ, T. & ROY, I. 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. *Curr Opin Microbiol,* 13**,** 321-6.

KHAN, F. I., NATRAJAN, B. R. & REVATHI, P. 2001. GreenPro: a new methodology for cleaner and greener process design. *Journal of Loss Prevention in the Process Industries,* 14**,** 307-328.

KHANNA, S. & SRIVASTAVA, A. K. 2005. Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry,* 40**,** 607-619.

KHOO, H. H. & TAN, R. B. H. 2010. Environmental impacts of conventional plastic and bio-based carrier bags. *The International Journal of Life Cycle Assessment,* 15**,** 338-345.

KIM, D. Y., KIM, H. W., CHUNG, M. G. & RHEE, Y. H. 2007. Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. *J Microbiol,* 45**,** 87-97.

KIM, S. & DALE, B. 2004. Life Cycle Assessment Study of Biopolymers (Polyhydroxyalkanoates) - Derived from No-Tilled Corn (11 pp). *The International Journal of Life Cycle Assessment,* 10**,** 200-210.

KIM, S. & DALE, B. 2008. Energy and greenhouse gas profiles of polyhydroxybutyrates derived from corn grain: a life cycle perspective. *Environ Sci Technol,* 42**,** 7690-7695.

KOLLER, M., GASSER, I., SCHMID, F. & BERG, G. 2011. Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms. *Engineering in Life Sciences,* 11**,** 222-237.

KOLLER, M., HESSE, P. J., BONA, R., KUTSCHERA, C., ATLIC, A. & BRAUNEGG, G. 2007. Various archae- and eubacterial strains as potential polyhydroxyalkanoates producers from whey lactose. *Macromolecular Bioscience,* 7**,** 218-226.

KOLLER, M., SANDHOLZER, D., SALERNO, A., BRAUNEGG, G. & NARODOSLAWSKY, M. 2013. Biopolymer from industrial residues: Life cycle assessment of poly(hydroxyalkanoates) from whey. *Resources, Conservation and Recycling,* 73**,** 64-71.

KROTSCHECK, C. & NARDOLAWSKY, M. 1996. The Sustainable Process Index A new dimension in ecological evaluation. *Ecological Engineering,* 6**,** 241-258.

KUBOI, R., UMAKOSHI, H. & KOMASAWA, I. 1995. Extractive cultivation of Escherichia coli using poly(ethylene glycol)/phosphate aqueous two-phase systems to produce intracellular B-galactosidase. *Biotechnol Prog,* 11**,** 202-207.

KULKARNI, N., VAIDYA, A. & RAO, M. 1999. extractive cultivation of recombinant escherichia coli using aqueous two-phase system for production and separation of extracellular xylanase. *Biochemical and Biophysical Research Communication,* 255**,** 274-278.

KUNASUNDARI, B. 2011. Isolation and recovery of microbial polyhydroxyalkanoates. *Express Polymer Letters,* 5**,** 620-634.

KWON, Y. J., KAUL, R. & MATTIASSON, B. 1995. extractive lactic acid fermentation in poly(ehtyleneimine)-based aqueous two-phase system. *Biotechnol Bioeng,* 50**,** 280-290.

KYLILI, A., CHRISTOFOROU, E. & FOKAIDES, P. A. 2016. Environmental evaluation of biomass pelleting using life cycle assessment. *Biomass and Bioenergy,* 84**,** 107-117.

LAFFERTY, R. M. & HEINZLE, E. 1978. Cyclic carbonic acid esters as solvents for poly-(β-hydroxybutyric acid. Google Patents.

LAKSHMAN, K. & SHAMALA, T. R. 2006. Extraction of polyhydroxyalkanoate from Sinorhizobium meliloti cells using Microbispora sp. culture and its enzymes. *Enzyme and Microbial Technology,* 39**,** 1471-1475.

LAYCOCK, B., HALLEY, P., PRATT, S., WERKER, A. & LANT, P. 2013. The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science,* 38**,** 536-583.

LEE, J.-Y., YOO, C., JUN, S.-Y., AHN, C.-Y. & OH, H.-M. 2010. Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology,* 101**,** S75-S77.

LEE, K., CHANG, H., CHANG, Y., KIM, B. & HAHN, S. 1993. The lysis of gram-negative Alcaligenes eutrophus and Alcaligenes latus by palmitoyl carnitine. *Biotechnology Techniques,* 7**,** 295-300.

LEE, S. Y. & CHOI, J.-I. 1998. Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by Alcaligenes latus. *Polymer Degradation and Stability,* 59**,** 387-393.

LEMOS, P. C., VIANA, C., SALGUEIRO, E. N., RAMOS, A. M., CRESPO, J. P. S. G., REISZCORR, GT & M., M. A. 1998. Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. *Enzyme and Microbial Technology,* 22**,** 662-671.

LEUCHAU, F., LING, T. C. & LYDDIATT, A. 2011. Selective partition of plasmid DNA and RNA from crude Escherichia coli cell lysate by aqueous two-phase systems. *Biochemical Engineering Journal,* 55**,** 230-232.

LI, C., OUYANG, F. & BAI, J. H. 2000. Extractive cultivation of Lactococcus lactis using a polyethylene glycol/ $MgSO_4$.7H2O aqueous two-phase system to produce nisin. *Biotechnology Letters,* 22**,** 843-847.

LI, L. J., JIN, Y. R., WANG, X. Z., LIU, Y., WU, Q., SHI, X. L. & LI, X. W. 2015. Ionic liquid and aqueous two-phase extraction based on salting-out coupled with high-performance liquid chromatography for the determination of seven rare ginsenosides in Xue-Sai-Tong injection. *J Sep Sci*.

LI, M., KIM, J. W. & PEEPLES, T. L. 2002a. Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. *Journal of Biotechnology,* 93**,** 15-26.

LI, M., KIM, J. W. & PEEPLES, T. L. 2002b. Kinetic enhancement of starch bioconversion in thermoseparating aqueous two-phase reactor systems. *Biochemical Engineering Journal,* 11**,** 25-32.

LI, M. & PEEPLES, T. L. 2004. Purification of hyperthermophilic archaeal amylolytic enzyme (MJA1) using thermoseparating aqueous two-phase systems. *J Chromatogr B Analyt Technol Biomed Life Sci,* 807**,** 69-74.

LI, M., WILLAUER, H. D., HUDDLESTION, J. G. & ROGERS, R. D. 2001. Temperature effect on polymer-based aqueous biphasic extraction technology in the paper pulping process. *Separation Science and Technology,* 36**,** 13.

LI, Q., CHEN, Q., LI, M. J., WANG, F. S. & QI, Q. S. 2011. Pathway engineering results the altered polyhydroxyalkanoates composition in recombinant Escherichia coli. *N Biotechnol,* 28**,** 92-5.

LI, R., ZHANG, H. & QI, Q. 2007. The production of polyhydroxyalkanoates in recombinant Escherichia coli. *Bioresour Technol,* 98**,** 2313-20.

LI, Z.-G., SUN, Y.-Q., ZHENG, W.-L., TENG, H. & XIU, Z.-L. 2013. A novel and environment-friendly bioprocess of 1,3-propanediol fermentation integrated with aqueous two-phase extraction by ethanol/sodium carbonate system. *Biochemical Engineering Journal,* 80**,** 68-75.

