EXTRACTIVE DISRUPTION PROCESS INTEGRATION USING ULTRASONICATION AND AQUEOUS TWO-PHASE SYSTEM FOR PROTEIN RECOVERY FROM MICROALGAE

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ABSTRACT

Microalgae emerge as the most promising protein source for aquaculture industry. However, the commercial production of microalgal protein at low cost remains challenging. The release of protein from microalgae is restricted by the presence of rigid thick cell wall. Another technical hurdle is that the whole protein recovery process involves several steps such as cell disruption, isolation and extraction; which is generally complicated, time-consuming and costly. To solve the technical hurdles, two experiments were designed in this study. The first experiment focused on the evaluation of a simple, economic, practical and scalable cell disruption technique for the protein recovery from microalgae. The effects of solvent types, alkali, and ultrasonication in cell disruption and protein solubility of microalgae (*Chorella sorokiniana, Chorella vulgaris, Chlamydomonas* sp. Tai-03 and *Scenedesmus* sp. Esp-07) were studied. To date, the notion of integrating microalgal cell disruption and protein recovery process into one step is yet to explore. Therefore, the feasibility of applying methanol/potassium ATPS in the extractive disruption integrated process for protein recovery was investigated in the second experiment. Parameters such as salt types, salt concentrations, methanol concentrations, NaCl addition were optimized. The possibility of upscaling and the effectiveness of using the recycled phase components at each recycling step were also studied. Based on the findings from the first experiment, it was found that alkaline treatment played a key role in cell disruption and protein solubilisation. From the industrial perspective, water is an excellent choice of solvent for simultaneous cell disruption and protein solubilisation due to low cost, ubiquitous availability and scalability. The combination of both alkaline and ultrasonication treatment showed the highest percentage of protein release and was thus proposed to be suitable for industrial application. The protein concentrations obtained from all the four microalgal strains after treated with the combination treatment were about 15-30% higher than alkaline

treatment and about 27-261% higher than ultrasonication treatment when using water as the solvent. Protein-rich strain of *C. sorokiniana* was selected for further study in the second experiment. The disruption method used in the second experiment was the combination of alkaline and ultrasonication treatment. Based on the results obtained from the integrated process, it was found that ATPS formed by 30% (w/w) K_3PO_4 and 20% (w/w) methanol with 3% (w/w) NaCl addition was optimum for protein recovery. In this system, the partition coefficient and yield were 7.28 and 84.23%, respectively. There were no significant differences in the partition coefficient and yield when the integrated process was scaled up to 100-fold. The recycled phase components can still be performed effectively at the 5^{th} cycle. In conclusion, the findings suggested that the integrated process is simple, environmental friendly and could be implemented at large scale.

ABSTRAK

Mikroalga muncul sebagai sumber protein yang paling menjanjikan untuk industri akuakultur. Walaupun begitu, pengeluaran komersial protein microalgal pada kos yang rendah kekal mencabar. Pembebasan protein daripada mikroalga adalah terhad dengan kehadiran dinding sel yang tegar dan tebal. Satu lagi halangan teknikal adalah bahawa keseluruhan proses pemulihan protein yang melibatkan beberapa langkah seperti gangguan sel, pengasingan dan pengeluaran; adalah pada umumnya rumit, memakan masa dan mahal. Untuk menyelesaikan halangan teknikal, dua eksperimen direkakan dalam kajian ini. Eksperimen pertama memberikan tumpuan kepada penilaian teknik gangguan sel yang mudah, ekonomi, praktikal dan membolehkan peningkatan skala besar untuk pemulihan protein dari mikroalga. Kesan-kesan jenis pelarut, rawatan alkali, dan rawatan ultrasonikasi dalam gangguan sel dan protein kebolehlarutan mikroalga (*Chorella sorokiniana, Chorella vulgaris, Chlamydomonas* sp. Tai-03 and *Scenedesmus* sp. Esp-07) telah dikaji. Setakat ini, idea untuk mengintegrasikan dan meringkaskan langkah gangguan sel microalgal dan protein proses pemulihan masih belum diterokai. Oleh itu, kemungkinan mengaplikasikan metanol / kalium ATPS dalam proses bersepadu gangguan ekstraktif untuk pemulihan protein disiasat dalam eksperimen kedua. Parameter seperti jenis garam, kepekatan garam, kepekatan metanol, penambahan NaCl telah dioptimumkan. Kemungkinan penskalaan dan keberkesanan menggunakan komponen fasa kitar semula dalam setiap langkah kitar semula juga telah dikaji. Berdasarkan keputusan daripada eksperimen pertama, didapati bahawa rawatan alkali memainkan peranan penting dalam gangguan sel dan kebolehlarutan protein. Dari perspektif industri, air adalah pilihan yang sangat baik sebagai pelarut untuk gangguan sel serentak dan kebolehlarutan protein kerana kos rendah, ketersediaan dan sesuai digunakan untuk skala besar. Gabungan kedua-dua rawatan alkali dan ultrasonikasi menunjukkan jumlah pembebasan protein yang paling tinggi dan dengan itu rawatan ini

dicadangkan sesuai untuk kegunaan industri. Kepekatan protein yang diperolehi daripada semua empat jenis microalgal selepas rawatan kombinasi adalah kira-kira 15-30% lebih tinggi daripada rawatan alkali dan lebih kurang 27-261% lebih tinggi daripada rawatan ultrasonikasi apabila menggunakan air sebagai pelarut. *C. sorokiniana* yang kaya dengan protein telah dipilih untuk kajian lebih lanjut dalam eksperimen kedua. Kaedah gangguan yang digunakan dalam eksperimen kedua adalah gabungan rawatan alkali dan ultrasonicakasi. Keputusan yang diperolehi daripada proses bersepadu menunjukkan bahawa ATPS dibentukkan oleh 30% (w/w) K_3PO_4 dan 20% (w/w) metanol, dengan 3% (w/w) NaCl tambahan adalah optimum untuk pemulihan protein. Dalam sistem ini, pekali penyekat dan hasil protein adalah 7.28 dan 84.23% masing-masing. Tiada perbezaan yang ketara dalam pekali partition dan hasil protein apabila proses bersepadu ini telah dipertingkatkan sehingga 100 kali ganda. Komponen fasa yang telah dikitarkan semula pada kitaran ke-5 masih boleh digunakan dengan berkesan. Dalam kesimpulan, keputusan eksperimen mencadangkan bahawa proses bersepadu adalah mudah, mesra alam dan boleh dilaksanakan pada skala yang besar.

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LIST OF SYMBOLS AND ABBREVIATIONS

A_B	:	The equilibrium concentration of the partitioned protein (μg/mL) in the bottom phase
A_T	:	The equilibrium concentration of the partitioned protein (μg/mL) in the top phase
ASE	:	Accelerated solvent extraction
ATPS	:	Aqueous two-phase system
BCA	:	Bicinchoninic acid
BSA	:	Bovine serum albumin
C	:	Protein concentration (μg/mL) obtained from the calibration curve
D	:	Dilution factor
DCW	:	Dry cell weight
H_2SO_4	:	Sulfuric acid
K	:	Partition coefficient
K_2HPO_4	:	Dipotassium phosphate
K_3PO_4	:	Tripotassium phosphate
KOH	:	Potassium hydroxide
m	:	The amount of biomass (mg)
NaCl	:	Sodium chloride
OVAT	:	One variable at a time
NaOH	:	Sodium hydroxide
PAA	:	Poly(acrylic acid)
PEG	:	Poly(ethylene glycol)
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFE	:	Supercritical fluid extraction

UAE	:	Ultrasound-assisted extraction
V	:	Volume (L) of the lysis buffer used to resuspend the biomass
V_B	:	Volume of bottom-phase
V_R	:	Volume ratio
V_T	:	Volume of top phase
Y_T	:	Yield of protein

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CHAPTER 1: INTRODUCTION

1.1 Background to the study

Fishmeal has long been used as the principle source of dietary protein in commercial fish feeds formulation (Roy & Pal, 2014) due to its compatibility with the protein requirement of fish. Fishmeal contains high-quality of protein, adequate balance of amino acid profile, high protein digestibility and excellent palatability (Ayadi, Rosentrater, & Muthukumarappan, 2012). Being the preferred foundation of aquaculture feed formulation, fishmeal contributes considerably to the variable production cost (Roy & Pal, 2014). According to the Food and Agriculture Organization of the United Nations (FAO), the aquaculture consumption of fishmeal is forecasted to be increased continually, which concomitant with a higher demand for protein feed sources (Norambuena et al., 2015; Taelman, De Meester, Van Dijk, Silva, & Dewulf, 2015). The increasing demands for protein source surpass the supply has consequently caused the inexorable upsurges in the price of fishmeal (Roy & Pal, 2014; Sirakov, Velichkova, Stoyanova, & Staykov, 2015). Apart from the rising cost, problems such as uncertain availability of fishmeal as a raw material to be incorporated in fish diet could also impede the sustainability of commercial fish farming (Kiron, Phromkunthong, Huntley, Archibald, & Scheemaker, 2012).

Alternatively, the most popular protein source replacement is the soybean meal (Bhosale, Bhilave, & Nadaf, 2010; Taelman et al., 2015), providing most of the protein needed to produce commercial fishmeal at cheaper cost. However, soybean meal is not regarded as an ideal fishmeal replacer for fish farming due to several limitations. These include low palatability, the presence of indigestible components and anti-nutritional substances. Meticulous processing methods are required to eliminate the anti-nutritional factors (Li, Mai, Jesse, & Wu, 2009; Norambuena et al., 2015). Furthermore, the deficiency in certain essential amino acids such as methionine, lysine and cystine would

cause significant changes in the nutritional quality of the fish produced and thus additional investment for the addition of synthetic amino acids is required (Stankovic, Dulic, & Markovic, 2011; Watanabe, 2002).

As a result, there is a pressing urge to seek for a new protein substitution that could supply comparable nutritional value at competitive cost and sustainable for the economic success of the aquaculture industry (Sirakov et al., 2015). Recent literature reported that microalgal protein emerges as the most promising plausible replacement for conventional protein source (Norambuena et al., 2015; Safi et al., 2014a; Solana, Rizza, & Bertucco, 2014). This is mainly due to their high protein content (Barreiro, Prins, Ronsse, & Brilman, 2013; Blackburn & Volkman, 2012; Gu et al., 2012) and nutritional quality (Norambuena et al., 2015) with excellent amino acids profile (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). As a major fraction of the chemical composition, the crude protein content of microalgae can be as high as 50%, which is approximately 25-fold higher than the most widely-cultivated soybean crops (Fernández, Fernández Sevilla, & Grima, 2013). Besides that, the farming of microalgae possess several advantages over conventional crops from an industrial perspective (Günerken et al., 2015) and has led to the increase of commercial interest.

1.2 Problem statements

Despite these advantages, the utilization of microalgae as a platform for protein source commercially is still on its infancy. At this juncture, extensive efforts have been dedicated to the development of feasible cultivation systems (Li et al., 2014) or efficient lipid extraction methods for biofuel production (Foley, Beach, & Zimmerman, 2011). Besides cultivation conditions and strain selection, protein production yield is greatly dependent on the cell disruption efficiency and protein extractability in solvent (Li et al., 2014). Nevertheless, there are still very few comprehensive studies exploring the

cost-effective and practical methods to disrupt and recover protein from the protein-rich strain of microalgae. Economic feasibility remains the most important aspect to consider when designing a downstream process. From a practical point of view, it is imperative to optimize the balance between products, processing cost and energy consumption in order to maximize the efficiency and profitability (Anastas & Zimmerman, 2003) of global aquaculture production at commercial level. Downstream processing costs typically contribute to a big portion of the total cost (Jegathese & Farid, 2014) and is rather complex but this constraint is not a valid argument against further development.

The mass production of microalgal protein remains an uphill battle due to the emergence of a few technical hurdles and economic bottlenecks especially in the downstream processing (Günerken et al., 2015). One of the main hurdles faced in the mass production of protein from microalgae is the low recovery rate. The presence of multiple layers of recalcitrant cell wall represents the major barrier in microalgae cell lysis (González-Fernández, Sialve, Bernet, & Steyer, 2012; Günerken et al., 2015). Proteins are located in different parts of cell and can be found in cell wall, cytoplasm, chloroplast and other intracellular organelles (Safi et al., 2015). This barrier must be sheared to facilitate the release of protein from microalgae. As the microalgal morphology is different from terrestrial plants, intracellular components cannot be extruded effectively from microalgae using mechanical press, a method which is designed specifically for product extrusion from terrestrial crops such as soy (Lee, Lewis, & Ashman, 2012). On top of that, there are many different species of microalgae grown under different cultivation conditions, thus varying greatly in their cell wall structure and chemical compounds concentration, making the predictions or extrapolations on disruption efficiency and recovery rate of intracellular compounds impossible (Günerken et al., 2015). In this regard, choosing a suitable cell disruption treatment prior to extraction is one of the most vital steps in downstream processing

(Günerken et al., 2015; Piasecka, Krzemińska, & Tys, 2014). The development of a simple and rapid cell disruption method is imperative for industrial application.

Another technical hurdle is that the process of protein recovery from microalgae involves a number of unit operational steps such as isolation and extraction of protein after cell disruption (Rito-Palomares & Lyddiatt, 2002). This may cause the loss of some quantity of target biomolecules in each operational step that could subsequently incurs a big overall loss (Mohammadi, Omidinia, & Taherkhani, 2008; Raja, Murty, Thivaharan, Rajasekar, & Ramesh, 2011). Additionally, the conventional discrete processes are generally time-consuming, tedious, complicated, require high energy input and would lead to difficulty for a smooth operation (Raja et al., 2011). For example, the existing biomolecules separation method using chromatography involves high costs, batch operation, low throughput and complex scale-up (Asenjo & Andrews, 2012). Whereas the elimination of cell debris and some contaminants by high speed centrifugation or cross-flow membrane filtration may be difficult to achieve at large (Rito-Palomares & Lyddiatt, 2002).

1.3 Significance of the study and research purpose

Owing to the growing significance of protein source for aquaculture sector, the effort to commercially harness protein from microalgae at large scale with minimum processing cost is gradually garnering worldwide attention. With the development of a cost-effective and sustainable downstream processing method, the idea of commercially harnessing protein from microalgae at industrial scale could be realized.

The effects of alkaline treatment and ultrasonication on cell disruption and protein release from microalgae have been poorly investigated. Halim et al. (2012) pointed out the difficulty to speculate the mechanism behind the chemical interaction in the cell wall and protein solubility, due to the limited understanding of the affinity of each

solvent for the cell wall composition of different microalgae (Halim, Harun, Danquah, & Webley, 2012).

As such, this study aimed to provide a new insight into the role of ultrasonication, alkaline hydrolysis, and the effects of different solvents on cell disruption and protein solubility for the facilitation of protein release from microalgae. The effects of single treatment versus combination treatment were also compared. Selection of disruption method for large scale application based on industrial perspective has also been highlighted.

Numerous researchers agreed that the notion of implementing extractive disruption process integration into downstream processing could be an excellent approach towards reducing production cost by simplifying the total number of unit operations in downstream processing (Gu, 2014; Mohammadi et al., 2008; Rito-Palomares & Lyddiatt, 2002). An interesting review written by Benavides et al. (2008) has provided a comprehensive information on the potential achievement of process integration based upon ATPS strategies (Benavides, Aguilar, Lapizco-Encinas, & Rito-Palomares, 2008). He expressed concerns about the restricted development of ATPS-based bioprocess, which is due to poor understanding and characterization of the effects of ATPS influential parameters on the partitioning of a particular compound (Benavides et al., 2008).

In view of this, the present study aimed to investigate the feasibility of using methanol/potassium salt ATPS in the integrated process of cell disruption and protein recovery from protein-rich strain of microalgae. to obtain the maximum protein recovery from microalgae, four influential variables such as types of potassium salt (K_2HPO_4 and K_3PO_4), potassium salt concentrations, methanol concentrations, the addition of sodium chloride (NaCl) on the partitioning behaviour of protein were optimized using “one variable at a time (OVAT)” optimization method. In addition, the

possibility of upscaling and the effectiveness of using the recycled phase components at each recycling step were also investigated.

CHAPTER 2: LITERATURE REVIEW

2.1 Microalgae

Microalgae are a group of structurally simple microorganisms, generally existing in unicellular or colonial forms (Cheah, Show, Chang, Ling, & Juan, 2014). They are mostly invisible to the naked eye, with sizes ranging from 1μm to 1mm, except when they form natural proliferations which is known as algal blooms (Blackburn & Volkman, 2012). Microalgae lack the various structures that characterize land plants, such as rhizoids, leaves, roots and stems (Cheah et al., 2014). In spite of lacking complex tissues and organs, microalgae represent the largest and most robust microorganisms on earth, ubiquitously distributed with good adaptation to a broad range of environmental conditions (Duong et al., 2015; Tan, Show, Chang, Ling, & Lan, 2015). They can even survive and thrive in extreme environments (Mata, Martins, & Caetano, 2010) such as saturated saline (Kumar, Dasgupta, Nayak, Lindblad, & Das, 2011), brackish water, coastal seawater, wastewater, seawater (Dragone, Fernandes, Vicente, & Teixeira, 2010; Kumar, Rao, & Arumugam, 2015).

One of the unique features of microalgae is the presence of chlorophyll and their ability to perform photosynthesis in a single cell with outstanding photosynthetic efficiency (Li, Horsman, Wu, Lan, & Dubois-calero, 2008; Singh & Sharma, 2012; Wang, Lan, & Horsman, 2012). Typically, microalgae require mainly light source, water, carbon dioxide and nutrients for efficient growth (Hosikian, Lim, Halim, & Danquah, 2010). They act like light-harvesting cell factories where carbon dioxide can be converted into biomass or a variety of bioactive components (Cheah et al., 2014). It was found that about 3-8% of radiant energy captured from light source can be transformed into biomass as compared to terrestrial crops with only about 0.5% (Verma, Mehrotra, Shukla, & Mishra, 2010). Being the prime source of bulk nutrients, carbon

and chemical energy for other organisms, microalgae are hence often regarded as the primary producers (Samarakoon & Jeon, 2012).

2.1.1 Classification of microalgae

These extensively diverse and abundant group of microorganisms (Singh & Sharma, 2012) have been categorized according to a few criteria such as pigments, chemical nature of storage products, cell wall constituents, as well as cytological and morphological characters (Dragone et al., 2010). Microalgae can be grouped into two basic types of cells namely prokaryotic microalgae and eukaryotic microalgae (Sambusiti, Bellucci, Zabaniotou, Beneduce, & Monlau, 2015). Prokaryotic cells lack membrane-bounded organelles and cyanobacteria are the only prokaryotic algae. On the contrary, eukaryotic microalgae have organelles such as plastids, mitochondria, nuclei, Golgi bodies and flagella (Dragone et al., 2010).

Microalgae can also be grouped nutritionally on the basis of their energy sources. There are three main types of growth conditions found in microalgae and are summarized in Table 2.1. Most microalgae are autotrophic, with the absolute requirement for light to perform photosynthesis, adequate supply of carbon dioxide and inorganic nutrients for optimal growth (Blackburn & Volkman, 2012). With the presence of these simple inorganic substances in their surroundings, autotrophs are capable of producing complex organic compounds such as carbohydrates, fats and proteins (Perez-Garcia, Escalante, de-Bashan, & Bashan, 2011). On the other hand, some microalgae are heterotrophic. Heterotrophs are in contrast with autotrophs; they cannot fix carbon and therefore need organic carbon compounds such as glucose, acetate, lactate and glutamate as carbon and energy source for growth. Microalgae which may have the dual capacity of both autotrophic and heterotrophic characteristics are known as mixotrophic. These phototrophic microalgae are able to adapt their

metabolism to heterotrophic conditions, depending on the availability of light intensity and organic compounds (Blackburn & Volkman, 2012).