LI, Z. J., CAI, L., WU, Q. & CHEN, G. Q. 2009. Overexpression of NAD kinase in recombinant Escherichia coli harboring the phbCAB operon improves poly(3-hydroxybutyrate) production. *Appl Microbiol Biotechnol,* 83**,** 939-47.

LIMA, C. A., JÚNIOR, A. C. V. F., FILHO, J. L. L., CONVERTI, A., MARQUES, D. A. V., CARNEIRO-DA-CUNHA, M. G. & PORTO, A. L. F. 2013. Two-phase partitioning and partial characterization of a collagenase from Penicillium aurantiogriseum URM4622: Application to collagen hydrolysis. *Biochemical Engineering Journal,* 75**,** 64-71.

LIU, Q., LUO, G., ZHOU, X. R. & CHEN, G. Q. 2011. Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by beta-oxidation pathway inhibited Pseudomonas putida. *Metab Eng,* 13**,** 11-7.

LIU, X.-W., WANG, H.-H., CHEN, J.-Y., LI, X.-T. & CHEN, G.-Q. 2009. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by recombinant Escherichia coli harboring propionyl-CoA synthase gene (prpE) or propionate permease gene (prpP). *Biochemical Engineering Journal,* 43**,** 72-77.

LIU, Y., WU, Z., ZHANG, Y. & YUAN, H. 2012. Partitioning of biomolecules in aqueous two-phase systems of polyethylene glycol and nonionic surfactant. *Biochemical Engineering Journal,* 69**,** 93-99.

LLADOSA, E., SILVÉRIO, S. C., RODRÍGUEZ, O., TEIXEIRA, J. A. & MACEDO, E. A. 2012. (Liquid+liquid) equilibria of polymer-salt aqueous two-phase systems for laccase partitioning: UCON 50-HB-5100 with potassium citrate and (sodium or potassium) formate at 23°C. *The Journal of Chemical Thermodynamics,* 55**,** 166-171.

LO´PEZ-MALO, A., GUERRERO, S. & ALZAMORA, S. M. 1999. Saccharomyces cerevisiae Thermal Inactivation Kinetics Combined with Ultrasound. *Journal of Food Protection,* 62**,** 1215-1217.

LU, X., ZHANG, J., WU, Q. & CHEN, G.-Q. 2003. Enhanced production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) via manipulating the fatty acid Î²-oxidation pathway inE. coli. *FEMS Microbiology Letters,* 221**,** 97-101.

LU, Y., LU, W., WANG, W., GUO, Q. & YANG, Y. 2013. The optimization of aqueous two-phase extraction of lysozyme from crude hen egg white using response surface methodology. *Journal of Chemical Technology & Biotechnology,* 88**,** 415-421.

MA, L., ZHANG, H., LIU, Q., CHEN, J., ZHANG, J. & CHEN, G. Q. 2009. Production of two monomer structures containing medium-chain-length polyhydroxyalkanoates by beta-oxidation-impaired mutant of Pseudomonas putida KT2442. *Bioresour Technol,* 100**,** 4891-4.

MAÑAS, P., PAGÁN, R., RASO, J., SALA, F. J. & CONDÓN, S. 2000. Inactivation of Salmonella Enteritidis, Salmonella Typhimurium, and Salmonella Senftenberg by Ultrasonic Waves under Pressure. *Journal of Food Protection,* 63**,** 451-456.

MENG, D. C., SHI, Z. Y., WU, L. P., ZHOU, Q., WU, Q., CHEN, J. C. & CHEN, G. Q. 2012. Production and characterization of poly(3-hydroxypropionate-co-4-hydroxybutyrate) with fully controllable structures by recombinant Escherichia coli containing an engineered pathway. *Metab Eng,* 14**,** 317-24.

MERCHUK, J. C., ANDREWS, B. A. & ASENJO, J. A. 1998. Aqueous two-phase systems for protein separation: Studies on phase inversion. *Journal of chromatography B,* 711**,** 285-293.

MILLER, S. A., BILLINGTON, S. L. & LEPECH, M. D. 2013. Improvement in environmental performance of poly(β-hydroxybutyrate)-co-(β-hydroxyvalerate) composites through process modifications. *Journal of Cleaner Production,* 40**,** 190-198.

MOKHTARANI, B., KARIMZADEH, R., AMINI, M. H. & MANESH, S. D. 2008. Partitioning of Ciprofloxacin in aqueous two-phase system of poly(ethylene glycol) and sodium sulphate. *Biochemical Engineering Journal,* 38**,** 241-247.

MORADI, M. & YAMINI, Y. 2012. Surfactant roles in modern sample preparation techniques: a review. *J Sep Sci,* 35**,** 2319-40.

MOREIRA, S., SILVERIO, S. C., MACEDO, E. A., MILAGRES, A. M., TEIXEIRA, J. A. & MUSSATTO, S. I. 2013. Recovery of Peniophora cinerea laccase using aqueous two-phase systems composed by ethylene oxide/propylene oxide copolymer and potassium phosphate salts. *J Chromatogr A,* 1321**,** 14-20.

MUDLIAR, S. N., VAIDYA, A. N., SURESH KUMAR, M., DAHIKAR, S. & CHAKRABARTI, T. 2007. Techno-economic evaluation of PHB production from activated sludge. *Clean Technologies and Environmental Policy,* 10**,** 255-262.

MUHR, A., RECHBERGER, E. M., SALERNO, A., REITERER, A., SCHILLER, M., KWIECIEŃ, M., ADAMUS, G., KOWALCZUK, M., STROHMEIER, K., SCHOBER, S., MITTELBACH, M. & KOLLER, M. 2013. Biodegradable latexes from animal-derived waste: Biosynthesis and characterization of mcl-PHA accumulated by Ps. citronellolis. *Reactive and Functional Polymers,* 73**,** 1391-1398.

MUMTAZ, T., YAHAYA, N. A., ABD-AZIZ, S., ABDUL RAHMAN, N. A., YEE, P. L., SHIRAI, Y. & HASSAN, M. A. 2010. Turning waste to wealth-biodegradable plastics polyhydroxyalkanoates from palm oil mill effluent – a Malaysian perspective. *Journal of Cleaner Production,* 18**,** 1393-1402.

NAIR, A. M., ANNAMALAI, K., KANNAN, S. K. & KUPPUSAMY, S. 2014. Characterization of polyhydroxyalkanoates produced by Bacillus subtilis isolated from soil samples. *Malaya J Biosci,* 1**,** 8-12.

NDUKO, J. M., SUZUKI, W., MATSUMOTO, K., KOBAYASHI, H., OOI, T., FUKUOKA, A. & TAGUCHI, S. 2012. Polyhydroxyalkanoates production from cellulose hydrolysate in Escherichia coli LS5218 with superior resistance to 5-hydroxymethylfurfural. *J Biosci Bioeng,* 113**,** 70-2.

NG, H. S., OOI, C. W., MOKHTAR, M. N., SHOW, P. L., ARIFF, A., TAN, J. S., NG, E. P. & LING, T. C. 2013. Extractive bioconversion of cyclodextrins by Bacillus cereus cyclodextrin glycosyltransferase in aqueous two-phase system. *Bioresour Technol,* 142**,** 723-6.