Table 2.1: Types of growth conditions for microalgae

Growth mode	Autotrophic	Heterotrophic	Mixotrophic
Carbon source	Inorganic	Organic	Inorganic and organic
Energy source	Light	Organic	Light and organic
Light availability requirements	Obligatory	No requirement	No obligatory
References	(Blackburn & Volkman, 2012; Perez-Garcia et al., 2011)	(Blackburn & Volkman, 2012)	(Blackburn & Volkman, 2012)

2.1.2 Major chemical composition of microalgae

Microalgae are classified as the futuristic raw material in biorefinery process, because of their relatively untapped potential to produce multiple valuable products (Trivedi, Aila, Bangwal, Kaul, & Garg, 2015; Yen et al., 2013). Even though they are small in size, these photosynthetic microorganisms have the capability of accumulating different types of metabolites which can subsequently be transformed into value-added products (Trivedi et al., 2015). The microalgal biomass is composed of three main components: proteins, carbohydrates and lipids (Jegathese & Farid, 2014). Studies on various microalgae demonstrated that proteins are always the major constituent of the microalgae biomass (typically 25-40% of the dry weight) (Blackburn & Volkman, 2012), followed by lipids and carbohydrates (Becker, 2007). The biomass composition of some microalgae species are shown in Table 2.2.

Table 2.2: Biomass composition of microalgae expressed on a dry matter basis

Microalgal species	Protein	Lipid	Carbohydrates
Botryococcus braunii	40	33	2
Chlamydomonas rheinhardii	48	21	17
Chlorella vulgaris	41–58	10–22	12–17
Dunaliella bioculata	49	8	4
Dunaliella tertiolecta	29	11	14
Euglena gracilis	39–61	14–20	14–18
Porphyridium cruentum	28–39	9–14	40–57
Prymnesium parvum	28–45	22–39	25–33
Scenedesmus dimorphus	8–18	16–40	21–52
Scenedesmus obliquus	50–56	12–14	10–17
Scenedesmus quadricauda	47	1.9	–
Spirulina platensis	42–63	4–11	8–14
Synechoccus sp.	63	11	15

[Adapted from (Sydney et al., 2010; Um & Kim, 2009)]

Proteins make up a large fraction of the biomass of the actively growing microalgae and are regarded as the valuable asset in microalgae (López et al., 2010). Microalgae have the ability to synthesis all types of essential amino acids which are mostly equivalent or even better than that of other high-quality plant protein (Safi et al., 2014a; Spolaore et al., 2006). It was reported that their protein quality value are greater than other vegetable sources, for example, wheat, rice, and legumes (Mata et al., 2010). Furthermore, the amino acid composition does not seem to be significantly affected by changes in environmental conditions (Blackburn & Volkman, 2012). Many metabolic studies have justified the benefits of using microalgae as a novel source of protein in food (Spolaore et al., 2006) mainly due to their abundance and complete amino acid profile.

2.1.3 Cell wall structure of microalgae

Microalgae are microscopic single cell microorganisms covered with relatively recalcitrant cell walls, which serve as a protection against invaders and harsh

environment (Günerken et al., 2015; Piasecka et al., 2014). Their cell envelopes are generally more rigid than the cell envelopes of other microorganisms or higher plants. It was reported that the tensile strength of the microalgal cell wall can be up to 9.5 MPa, which is about three times higher than that of carrot, *Daucus carota* (Lee et al., 2012). These complex cell walls are typically tri-layered structures which composed of polysaccharides such as pectin, cellulose, mannose, xylan; minerals namely calcium or silicates; as well as proteins such as glycoprotein with high mechanical strength and chemical resistance (Kim et al., 2013; Safi et al., 2015). An additional of tri-laminar sheath (TLS) containing algaenan may also be found in certain microalgal species, making them highly resistance to degradation (Versteegh & Blokker, 2004). Besides that, the intracellular compounds are mostly found in globules or bound to complex membranes, making the extraction of cell contents a great challenge (Günerken et al., 2015; Wang, Yuan, Jiang, Jing, & Wang, 2014).

2.1.4 Advantages of microalgae

Microalgal farming could be potentially more cost-effective compared to conventional farming (Li et al., 2008). The mass production of nutrient-rich microalgae has led to the increase of commercial interest due to the fact that microalgae possess a number of attractive attributes which offer several advantages over conventional crops from an industrial perspective (Li et al., 2008; Mata et al., 2010). In comparison to land-based crops, microalgae are tiny in size, have higher photosynthetic efficiency (Perrine, Negi, & Sayre, 2012), short harvesting cycle (less than ten days) (Chen et al., 2013), possess high metabolites content, high disease resistance ability, high growth rate, high biomass density (Roy & Pal, 2014) and rich in high quality nutritional content (Norambuena et al., 2015).

The biomass productivity of microalgae was estimated to be 50 times more than of switch grass (Li et al., 2008). This is because their simple cellular structure enable them to be more efficient in converting solar energy (Mata et al., 2010). Most of them reproduce vegetatively (Blackburn & Volkman, 2012) with biomass doubling times are commonly within 24 h or can be as short as 3.5 h during exponential growth (Chisti, 2007). Microalgae can be cultured on non-arable land (Ferreira et al., 2013) and there is less seasonal and weather restrictions (Duong et al., 2015) in the microalgal cultivation when compared with terrestrial plants.

Microalgae farming is amenable to mass culture with minimum land space requirement (Sambusiti et al., 2015). They are characterized by a high productivity per unit area compared to conventional farming (Chisti, 2007; Mata et al., 2010). Additionally, microalgal farming does not compete with conventional agriculture for resources (Dragone et al., 2010), requiring lesser fertilizer, simple nutrient input (Verma et al., 2010) and generally consumes less freshwater than conventional agriculture. Furthermore, microalgal farming able to consume nutrients contained in wastewater for growth, therefore phosphates and nitrates can be effectively eliminated from wastewater (Solana et al., 2014).

Microalgae could aid in combating the greenhouse effect and global warming. With the capability of tolerating high carbon dioxide content, they are highly efficient in fixing carbon dioxide (Cheah et al., 2014; Li et al., 2008; Yang et al., 2015). Attributed by the high rate of carbon dioxide sequestration ability, the emissions of industrial exhaust gases such as carbon dioxide can be mitigated efficiently by microalgae (Cheah et al., 2014). The major advantages of microalgal farming are illustrated in Fig. 2.1.

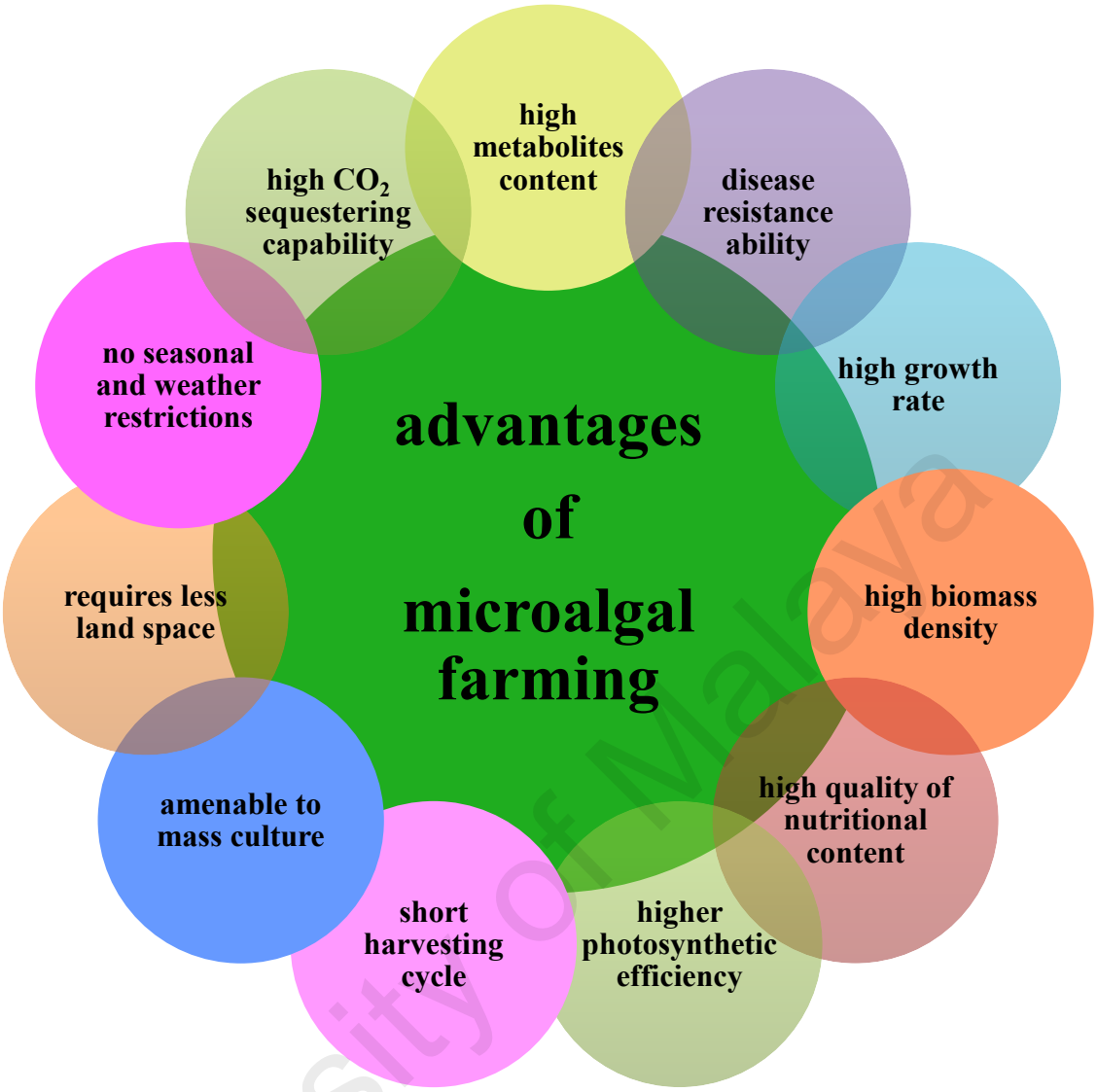

Figure 2.1: Advantages of microalgal farming

2.1.5 Application of microalgae for aquaculture industry

Microalgae are the primary bases of the entire aquatic food chain that produce the food resources to support the rearing of all stages of marine production. Fish are adapted to consume microalgae. Since fishmeal is one of the most expensive components in conventional fish feeding, it would be of commercial relevant if the protein source could be supplanted by microalgal protein (Roy & Pal, 2014). The high protein content and amino acid profiles are the main reason that microalgae are recognised as the valuable ingredients for aquatic animal feeds. Microalgae can be

incorporated into the animal feed to enhance the nutritional value of animal feed (Spolaore et al., 2006).