NG, H. S., TAN, C. P., MOKHTAR, M. N., IBRAHIM, S., ARIFF, A., OOI, C. W. & LING, T. C. 2012. Recovery of Bacillus cereus cyclodextrin glycosyltransferase and recycling of phase components in an aqueous two-phase system using thermo-separating polymer. *Separation and Purification Technology,* 89**,** 9-15.

NIKEL, P. I., DE ALMEIDA, A., MELILLO, E. C., GALVAGNO, M. A. & PETTINARI, M. J. 2006. New recombinant Escherichia coli strain tailored for the production of poly(3-hydroxybutyrate) from agroindustrial by-products. *Appl Environ Microbiol,* 72**,** 3949-54.

NIKEL, P. I., GIORDANO, A. M., DE ALMEIDA, A., GODOY, M. S. & PETTINARI, M. J. 2010. Elimination of D-lactate synthesis increases poly(3-hydroxybutyrate) and ethanol synthesis from glycerol and affects cofactor distribution in recombinant Escherichia coli. *Appl Environ Microbiol,* 76**,** 7400-6.

NIKEL, P. I., PETTINARI, M. J., GALVAGNO, M. A. & MENDEZ, B. S. 2008. Poly(3-hydroxybutyrate) synthesis from glycerol by a recombinant Escherichia coli arcA mutant in fed-batch microaerobic cultures. *Appl Microbiol Biotechnol,* 77**,** 1337-43.

NILSSON, A., JOHANSSON, H. O., MANNESSE, M., EGMOND, M. R. & TJERNELD, F. 2002. Partitioning of peptide-tagged proteins in aqueous two-phase systems using hydrophobically modified micelle-forming thermoseparating polymer. *Biochim Biophys Acta,* 1601**,** 138-148.

NODA, I. 1998. Process for recovering polyhydroxyalkanotes using air classification. Google Patents.

NOEL, B. J. 1962. *Process for preparing poly-b-hydroxybutyric acid*. United States patent application.

NOMURA, C. T., TAGUCHI, K., GAN, Z., KUWABARA, K., TANAKA, T., TAKASE, K. & DOI, Y. 2005. Expression of 3-ketoacyl-acyl carrier protein reductase (fabG) genes enhances production of polyhydroxyalkanoate copolymer from glucose in recombinant Escherichia coli JM109. *Appl Environ Microbiol,* 71**,** 4297-306.

OJUMU, T. V. & SOLOMON, B. O. 2004. Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. *Biotechnology,* 3**,** 18-24.

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**,** 68-73.

ORDOÑEZ, J. A., AGUILERA, M. A., GARCIA, M. L. & SANZ, B. 1987. Effect of combined ultrasonic and heat treatment (thermoultrasonication) on the survival of a strain of Staphylococcus aureus. *Journal of Dairy Research,* 54**,** 61-67.

PANDEY, S. K. & BANIK, R. M. 2011. Extractive fermentation for enhanced production of alkaline phosphatase from Bacillus licheniformis MTCC 1483 using aqueous two-phase systems. *Bioresour Technol,* 102**,** 4226-31.

PARK, S. J., CHOI, J.-I. & LEE, S. Y. 2005. Engineering of Escherichia coli fatty acid metabolism for the production of polyhydroxyalkanoates. *Enzyme and Microbial Technology,* 36**,** 579-588.

PARK, S. J. & YUP LEE, S. 2004. New FadB homologous enzymes and their use in enhanced biosynthesis of medium-chain-length polyhydroxyalkanoates in FadB mutant Escherichia coli. *Biotechnol Bioeng,* 86**,** 681-6.

PAWELZIK, P., CARUS, M., HOTCHKISS, J., NARAYAN, R., SELKE, S., WELLISCH, M., WEISS, M., WICKE, B. & PATEL, M. K. 2013. Critical aspects in the life cycle assessment (LCA) of bio-based materials – Reviewing methodologies and deriving recommendations. *Resources, Conservation and Recycling,* 73**,** 211-228.

PEREIRA, M., WU, Y.-T., VENÂNCIO, A. & TEIXEIRA, J. 2003. Aqueous two-phase extraction using thermoseparating polymer: a new system for the separation of endo-polygalacturonase. *Biochemical Engineering Journal,* 15**,** 131-138.

PERSSON, J., JOHANSSON, H.-O. & TJERNELD, F. 2000a. Biomolecule separation using temperature-induced phase separation with recycling of phase-forming polymers. *Industrial & Engineering Chemistry Research,* 39**,** 9.

PERSSON, J., JOHANSSON, H. O. & TJERNELD, F. 1999a. Purification of protein and recycling of polymers in a new aqueous two-phase system using two thermoseparating polymers. *Journal of Chromatography A,* 864**,** 31-48.

PERSSON, J., KAUL, A. & TJERNELD, F. 2000b. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide–propylene oxide copolymers. *Journal of Chromatography B,* 743**,** 115-126.

PERSSON, J., NYSTROM, L., AGELAND, H. & TJERNELD, F. 1998. Purification of recombinant apolipoprotein A-1Milano expressed in Escherichia coli using aqueous two-phase extraction followed by temperature-induced phase separation. *Journal of Chromatography B,* 711**,** 97-109.

PERSSON, J., NYSTROM, L., AGELAND, H. & TJERNELD, F. 1999b. Purification of recombinant proteins using thermoseparating aqueous-two phase system and polymer recycling. *Journal of Chemical Technology & Biotechnology,* 74**,** 238-243.

PHILIP, S., KESHAVARZ, T. & ROY, I. 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *Journal of Chemical Technology & Biotechnology,* 82**,** 233-247.

PIETRINI, M., ROES, L., PATEL, M. K. & CHIELLINI, E. 2007. Comparative Life Cycle Studies on Poly(3-hydroxybutyrate)-Based Composites on Potential Replacement for Conventional Petrochemical Plastics. *Biomacromolecules,* 8**,** 2210-2218.

PLASTICSEUROPE. 2015. *Plastics - the Facts 2015. An analysis of European latest plastics production, demand and waste data* [Online]. http://www.plasticseurope.org/documents/document/20151216062602-plastics_the_facts_2015_final_30pages_14122015.pdf: PlasticsEurope. [Accessed 5/12/2016 2016].

PORTO, C. S., PORTO, T. S., NASCIMENTO, K. S., TEIXEIRA, E. H., CAVADA, B. S., LIMA-FILHO, J. L. & PORTO, A. L. F. 2011. Partition of lectin from Canavalia grandiflora Benth in aqueous two-phase systems using factorial design. *Biochemical Engineering Journal,* 53**,** 165-171.

POSADA, J. A., NARANJO, J. M., LÓPEZ, J. A., HIGUITA, J. C. & CARDONA, C. A. 2011. Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol. *Process Biochemistry,* 46**,** 310-317.

RABELO, A. P. B., TAMBOURGI, E. B. & JR., A. P. 2004. Bromelain partitioning in two-phase aqueous systems containing PEO-PPO-PEO block copolymers. *Journal of Chromatography B,* 807**,** 61-68.

RAI, R., KESHAVARZ, T., ROETHER, J. A., BOCCACCINI, A. R. & ROY, I. 2011. Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Materials Science and Engineering: R: Reports,* 72**,** 29-47.

RAMSAY, J. A., BERGER, E., RAMSAY, B. A. & CHAVARIE, C. 1990. Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. *Biotechnology Techniques,* 4**,** 221-226.