Numerous nutritional and toxicological analyses have revealed the suitability of using microalgae as the replacement for traditional protein source (Blackburn & Volkman, 2012). The accessible and relatively economical food component features of microalgae (Sirakov et al., 2015) could ensure long-term environmental and economical sustainability in aquaculture industry.

2.2 Cell disruption

The release of cell contents from microalgae is hindered by the intrinsic rigidity of cell walls and the presence of membranes. Furthermore, the intracellular products such as protein are usually embedded within a multilayer of cell, resulting in low extraction yield from microalgae (Günerken et al., 2015; Safi et al., 2014b; Wang et al., 2014). To obtain the maximum recovery of cell contents from microalgae, employing an appropriate cell disruption technique to facilitate the release of the cell contents prior to extraction is undoubtedly one of the most crucial preliminary steps in downstream processing (Günerken et al., 2015; Piasecka et al., 2014). The complex cell wall structure must be sheared to allow access to the internal components that are entrapped within the thick cell wall (Safi et al., 2014b). The pretreatment of the cell wall is essential to enhance assimilation and bioavailability of the intracellular compounds in the extraction solvent (Safi et al., 2015). The internal components can then be liberated into the liquid medium, making them readily available for further separation and extraction processes.

However, selecting an ideal disruption method to facilitate the release of intracellular contents is quite challenging as this process would have a significant impact on the efficiency of the subsequent steps in downstream processing. In addition, cell disruption

is an energy intensive process and is relatively influential on the total production cost. This process could thereby affect the economy and yields of bio-products (Günerken et al., 2015; Lee et al., 2012). On the other hand, the quantity and quality of the functional compounds in the extract also depends on the effectiveness of the cell disruption method (Rakesh et al., 2015; Safi et al., 2014a). The nature and composition of the structural cell wall has an important effect on the disruption efficiency and the extraction yield of intracellular biomolecules (Borowitzka & Moheimani, 2013; Safi et al., 2014a). Unlike higher plants, the cell walls of microalgae vary considerably (Wang et al., 2014). Some cells are more difficult to rupture. For example, lipid extraction from *Chlorella* or *Nannochloropsis* is much more difficult than *Dunaliella* due to the presence of a thick resistant cell wall (Borowitzka & Moheimani, 2013). In contrast, a more energy efficient disruption method can be chosen to disrupt the fragile cell walled microalgae such as *Porphyridium cruentum* which lacks of a well-defined cell wall (Günerken et al., 2015; Safi et al., 2014a). It has also been observed experimentally that the decrease in protein recovery mirrors the increasing rigidity of the extracellular coverings in microalgae (Safi et al., 2014a). Therefore, the choice of disruption technique is highly specific and strongly depends on the microalgal strain and the characteristics of the cell wall structure (Günerken et al., 2015; Safi et al., 2014a).

An effective disruption method is characterized by the high selective and efficient release of the specific intracellular content from cells with minimum micronization of cell debris and low risk of contamination (Harrison, 1991). There are a few factors which collectively determine the suitability of implementing a cell disruption process in downstream processing. In general, a good cell disruption technique should be characterized by simple, easy handling, low energy demand, high disruption yields within short operating time. The disruption technique should also be applicable to large

scale process by using economical and less toxic disruption reactants without causing excessive production of liquid wastes (Steriti, Rossi, Concas, & Cao, 2014).

2.2.1 Chemical method: alkaline treatment

The main advantage of disrupting cells using chemical method is that it does not require a large amount of heat or electricity (Kim et al., 2013), making the process simple and scalable (Steriti et al., 2014). In chemical method, the disruption efficiency is highly dependent on the choice of solvent mixture. In other words, the chemical disruption of cell wall relies on the selective interaction of a chemical with the cell wall (Young, Nippen, Titterbrandt, & Cooney, 2014). The permeability of cells can be increased by diverse chemicals such as acids, bases and surfactants, which are involved in the degradation of the chemical linkages on the cell wall. Cells rupture occurs if the permeability of cells exceeds a certain limit (Kim et al., 2013). Cell lysis is crucial for the solubilisation of protein from microalgal cell (Ursu et al., 2014). The hydrolysis of protein takes place when subjected to certain chemical agents such as alkaline, acid or enzymes. Those chemical agents increase the protein solubility by breaking up protein into smaller fragments (Boye & Barbana, 2012).

Protein solubility can be varied at different pH. Highly acidic and alkaline conditions enhance the solubility of protein by inducing net charges on the amino acid residues (Damodaran, 1996). Alkaline and acid hydrolysis can be achieved through the addition of NaOH, KOH, HCl, and H_2SO_4 (Sathish & Sims, 2012). However, alkaline treatment is more widely applicable for microalgae (Chen & Vaidyanathan, 2013; Kim et al., 2013). It has been reported that more proteins were extracted in alkaline solution, compared to acidic solution (Safi et al., 2014b; Ursu et al., 2014). For example, without the aid of enzyme, 15-30% of protein can be extracted from microalgae meals under alkaline condition (Sari, Bruins, & Sanders, 2013).

Previous studies revealed that the alkaline solution was effective in serving the dual purpose of disrupting cell wall and solubilizing protein from microalgae (Gerde et al., 2013; Ursu et al., 2014). Alkaline treatment could act in synergy with the mechanical characteristics of the microalgal cell envelope, resulting in a significant increase of the protein release in the aqueous phase (Safi et al., 2014a; Safi et al., 2014b). Also, the alkaline solution could improve the solubility of protein by inducing net electrical charges on the amino acid residues (Damodaran, 1996). Apart from this, alkaline treatment was also reported to be the most commonly used method for protein extraction from oat flour. It was found that a good protein yield from oat bran can only be achieved under more alkaline conditions (Guan & Yao, 2008).

2.2.2 Mechanical method: ultrasonication treatment

Mechanical methods directly destroy the cells via physical force. The main advantage of these physical pretreatment methods is that they can be universally applied to any type of microalgae, regardless of the species (Kim et al., 2013). Among all the mechanical methods, ultrasonication is proposed to be able to rupture the microalgal cells mildly (Vanthoor-Koopmans, Wijffels, Barbosa, & Eppink, 2013). The effect of ultrasonication on extraction yield is attributed to the micro-scale eddies and heightened mass transfer, produced by cavitation and bubble collapse that can induce stress on microalgal cells. Tiny unsteady cavitation bubbles around cells in liquid medium are induced upon the exposure of microalgae to the high intensity of ultrasonic waves. Implosion of bubbles emits shockwaves that could generate chemical and mechanical energy for the shattering of the rigid cell wall and consequently causing the release of desired intracellular compounds into the solution (Dragone et al., 2010; Safi et al., 2014b). Guldhe et al. also agreed that the cavitational effect in ultrasonication could enhance the extraction of chemical compounds from microalgal cells by facilitating

solvent access through cell disruption and proper mass transfer (Guldhe, Singh, Rawat, Ramluckan, & Bux, 2014).

As one of the most promising mechanical cellular disruption methods, ultrasonication was found to be able to disrupt the cell wall effectively and thus increasing the extraction yield of various intracellular compounds from microalgae such as protein (Safi et al., 2014b), lipids (Natarajan, Ang, Chen, Voigtmann, & Lau, 2014) and pigments (Grimi et al., 2014), just to name a few. A recent experiment showed that ultrasonic treatment was able to disintegrate the microalgal cells successfully, indicated by the increased concentrations of protein and carbohydrate released into the solution (Keris-Sen, Sen, Soydemir, & Gurol, 2014). It was also reported that the application of ultrasonication has been proven to be efficient in disrupting various microalgal strains through the destruction of both cell walls and membranes (Piasecka et al., 2014). To protect against overheating during the process, samples are usually placed in an ice bath to absorb the ultrasonic heat (Piasecka et al., 2014). Alternatively, the operating temperature can be regulated externally by circulating cold water during the disruption process (Tiwari, 2015).

Ultrasonication is generally used in conjunction with solvents in cell disruption (Prabakaran & Ravindran, 2011; Tiwari, 2015). Many researchers have investigated the advantages of using ultrasound-assisted extraction (UAE) compared with the other methods. UAE belongs to the environmental friendly and energy-efficient technique (Barba, Brianceau, Turk, Boussetta, & Vorobiev, 2015). It does not consume as much energy as compared to the other cell disruption techniques such as high pressure homogenization (Vanthoor-Koopmans et al., 2013). This technique induces cell damage without requiring the addition of beads as required by bead milling (Middelberg, 1995) or offers clean extraction by avoiding the generation of fine cell debris as in high pressure homogenization which could increase the difficulty of separation in the

subsequent purification step (Günerken et al., 2015). UAE could reduce the solvent consumption and increase the penetration of solvent into cellular materials within short extraction time (Show, Lee, Tay, & Chang, 2014). The synergistic disruptive effects of the ultrasonic vibration and chloroform/methanol mixture on microalgal cells enabled approximately 1.5-2.0-fold increase in lipid extraction yields (Keris-Sen et al., 2014). Researchers also found that ultrasonication able to enhance the microalgal protein solubilisation (Gerde et al., 2013; Safi et al., 2014a). The extraction of a range of compounds from various matrices using UAE has also been reported in several studies such as the extraction of oil from soybean flakes (Li, Pordesimo, & Weiss, 2004) and polyphenols from tea leaves (Both, Chemat, & Strube, 2014). In general, ultrasonication is comparatively easy to use, versatile, flexible, and requires low investment compared to other novel extraction techniques such as pressurized solvent extraction, supercritical fluid extraction (SFE) or accelerated solvent extraction (ASE) (Tiwari, 2015). In addition, ultrasonic devices are applicable for laboratory scale use or can be scaled-up and operated continuously (Gerde, Montalbo-Lomboy, Yao, Grewell, & Wang, 2012).