RAYNOLDS, M., FRASER, R. & CHECKEL, D. 2000. The relative mass-energy-economic (RMEE) method for system boundary selection Part 1: A means to systematically and quantitatively select LCA boundaries. *The International Journal of Life Cycle Assessment,* 5**,** 37-46.

ROGERS, R. D., BOND, A. H., BAUER, C. B., ZHANG, J. & GRIFFIN, S. T. 1996. Metal ion separation in polyethylene glycol-based aqueous two-phase system: Correlation of partitioning behavior with available thermodynamic hydration data. *Journal of Chromatography B,* 680**,** 221-229.

ROSA, P. A., AZEVEDO, A. M. & AIRES-BARROS, M. R. 2007. Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. *J Chromatogr A,* 1141**,** 50-60.

ROSA, P. A. J., FERREIRA, I. F., AZEVEDO, A. M. & AIRES-BARROS, M. R. 2010. Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals. *Journal of Chromatography A,* 1217**,** 2296-2305.

ROSS, K. C. & ZHANG, C. 2010. Separation of recombinant β-glucuronidase from transgenic tobacco by aqueous two-phase extraction. *Biochemical Engineering Journal,* 49**,** 343-350.

SALEHIZADEH, H. & VAN LOOSDRECHT, M. C. M. 2004. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnology Advances,* 22**,** 261-279.

SANTOS, V. C., HASMANN, F. A., CONVERTI, A. & PESSOA, A. 2011. Liquid–liquid extraction by mixed micellar systems: A new approach for clavulanic acid recovery from fermented broth. *Biochemical Engineering Journal,* 56**,** 75-83.

SHARPE, P. T. 1985. The partition of charged liposomes in aqueous two-phase systems. *Molecular and Cellular Biochemistry,* 68**,** 151-159.

SHONNARD, D. R. & HIEW, D. S. 2000. Comparative Environmental Assessments of VOC Recovery and Recycle Design Alternatives for a Gaseous Waste Stream. *Environmental Science & Technology,* 34**,** 5222-5228.

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**,** 577-584.

SHOW, P. L., TAN, C. P., ANUAR, M. S., ARIFF, A., YUSOF, Y. A., CHEN, S. K. & LING, T. C. 2012a. Primary recovery of lipase derived from Burkholderia cenocepacia strain ST8 and recycling of phase components in an aqueous two-phase system. *Biochemical Engineering Journal,* 60**,** 74-80.

SHOW, P. L., TAN, C. P., SHAMSUL ANUAR, M., ARIFF, A., YUSOF, Y. A., CHEN, S. K. & LING, T. C. 2012b. Extractive fermentation for improved production and recovery of lipase derived from Burkholderia cepacia using a thermoseparating polymer in aqueous two-phase systems. *Bioresour Technol,* 116**,** 226-33.

SILVÉRIO, S. C., RODRÍGUEZ, O., TAVARES, A. P. M., TEIXEIRA, J. A. & MACEDO, E. A. 2013. Laccase recovery with aqueous two-phase systems: Enzyme partitioning and stability. *Journal of Molecular Catalysis B: Enzymatic,* 87**,** 37-43.

SINGH, S. & MOHANTY, A. 2007. Wood fiber reinforced bacterial bioplastic composites: Fabrication and performance evaluation. *Composites Science and Technology,* 67**,** 1753-1763.

SINHA, J., DEY, P. K. & PANDA, T. 2000. Aqueous two-phase system: the system of choice for extractive fermentation. *Appl Microbiol Biotechnol,* 54**,** 476-486.

SUJATHA, K. & SHENBAGARATHAI, R. 2006. A study on medium chain length-polyhydroxyalkanoate accumulation in Escherichia coli harbouring phaC1 gene of indigenous Pseudomonas sp. LDC-5. *Lett Appl Microbiol,* 43**,** 607-14.

TAGUCHI, S., YAMADA, M., MATSUMOTO, K., TAJIMA, K., SATOH, Y., MUNEKATA, M., OHNO, K., KOHDA, K., SHIMAMURA, T., KAMBE, H. & OBATA, S. 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. *Proc Natl Acad Sci U S A,* 105**,** 17323-7.

TAMER, I. M., MOO-YOUNG, M. & CHISTI, Y. 1998. Disruption of Alcaligenes latus for Recovery of Poly(β-hydroxybutyric acid): Comparison of High-Pressure Homogenization, Bead Milling, and Chemically Induced Lysis. *Industrial & Engineering Chemistry Research,* 37**,** 1807-1814.

TAMER, M. I. & MOO-YOUNG, M. 1998. Optimization of poly(β-hydroxybutyric acid) recovery from Alcaligenes latus: combined mechanical and chemical treatments. *Bioprocess Engineering,* 19**,** 459-468.

TANAKA, H., KUBOI, R., KOMASAWA, I. & TSUCHIDO, T. 1993. Control of autolysis of Bacillus subtilis for selective production of the intracellular enzyme glucose-6-phosphate dehydrogenase in aqueous two-phase systems. *Journal of Fermentation and Bioengineering,* 75**,** 424-429.

TANAMOOL, V., IMAI, T., DANVIRUTAI, P. & KAEWKANNETRA, P. 2011. Biosynthesis of polyhydroxyalkanoate (PHA) by Hydrogenophaga sp. isolated from soil environment during batch fermentation. *J Life Sci,* 5**,** 1003-1012.

TAYLOR, M. T., BELGRADER, P., POURAHMADI, F., MCMILLAN, W. A., CHANG, R. & DORITY, D. B. 2002. Apparatus and method for rapid ultrasonic disruption of cells or viruses. Google Patents.

TOMA, M., VINATORU, M., PANIWNYK, L. & MASON, T. J. 2001. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. *Ultrasonics Sonochemistry,* 8**,** 137-142.

TOMIZAWA, S., HYAKUTAKE, M., SAITO, Y., AGUS, J., MIZUNO, K., ABE, H. & TSUGE, T. 2011. Molecular weight change of polyhydroxyalkanoate (PHA) caused by the PhaC subunit of PHA synthase from Bacillus cereus YB-4 in recombinant Escherichia coli. *Biomacromolecules,* 12**,** 2660-6.

TOU, B. S. Y., NEO, K. E., TEY, B. T. & NG, M. Y. T. 2014. Effect of phase inversion and separation on hepatitis B core antigen extraction from unclarified bacterial feedstock using aqueous two-phase system. *Separation and Purification Technology,* 130**,** 45-55.

TRAUSSNIG, H., KLOIMSTEIN, E., KROATH, H. & ESTERMANN, R. 1990. Extracting agents for poly-D(-)-3-hydroxybutyric acid. Google Patents.

TRIPATHI, L., WU, L. P., MENG, D., CHEN, J. & CHEN, G. Q. 2013. Biosynthesis and characterization of diblock copolymer of p(3-hydroxypropionate)-block-p(4-hydroxybutyrate) from recombinant Escherichia coli. *Biomacromolecules,* 14**,** 862-70.

UMAKOSHI, H., YANO, K., KUBOI, R. & KOMASAWA, I. 1996. extractive cultivation of recombinant escherichia coli using aqueous two-phase systems for production and separation of intracellular heat shock proteins. *Biotechnol Prog,* 12**,** 51-56.

VALAPPIL, S. P., MISRA, S. K., BOCCACCINI, A. R., KESHAVARZ, T., BUCKE, C. & ROY, I. 2007. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV. *J Biotechnol,* 132**,** 251-8.

VAN HEE, P., ELUMBARING, A. C. M. R., VAN DER LANS, R. G. J. M. & VAN DER WIELEN, L. A. M. 2006. Selective recovery of polyhydroxyalkanoate inclusion bodies from fermentation broth by dissolved-air flotation. *Journal of Colloid and Interface Science,* 297**,** 595-606.

VAN WEGEN, R. J., LING, Y. & MIDDELBERG, A. P. J. 1998. Industrial Production of Polyhydroxyalkanoates Using Escherichia Coll: An Economic Analysis. *Chemical Engineering Research and Design,* 76**,** 417-426.

VENKATESWAR REDDY, M., NIKHIL, G. N., VENKATA MOHAN, S., SWAMY, Y. V. & SARMA, P. N. 2012. Pseudomonas otitidis as a potential biocatalyst for polyhydroxyalkanoates (PHA) synthesis using synthetic wastewater and acidogenic effluents. *Bioresour Technol,* 123**,** 471-9.

VINATORU, M. 2001. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry,* 8**,** 303-313.

WALTER, H., BROOKS, D. E. & FISHER, D. 1985. *Partitioning in aqueous two-phase systems: theory, methods, uses and application to biotechnology,* Orlando, Academic Press.

WANG, Q., LIU, C., XIAN, M., ZHANG, Y. & ZHAO, G. 2012a. Biosynthetic pathway for poly(3-hydroxypropionate) in recombinant Escherichia coli. *J Microbiol,* 50**,** 693-7.

WANG, Q., TAPPEL, R. C., ZHU, C. & NOMURA, C. T. 2012b. Development of a new strategy for production of medium-chain-length polyhydroxyalkanoates by recombinant Escherichia coli via inexpensive non-fatty acid feedstocks. *Appl Environ Microbiol,* 78**,** 519-27.

WIENDAHL, M., OELMEIER, S. A., DISMER, F. & HUBBUCH, J. 2012. High-throughput screening-based selection and scale-up of aqueous two-phase systems for pDNA purification. *J Sep Sci,* 35**,** 3197-207.

WILLAUER, H. D., HUDDLESTON, J. G., LI, M. & ROGERS, R. D. 2000. Investigation of aqueous biphasic systems for the separation of lignins from cellulose in the paper pulping process. *Journal of Chromatography B,* 743**,** 127-135.

WONG, M. S., CAUSEY, T. B., MANTZARIS, N., BENNETT, G. N. & SAN, K. Y. 2008. Engineering poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer composition in E. coli. *Biotechnol Bioeng,* 99**,** 919-28.

WRIGLEY, D. M. & LLORCA, N. G. 1992. Decrease of Salmonella typhimurium in Skim Milk and Egg by Heat and Ultrasonic Wave Treatment. *Journal of Food Protection,* 55**,** 678-680.

YANG, X., DU, M., LEE, D.-J., WAN, C., CHEN, C. & WAN, F. 2012a. Enriching polyhydroxyalkanoates (PHA) producing microorganisms by complex organics from quickly alkaline fermentation liquor. *Journal of the Taiwan Institute of Chemical Engineers,* 43**,** 953-957.

YANG, Y. H., BRIGHAM, C. J., SONG, E., JEON, J. M., RHA, C. K. & SINSKEY, A. J. 2012b. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing a predominant amount of 3-hydroxyvalerate by engineered Escherichia coli expressing propionate-CoA transferase. *J Appl Microbiol,* 113**,** 815-23.

YOUNG, D. & CABEZAS, H. 1999. Designing sustainable processes with simulation: The waste reduction (WAR) algorithm. *Comput Chem Eng,* 23**,** 1477-1491.

YOUNG, D., SCHARP, R. & CABEZAS, H. 2000. The waste reduction (WAR) algorithm: environmental impacts, energy consumption, and engineering economics. *Waste Management,* 20**,** 605-615.

YU, J. & CHEN, L. X. L. 2006. Cost-Effective Recovery and Purification of Polyhydroxyalkanoates by Selective Dissolution of Cell Mass. *Biotechnology Progress,* 22**,** 547-553.

YU, J. & CHEN, L. X. L. 2008. The Greenhouse Gas Emissions and Fossil Energy Requirement of Bioplastics from Cradle to Gate of a Biomass Refinery. *Environmental Science & Technology,* 42**,** 6961-6966.

ZHENG, H., YIN, J., GAO, Z., HUANG, H., JI, X. & DOU, C. 2011. Disruption of Chlorella vulgaris Cells for the Release of Biodiesel-Producing Lipids: A Comparison of Grinding, Ultrasonication, Bead Milling, Enzymatic Lysis, and Microwaves. *Applied Biochemistry and Biotechnology,* 164**,** 1215-1224.

ZHONG, Z. W., SONG, B. & HUANG, C. X. 2009. Environmental Impacts of Three Polyhydroxyalkanoate (PHA) Manufacturing Processes. *Materials and Manufacturing Processes,* 24**,** 519-523.

ZHU, J. H., YAN, X. L., CHEN, H. J. & WANG, Z. H. 2007. In situ extraction of intracellular L-asparaginase using thermoseparating aqueous two-phase systems. *J Chromatogr A,* 1147**,** 127-34.

ZIJLSTRA, G. M., GOOIJER, C. D. & TRAMPER, J. 1998. Extractive bioconversions in aqueous two-phase systems. *Current Opinion in Biotechnology,* 9**,** 171-176.

ZIMMERMANN, H. J. 1978. Fuzzy programming and linear programming with several objective function. *Fuzzy Sets and Systems,* 1**,** 45-55.

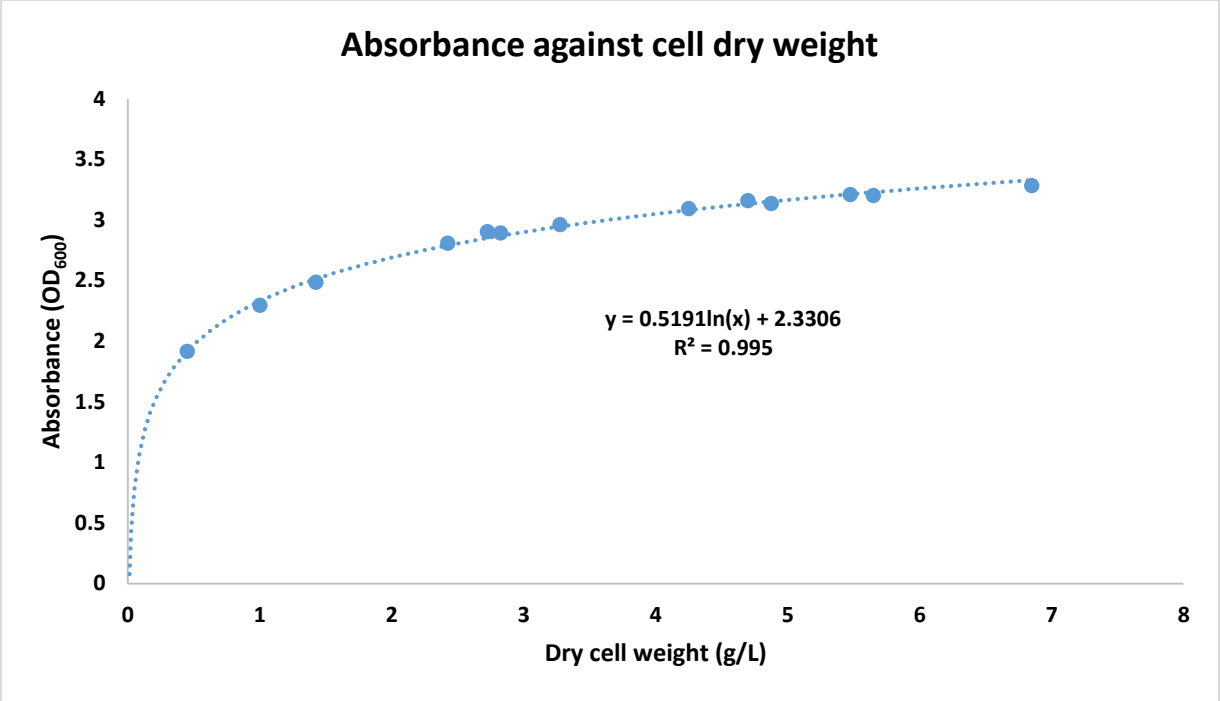
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Chapter 10