2.3 Aqueous two-phase systems (ATPS)

ATPS phase separation technique was first developed by a Swedish biochemist, P. A. Albertsson in 1986 (Albertsson, 1986). Since then, ATPS has become a powerful tool for the separation of biomaterials (Albertsson, 1986; Hatti-Kaul, 2000) and has been extensively exploited to process different biomaterials such as proteins, enzymes, nucleic acids, cell organelles, virus particles, microorganism, plant and animal cells (Platis & Labrou, 2009; Raja et al., 2011). Previous studies reported that ATPS was able to separate intracellular protein from cell debris (Asenjo & Andrews, 2012) or other soluble cell components (Gu, 2014; Mohammadi et al., 2008). In the process of extracting papain from wet Carica papaya latex, ATPS showed higher recovery (88%)

with a much shorter processing time than traditional procedure involving a two-step salt precipitation (49%) (Nitsawang, Hatti-Kaul, & Kanasawud, 2006). The extraction of selective proteins namely photosynthetic pigment C-phycocyanin (Sørensen, Hantke, & Eriksen, 2013) or non-chlorophyll accessory pigments such as fucoxanthin (Gómez-Loredo, Benavides, & Rito-Palomares, 2014) from microalgae using ATPS have also been studied recently. This well-established method has more versatility over the conventional solvent extraction methods in the downstream processing of biomolecules (Raja et al., 2011). For instance, this system is not only able to extract protein by partitioning the desired protein and non-protein component or contaminant protein to different phases respectively, but also capable of concentrating the target protein by partitioning them into the smaller volume of the extraction phase (Zhao, Peng, Gao, & Cai, 2014). A schematic illustration of product recovery using ATPS is shown in Fig. 2.2.

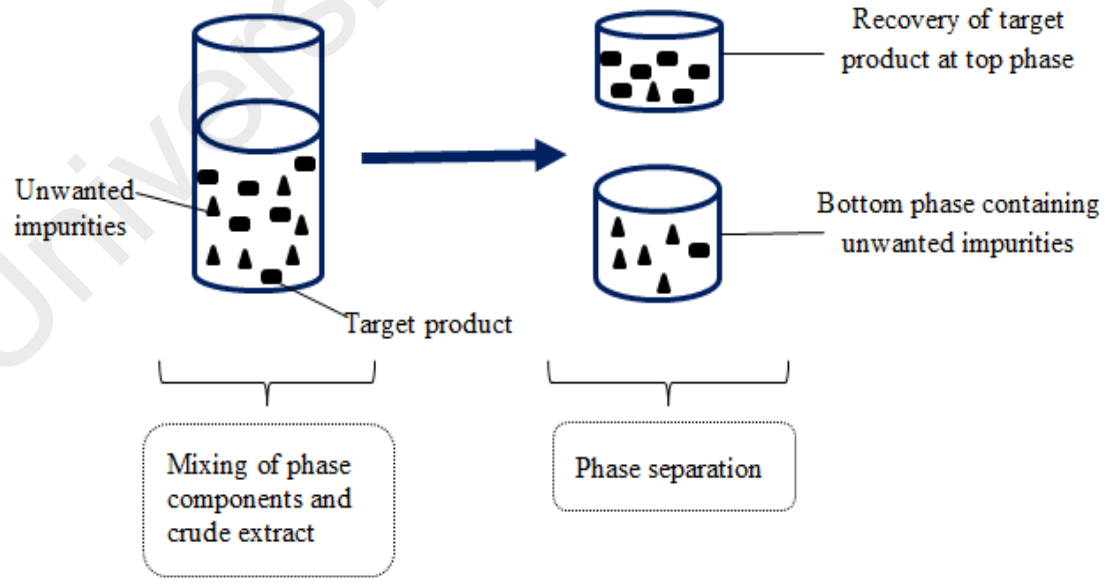

Figure 2.2: Schematic view of recovery of target product based on ATPS concept

2.3.1 Advantages of ATPS

ATPS is considered as an efficient and economical potential industrial technology. There are many advantages of using ATPS for bioseparation. These include simplicity in operation, high effectiveness, low cost (Goja, Yang, Cul, & Li, 2013; Raja et al., 2011; Zhao et al., 2014), rapid mass transfer and phase equilibrium, benign, low interfacial tension of phases (Goja et al., 2013), rapid separation with little denaturation, high selectivity, biocompatibility and low energy requirement (Quental et al., 2015; Raja et al., 2011; Rito-Palomares, 2004; Soares, Azevedo, Van Alstine, & Aires-Barros, 2015). ATPS has been proven to provide a biocompatible environment for the separation of proteins and enzymes from protein mixtures or cell extracts due to the presence of high water content in both phases (Agasøster, 1998; Albertsson, 1986). The extremely low interfacial tension of ATPS (between 0.0001 and 0.1 dyne/cm) creates high interfacial contact area of the dispersed phases, which in turn, enhances the efficiency of the mass transfer (Albertsson, 1986).

Chemical cost is considered one of the dominant cost factors for large-scale bioseparation process. The use of inexpensive phase components in ATPS makes the whole downstream processing more economical. Problem of downstream pollution may also be avoided by recycling the ATPS phase components (Hatti-Kaul, 2000). Extraction using this technological simple process is relatively rapid and the processing capacity of ATPS is quite high. Due to the simplicity and reliability of scaling-up approach, the extent of ATPS extraction to industrial scale application is feasible and practical (Goja et al., 2013; Zhao et al., 2014).

2.3.2 Parameters

In ATPS, the partition profile of the solutes depends on the different physicochemical interactions between the biomaterial and the phase forming

components (Gómez-Loredo, González-Valdez, & Rito-Palomares, 2015). Interactions such as hydrogen bond, van der Waals' forces, electrostatic interactions, steric effects, hydrophobicity, biospecific affinity interactions and conformational effects between the phase components and the substances contribute to the partitioning of the particular substance (Albertsson, 1986). As a result, the partitioning efficiency of solutes in ATPS can be manipulated and improved significantly by altering various parameters such as concentration of the phase component, biomolecule size, affinity of the molecules for the phase forming component, pH (Goja et al., 2013), system temperature (Raja et al., 2011) and the presence of additives (e.g. NaCl) (Rito-Palomares, 2004).

2.3.3 Alcohol/salt ATPS

Various compounds such as polyethylene glycol (PEG), dextran, salts, and ionic liquids can be used for the formation of ATPS. ATPS formed by alcohol and salt is simple, low-cost, characterized by low viscosity, short phase-separation time and high polarity (Tan, Huo, & Ling, 2002). Additionally, the phase-forming components can be recovered easily for reuse after aqueous two-phase extraction (Tan et al., 2002). Alcohol can be recovered using evaporation method whilst salt can be recycled by dilution crystallization method (Ooi et al., 2009; Tan, Li, & Xu, 2013). These characteristics contribute to the easy upscaling of alcohol/salt ATPS for industrial scale (Tan et al., 2013). However, extensive research concerning the recycling of the phase components from this type of ATPS is still limited and thus requires further investigation.

2.3.4 ATPS-based integrated process: Extractive disruption

Downstream processing represents a major economic limitation to the mass production of bioproducts from microalgae at lower cost (Günerken et al., 2015). As part of the downstream processing, isolation and extraction continue to be a significant

challenge towards the commercial production of microalgal protein. The development of competent and vigorous new downstream strategy is crucial to favour the economic feasibility of the process (Jegathese & Farid, 2014). Aspects such as recovery, cost, throughput and compatibility need to be considered when developing an economically practical downstream process (Naganagouda & Mulimani, 2008). For environmental benefits and long-term sustainability, all the processing stages should be simplified without the involvement of extensive energy input. Furthermore, the processes should be easily adopted and implemented in the existing industry as a strategy to sustainable aquaculture (Jegathese & Farid, 2014).

In the recent years, the notion to implement process integration into the industrial scale of bioproducts recovery process is of great practical and economic interest (Benavides et al., 2008; Fresewinkel et al., 2014). Through process integration, several downstream processes such as separation, concentration and extraction can be integrated into one single step and this could reduce the overall production cost (Gu, 2014; Mohammadi et al., 2008). Previous study demonstrated that the attempt to recover intracellular protein from bakers' yeast using process integration wherein cell disruption and ATPS were integrated into one single step was realizable (Rito-Palomares & Lyddiatt, 2002). Integrated method offers considerable potential benefit for the recovery of intracellular protein (Benavides et al., 2008). Studies showed that the direct integration of cell disruption with primary recovery unit operations enable faster processing with less opportunity for target modification or degradation. This could thus enhance both the yield and molecular quality of protein products (Buyel, Twyman, & Fischer, 2015; Rito-Palomares & Lyddiatt, 2002). Besides reducing the number of operational steps, this method could also lessen waste, reduce energy consumption and consequently decrease the overall cost (Goja et al., 2013; Zhao et al., 2014).

The flow diagrams of conventional discrete process and integrated process (extractive disruption) are illustrated in Fig. 2.3.