APPENDIX

Table 7.1: Results of UV-vis spectrophotometer standard curve

Cell (ml)	Water (ml)	Absorbance (OD_{600})	True Absorbance (OD_{600})	Petri dish (g)	Petri dish + Cell (g)	DCW (g)	Concentration (g/L)
4.0	0.0	3.221	3.203	8.755	8.778	0.023	5.650
3.0	1.0	3.111	3.094	8.751	8.768	0.017	4.250
2.0	2.0	2.923	2.905	8.746	8.757	0.011	2.725
0.6	3.4	2.504	2.486	8.805	8.811	0.006	1.425
1.2	2.8	2.828	2.810	9.093	9.103	0.010	2.425
1.4	2.6	2.912	2.894	8.807	8.818	0.011	2.825
2.5	1.5	3.178	3.160	8.799	8.818	0.019	4.700
1.6	2.4	2.980	2.962	9.096	9.109	0.013	3.275
2.8	1.2	3.228	3.210	9.086	9.108	0.022	5.475
3.5	0.5	3.303	3.285	7.837	7.864	0.027	6.850
2.3	1.7	3.154	3.136	7.479	7.498	0.019	4.875
0.3	3.7	1.935	1.917	7.465	7.467	0.002	0.450
0.5	3.5	2.314	2.296	7.452	7.456	0.004	1.000



Footnote: OD_{600} for defined medium is 0.01785.