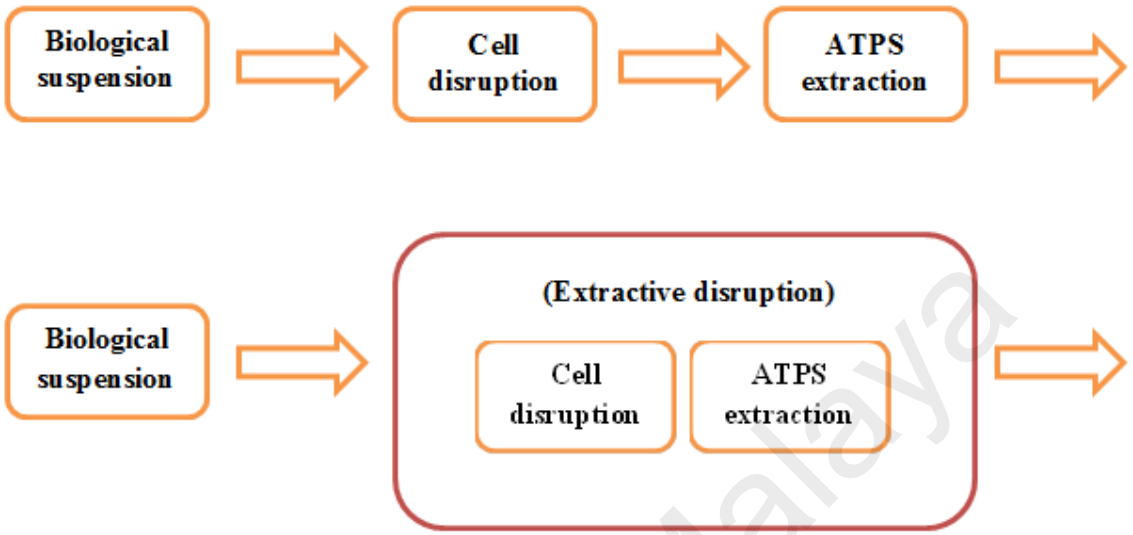

Figure 2.3: Simplified representation of two flow diagrams in downstream processing for comparison. The upper flow diagram represents the conventional discrete process in which the cell disruption process is followed by aqueous two-phase extraction. The lower diagram represents the integrated process (extractive disruption) as proposed in this study.

2.4 Concluding remark

Although different extraction methods are available for protein recovery, ATPS which consists of low-cost phase forming components such as alcohol/salt that feature potential recycling capability is still a better option. This system confers many advantages over other recovery strategies (Benavides et al., 2008). In numerous studies, ATPS has shown great potential for the efficient separation and extraction of biological compounds from various sources. Therefore, the direct integration of alcohol/salt ATPS with cell disruption process is deemed as a potential and practical method to be applied in the downstream processing. Nonetheless, the adoption of the ATPS-based extractive disruption strategy in the microalgal industry is still in the infant stage. Therefore, the possibility of applying this strategy on protein recovery from microalgae is worth to be studied.

CHAPTER 3: MATERIALS AND METHODS

To achieve the research objectives, two experimental processes were designed in this study.

1. Cell disruption

   The first experiment was mainly focus on the disruption process. The role of ultrasonication, alkaline treatment and the effects of different solvents in rupturing the cell wall and protein solubility of microalgae were studied. This experiment formed a basis towards developing a more efficient and versatile microalgal disruption and protein extraction process.

2. Extractive disruption integrated process

   An extended study which involved the integration of cell disruption and aqueous two-phase extraction into one step was carried out in the second experiment. The feasibility of applying methanol/potassium salt ATPS in the integrated process was studied. The effects of different ATPS variables such as types of salt, the concentrations of methanol and potassium salt, the addition of sodium chloride (NaCl) on the partitioning behaviour of protein were determined to obtain maximum protein yield from microalgae under optimum conditions. Besides that, the possibility of upscaling and the effectiveness of using the recycled phase components at each recycling step were also explored.

These two experimental processes were summarized in the flow diagrams and illustrated in Figs. 3.1-3.2.

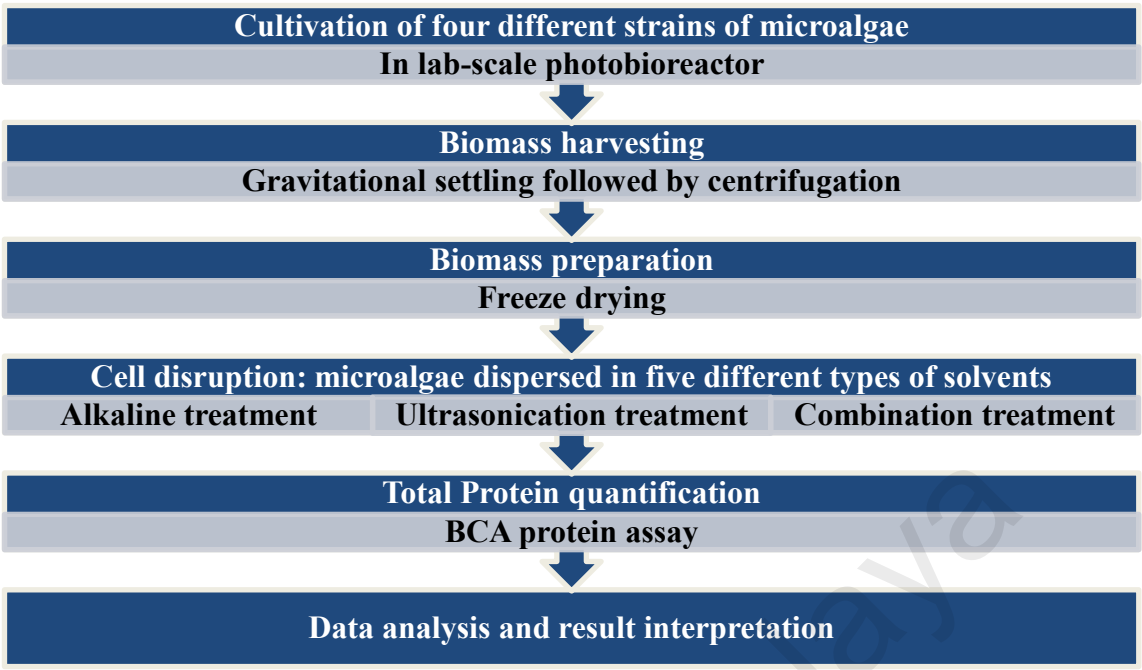

Figure 3.1: Flow diagram of the first experimental process from microalgae cultivation to protein quantification

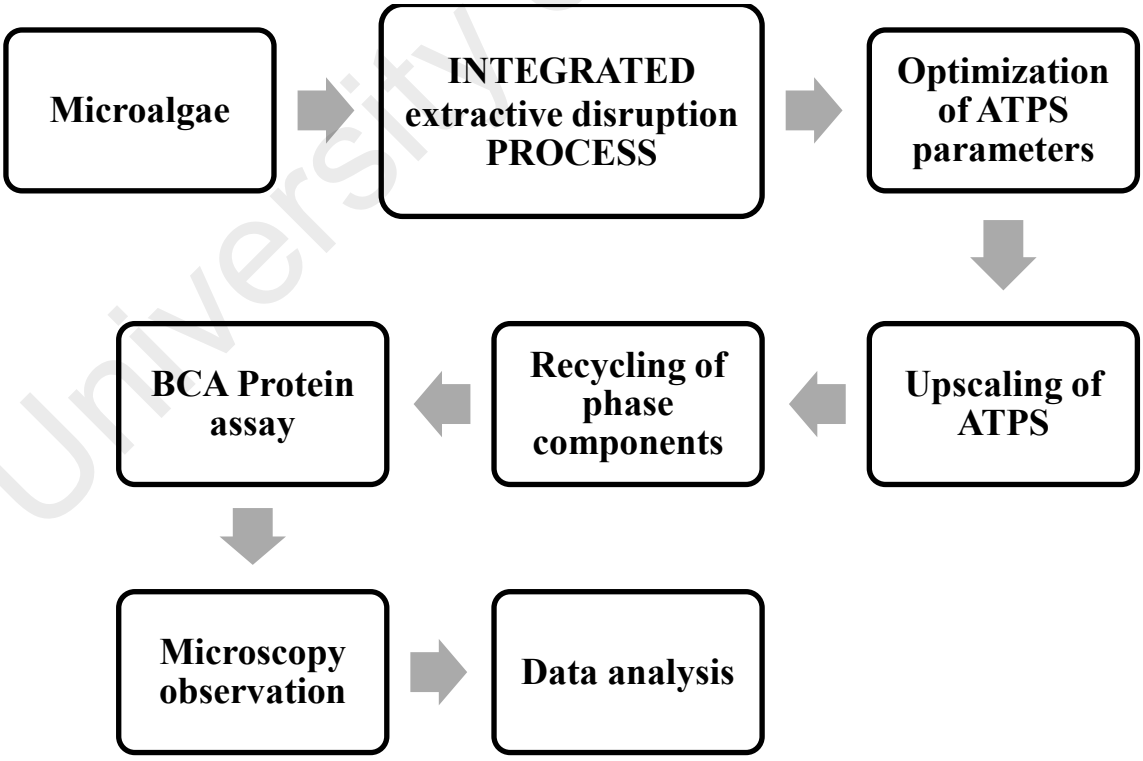

Figure 3.2: Flow diagram of the second experimental process for the extractive disruption of microalgal protein

3.1 Materials

BCA protein test kit with bovine serum albumin (BSA) standards included in (71285-3) and ethanol were purchased from Merck. Chemicals such as methanol, 1-propanol, 2-propanol and potassium hydroxide (KOH), NaCl, tripotassium phosphate (K_3PO_4) and dipotassium phosphate (K_2HPO_4) were purchased from Sigma Aldrich.

3.2 Microalgae

Four different strains of green microalgae were provided by National Cheng Kung University, Taiwan for the present study. These include *Chlorella sorokiniana* and *Chlorella vulgaris* which belong to the division of *Chlorophyta* and *Trebouxiophyceae* class; whereas both *Scenedesmus* sp. Esp-07 and *Chlamydomonas* sp. Tai-03 are from the same division of *Chlorophyta* and *Chlorophyceae* class. The microalgae were cultivated in a laboratory-scale photobioreactor to produce maximum biomass. Next, the frozen paste of crude microalgal biomass was dried using freeze dryer. Then the biomass was manually ground into fine powder using a mortar. This freeze drying process mainly served to dry the microalgal biomass and was not part of the disruption process. Freeze drying is broadly used for dewatering of microalgal biomass. It is a gentle processing technique which enables the preservation of all the cell constituents without causing damage to the cell wall (Brennan & Owende, 2010; Guldhe et al., 2014). Besides ease of storage, the microalgal stocks were prepared and used in dried biomass form throughout the study. This was to standardize the starting materials and avoid measurement errors associated with liquid carry-over in microalgae fresh weight (Slocombe, Ross, Thomas, McNeill, & Stanley, 2013). As all samples were being treated equally, this shall normalize the effect of freeze drying, hence the results would be strictly dependent on the subsequent differential treatments for comparison.