Figure 7.1. UV-vis spectrophotometer standard curve

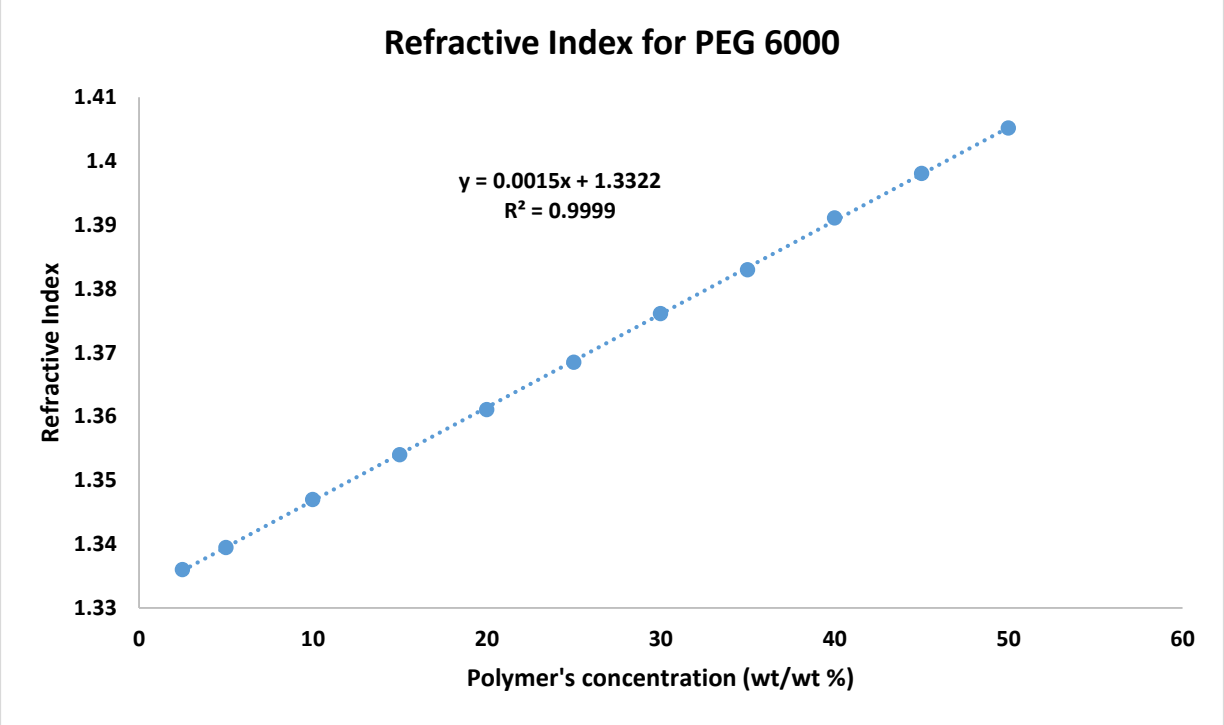


Figure 7.2. Refractive index for different concentration of PEG 6000

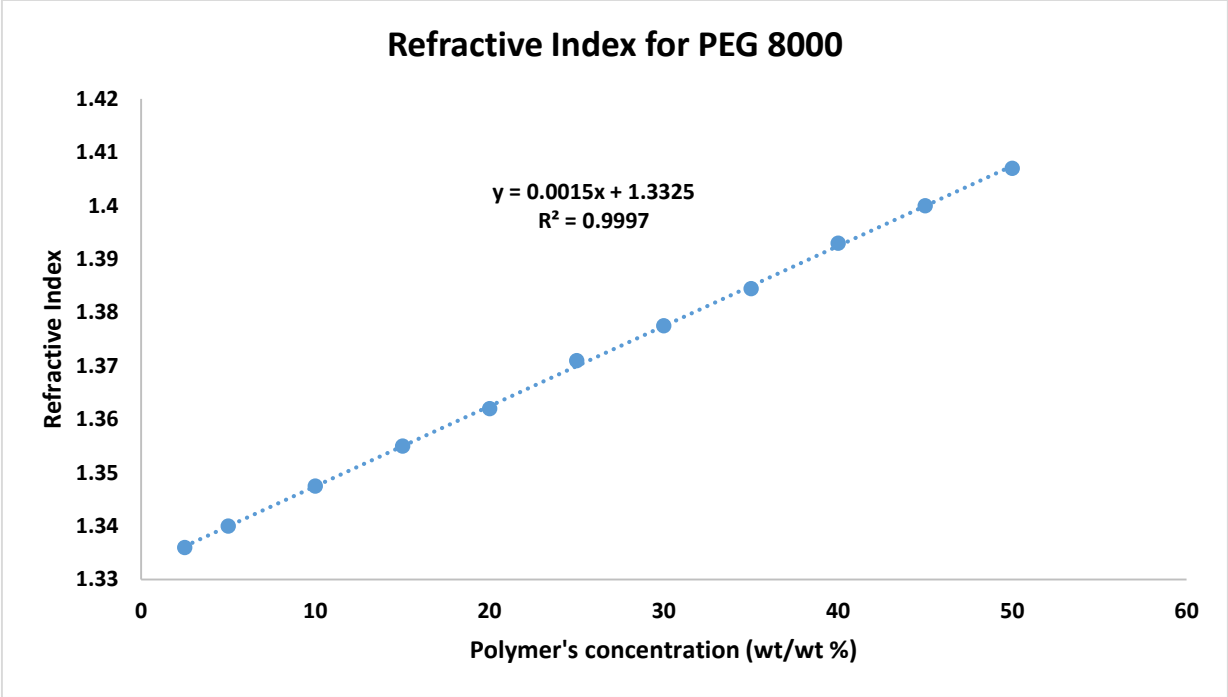


Figure 7.3. Refractive index for different concentration of PEG 8000

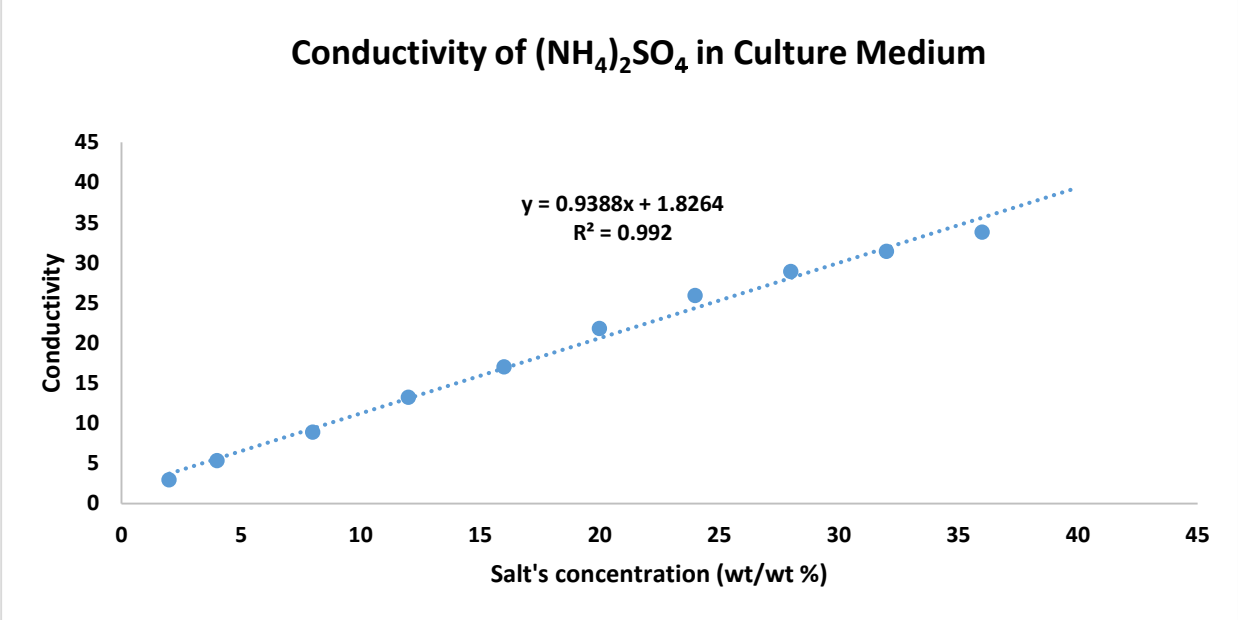


Figure 7.4. Conductivity of ammonium phosphate in culture medium

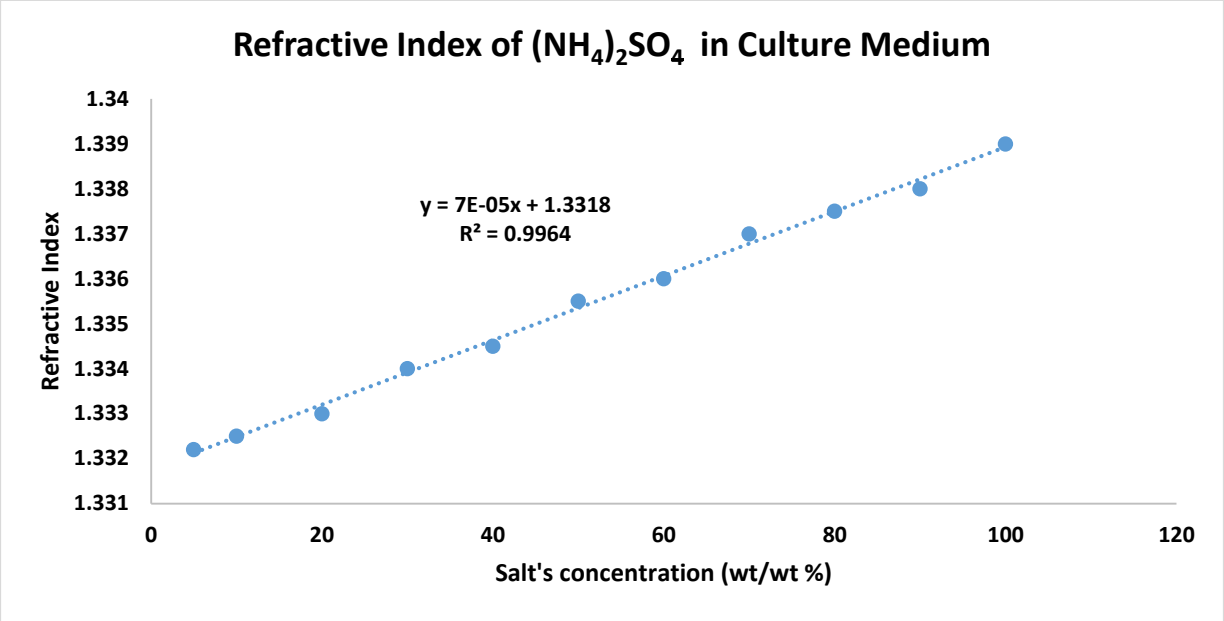


Figure 7.5. Refractive index of ammonium phosphate in culture medium

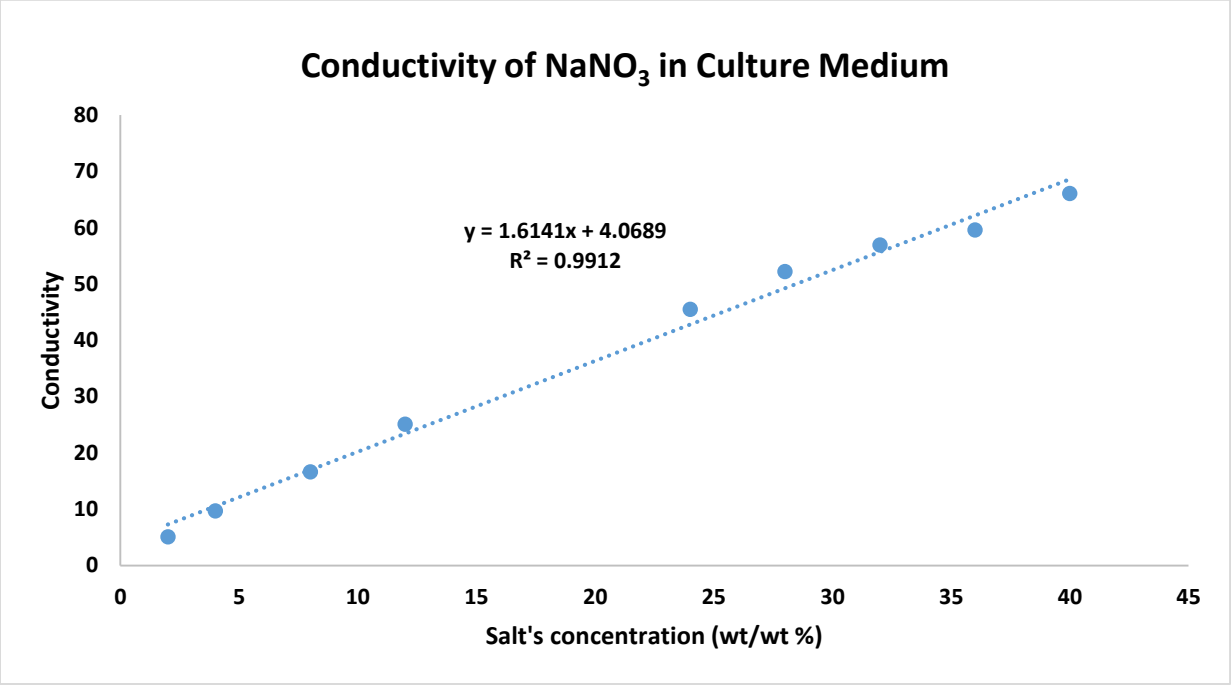
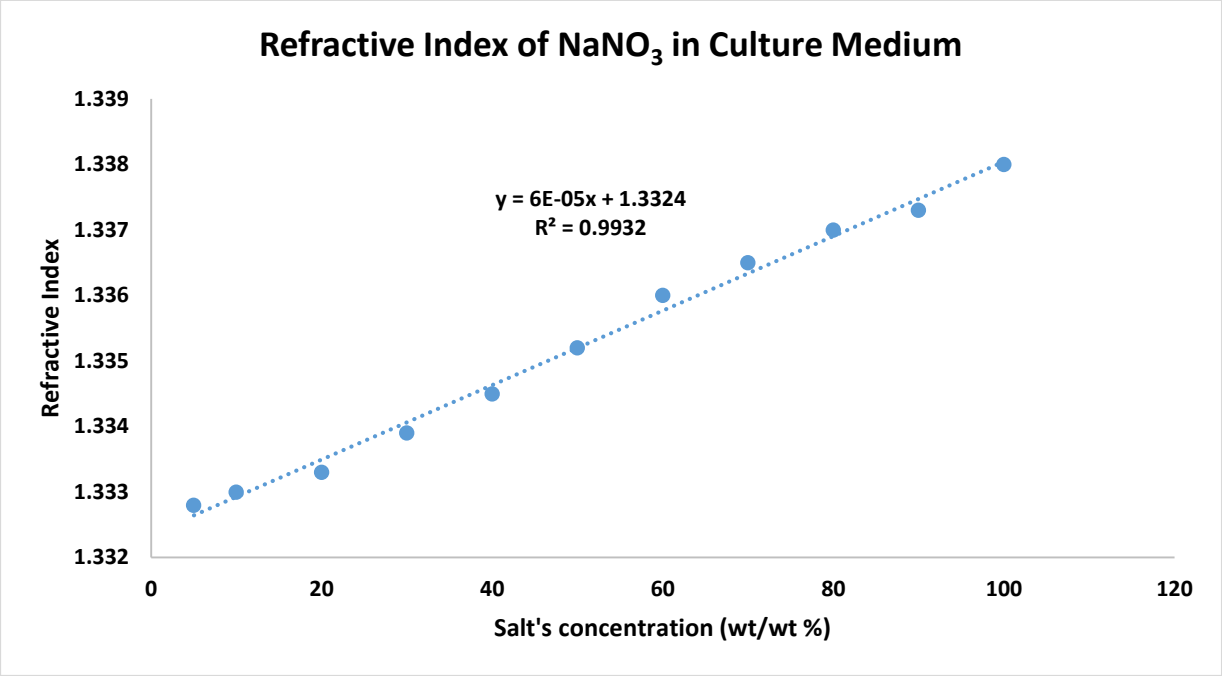
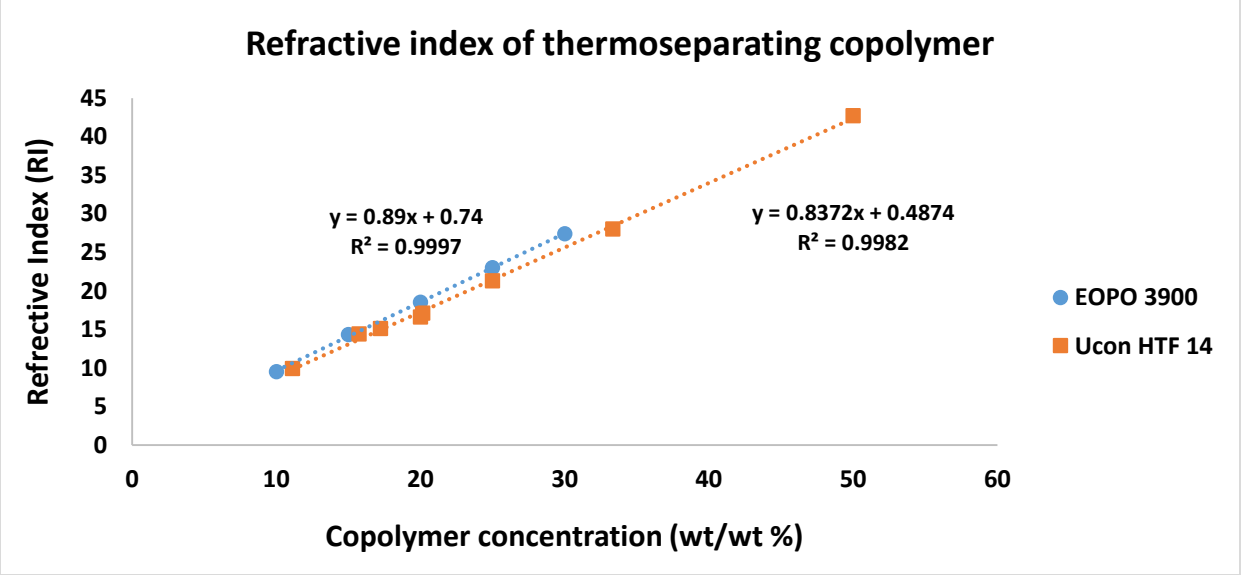


Figure 7.6. Conductivity of sodium nitrate in culture medium



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3736 Figure 7.7. Refractive index of sodium nitrate in culture mediu



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3738 Figure 7.8. Refractive index for different concentration of thermoseparating copolymer

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