3.3 Cell disruption

Five different types of solvents were chosen to examine their efficacy to facilitate protein release in the disruption process. The solvents were as follows: methanol, ethanol, 1-propanol, 2-propanol and water for comparison.

3.3.1 Control

To compare with the other disruption treatments, a set of blanks was prepared whereby cell disruption was not performed for this control group. Microalgal biomass weighed 0.1g was dispersed in 10 mL of each solvent without 0.5N KOH, respectively. The mixture was vortexed for 1 min. After that, the supernatant was recovered by centrifugation at 4000 rpm for 5 min (Eppendorf Centrifuge 5810 R, Germany) for protein analysis.

3.3.2 Ultrasonication

Microalgal biomass weighed 0.1g was dispersed in 10 mL of each solvent without 0.5N KOH, respectively. The test tubes were vortexed for 1 min and then sonicated at 37 kHz for 20 min (Thermo-3D, Australia). After centrifugation at 4000 rpm for 5 min, the pellet was discarded and supernatant which contained protein was collected for protein analysis.

3.3.3 Alkaline treatment

Microalgal biomass weighed 0.1g was dispersed in 10 mL of each solvent with 0.5N KOH respectively. The mixture was mixed thoroughly and vortexed for 1 min. After centrifugation at 4000 rpm for 5 min, pellet was discarded and supernatant which contained protein was collected for protein analysis.

3.3.4 Combination of alkaline and ultrasonication treatment

Microalgal biomass weighed 0.1g was homogenized in 10 mL of each solvent with 0.5N KOH, respectively. The mixture was vortexed for 1 min and then subjected to ultrasonication for 20 min. After centrifugation at 4000 rpm for 5 min, the pellet was discarded and supernatant which contained protein was collected for protein analysis.

3.4 Extractive disruption integrated process

3.4.1 Phase diagram

The phase diagrams for methanol/K_2HPO_4 and methanol/K_3PO_4 were constructed according to the turbidometric titration method which has been described in detail by Albertsson (Albertsson, 1986). In this experiment, a mixture of methanol and potassium salt of known amount was titrated drop-wise with the appropriate amount of distilled water until the mixture turned clear. This indicated the formation of one phase. Drop-wise titrations were carried out on an electrical balance. The mixture was mixed constantly after each droplet. The total weight of water added was measured and the resultant compositions of methanol and potassium salt at the point of transition were calculated. The above procedures were repeated to obtain sufficient binodal points. Phase diagrams were then plotted at varying methanol and salt concentrations based on the binodal points (Hatti-Kaul, 2000).

3.4.2 Integrated process: Cell disruption and aqueous two-phase extraction for microalgal protein recovery

This section describes the procedures of process integration in which both ultrasound-assisted disruption and aqueous two-phase extraction were integrated into one process and carried out simultaneously. Appropriate amounts of potassium salt, water and methanol were weighed into a 15 mL centrifuge tube. Subsequently, 0.2% (w/w) of dry microalgal biomass was added to each system to obtain a final total weight

of 10 g. All components of the system were measured by weight. After thorough mixing by a vortex mixer, each mixture was exposed to ultrasonication for 20 min. Subsequently, the mixture was centrifuged at 4000 rpm for 5 min for the complete formation of biphasic system. The volume ratio of each system was recorded and protein concentrations in the top and bottom layer of ATPS were estimated respectively by BCA assay. All experiments were carried out at room temperature for at least three independent trials.

3.4.3 Optimization of ATPS

To achieve maximum protein recovery from microalgae, four parameters such as types of salt (K_2HPO_4 and K_3PO_4), salt concentrations, methanol concentrations and NaCl addition (0–5%) (w/w) were optimized systematically. The concentration of the salt and methanol were selected based on the phase diagram (above the binodal curve) constructed in Section 3.4.1, in which the mixture of both components able to form two immiscible aqueous phases. The method of ATPS optimization adopted in this study was "one variable at a time (OVAT)" in which significant factors of the process were identified and altered by keeping all other factors constant (Raja et al., 2011). The volume ratio of each system was recorded and protein concentrations in the top and bottom layer of ATPS were estimated, respectively, by BCA assay. All experiments were carried out at room temperature for at least three independent trials.

3.4.4 Upscaling of ATPS

After optimization, the chosen system was scaled up to a final total weight of 1000g. The sample preparation steps and experiment were carried out following the same procedures and under the same operating conditions as in small scale. The volume ratio of each system was recorded and protein concentrations in the top and bottom layer of

ATPS were estimated respectively by BCA assay. All experiments were carried out at room temperature for at least three independent trials.

3.5 Recycling of phase components

The effectiveness of using recovered phase components of the integrated process was evaluated in this section. After centrifugation, the methanol-rich top phase which was separated from the salt-rich bottom phase was then subjected to evaporation for recycling. The recycled methanol was then mixed with the bottom phase recovered from the primary system to form a secondary two-phase system. Separation of cell debris was performed between cycles. Appropriate amounts of fresh methanol, water and potassium salt were added in to account for phase components losses from the system, when deemed necessary. Five successive recovery operations were performed by repeating the same procedures (Ng et al., 2012). The volume ratio of each system was recorded and protein concentrations in the top and bottom layer of ATPS were estimated, respectively, by BCA assay. All experiments were carried out at room temperature for at least three independent trials.

3.6 Bicinchoninic acid (BCA) assay

The total protein concentrations were estimated by the BCA protein assay according to the user manual of Merck protein assay kit. A total of 25 µL of sample was mixed with 200 µL of the working reagent in a 96-well plate and subsequently incubated for 30 min at 37°C. The absorbance was measured at 562 nm using microplate absorbance reader (BioTek Epoch Microplate Spectrophotometer, USA). BSA was used as a protein standard to construct a calibration curve. To eliminate interference from the phase components, samples were analyzed against a set of blanks containing the identical phase system without microalgae. All the protein content estimations were carried out in

triplicate. The spectrophotometric absorbance readings were converted to protein concentrations (μg/mL) using an equation generated from the BSA standard curve (Smith et al., 1985).

3.7 Morphological observation of microalgae cells

Microscope slides of microalgal cells before and after subjected to process integration were prepared for microscopy observation. A drop of water containing microalgal cells was placed onto a microscope slide and checked under a light microscope (Olympus CX21, Japan). At magnification of 1000x, the microscopy images were captured to evaluate the morphological changes of the treated cells compared with the untreated cells (control).

3.8 Calculations

- The protein content of the biomass was calculated using the following equation (López et al., 2010):

  $$\text{protein}\left(\%, \frac{w}{w}\right) = \frac{CVD \times 100}{m} \quad (1)$$

  where,

  C denotes protein concentration (μg/mL) obtained from the calibration curve; V symbolizes volume (L) of the lysis buffer used to resuspend the biomass; D is the dilution factor; m represents the amount of biomass (mg).

- The partition coefficient (K) of the protein is defined as the ratio of the protein concentration in the two phases (Lin et al., 2013):

  $$K = A_T/A_B \quad (2)$$

  where,

A_T and A_B represent the equilibrium concentration of the partitioned protein (μg/mL) in the top phase and bottom phase, respectively.

- The volume ratio (V_R) was calculated according to the following equation (Lin et al., 2013):

$$V_R = V_T / V_B \quad (3)$$

where,

V_T symbolizes the volume of top phase; V_B denotes the volume of bottom-phase.

- Yield of protein in top phase was determined to evaluate the recovery performance (Lin et al., 2013; Ooi et al., 2009):

$$Y_T(\%) = \frac{100}{1 + [1/(V_R * K)]} \quad (4)$$

where,

V_R is the volume ratio; K is the partition coefficient.

3.9 Statistical analysis

All the results were expressed as mean ± SD of three independent trials. Data were statistically analysed at a 95% confidence level either using one-way analysis of variance (ANOVA) or two-sample t-Test.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Cell disruption

The percentages of protein released from four different strains of microalgae (*C. sorokiniana, C. vulgaris, Chlamydomonas* sp. Tai-03*, Scenedesmus* sp. Esp-07) in the respective solvents after each cell disruption treatment are presented in Figs. 4.1-4.4. In this study, three different types of cell disruption methods were compared. Control (in single solvent without sonication or KOH) was served as the baseline reference. Surprisingly, osmosis phenomenon was observed in all the green microalgae which were known to have rigid cell walls. Dispersion of green microalgae into single solvent alone released approximately 0.1-3.5% (w/w) of protein (Figs. 4.1-4.4). This indicated that all the solvents could penetrate the thick cell wall structure of the microalgae, but at different diffusion rates.

Blanks for respective solvents were prepared following the same way as the sample preparation steps, except without the presence of microalgal cells. However, it was noticed that 2-propanol (without protein) was incompatible with the BCA kit, producing false positive result upon mixing with the BCA reagent resulting in the formation of intense purple colour. This would cause an overestimation of the protein content and thus the results of samples using 2-propanol as the solvent were not included in this study.

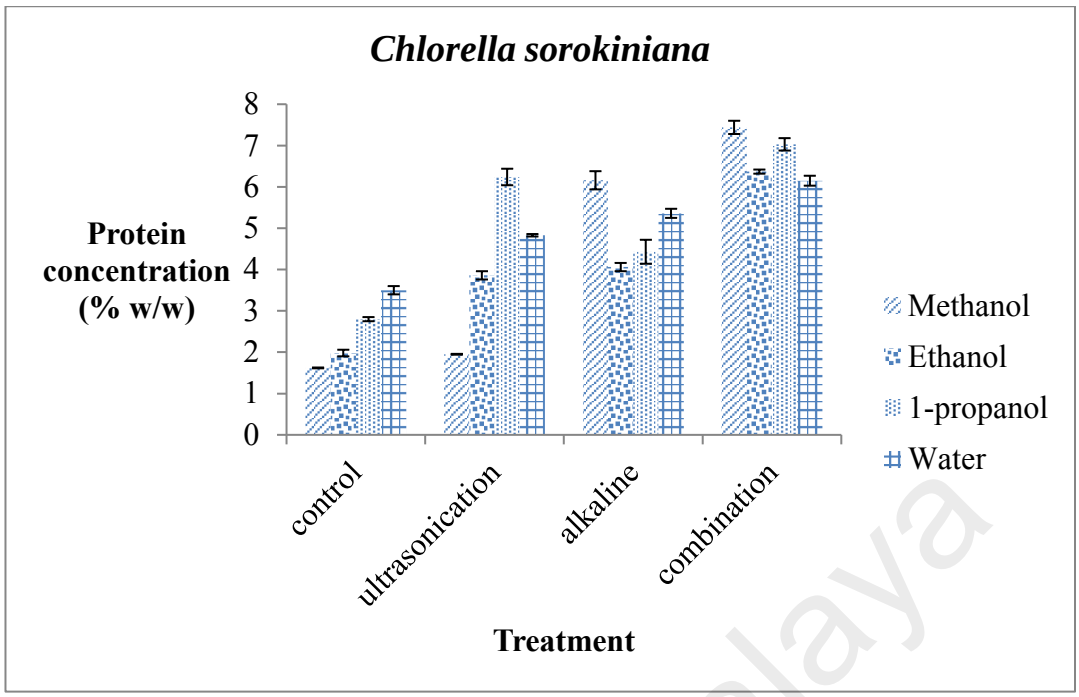

Figure 4.1: Total protein content of *Chlorella sorokinania* subjected to different disruption procedures. Result pooled from three independent trials, error bars indicate mean value ± SD.

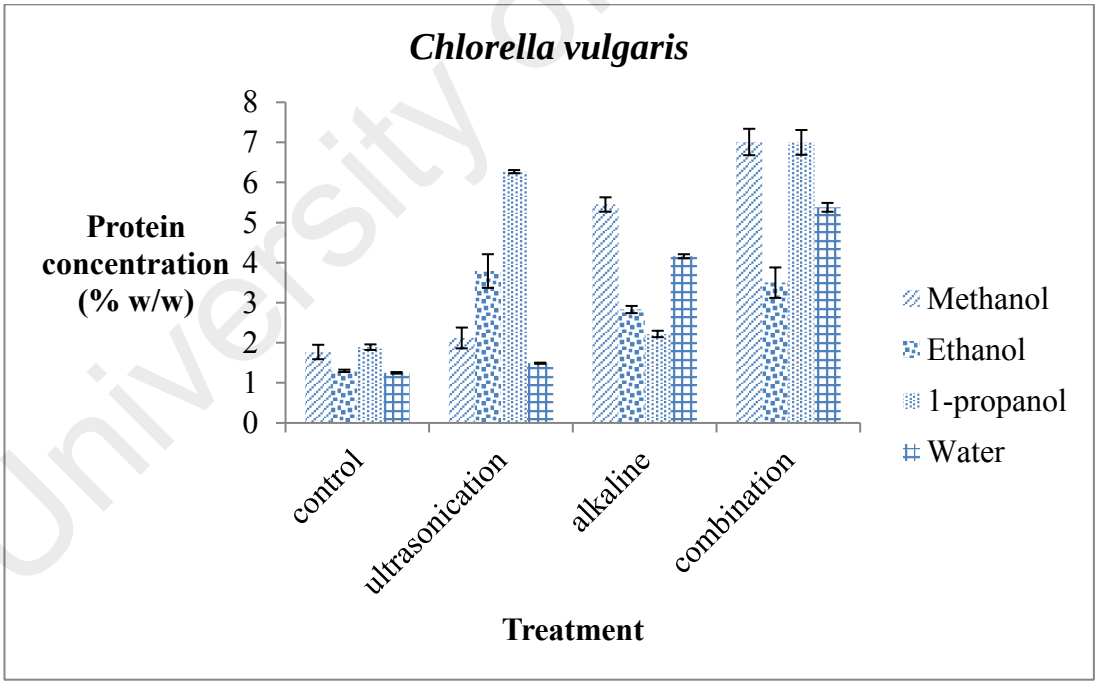

Figure 4.2: Total protein content of *Chlorella vulgaris* subjected to different disruption procedures. Result pooled from three independent trials, error bars indicate mean value ± SD.

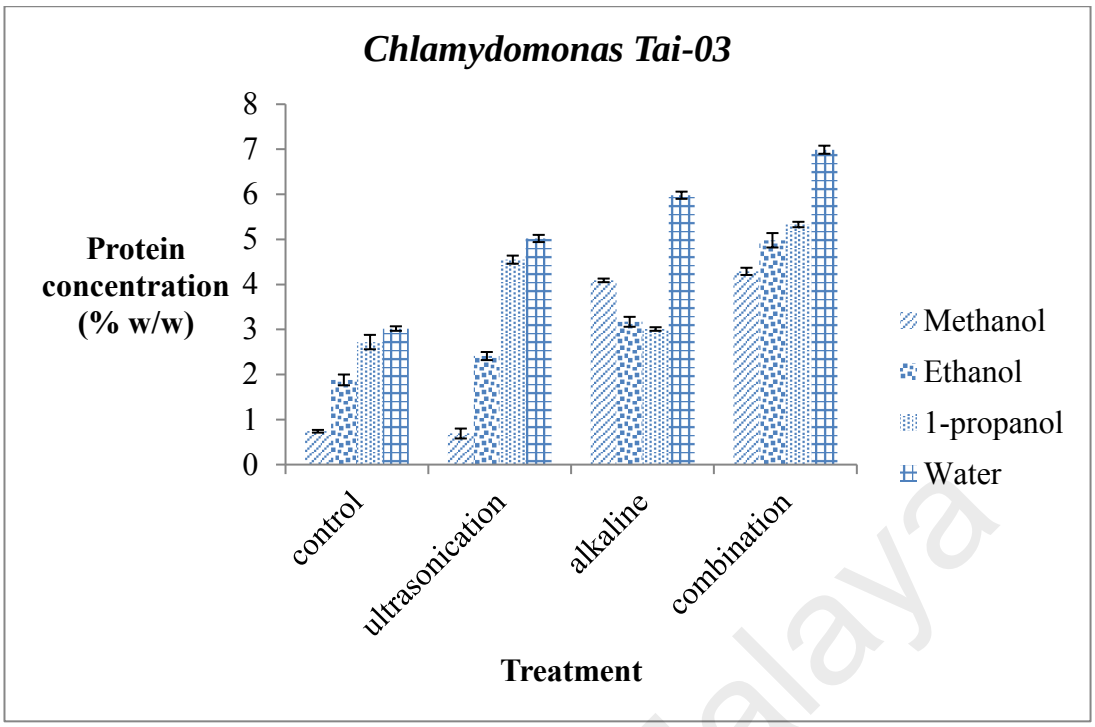

Figure 4.3: Total protein content of *Chlamydomonas* sp. Tai-03 subjected to different disruption procedures. Result pooled from three independent trials, error bars indicate mean value ± SD.

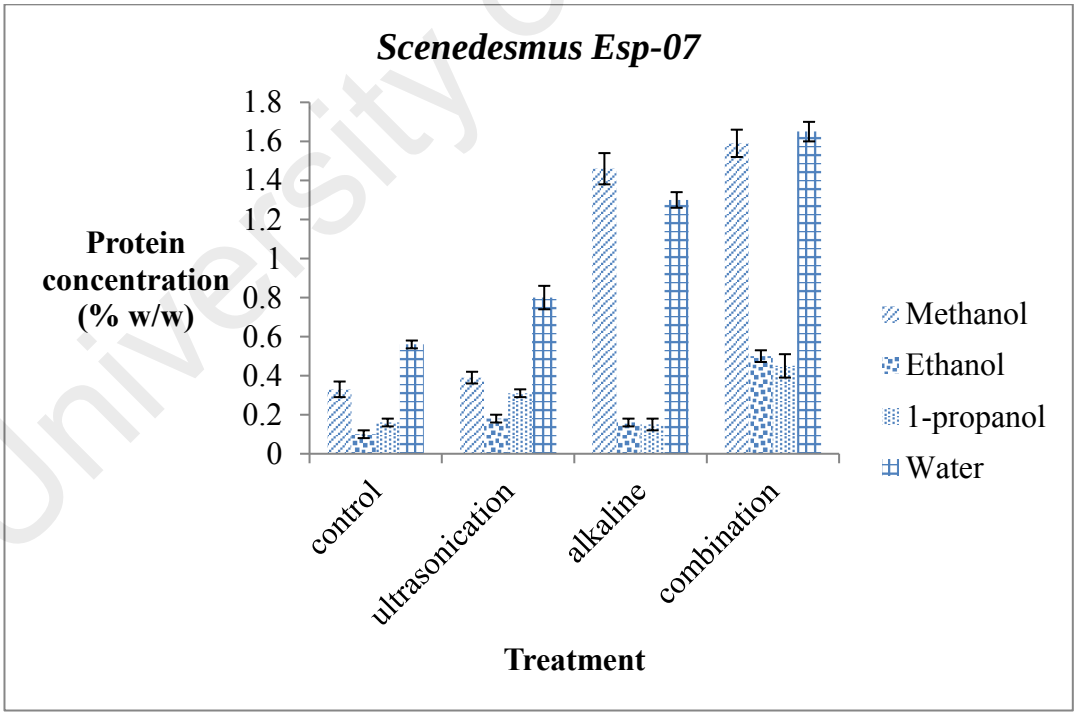

Figure 4.4: Total protein content of *Scenedesmus* sp. Esp-07 subjected to different disruption procedures. Result pooled from three independent trials, error bars indicate mean value ± SD.

4.1.1 Factors affecting protein release

The overall protein release from *Scenedesmus* sp. Esp-07 using ultrasonication, alkaline treatment, and combination of both was the lowest as compared to the other three strains (Fig. 4.4). A possible explanation to this could be due to the nature of its cell wall structure, making *Scenedesmus* sp. Esp-07 less susceptible to these treatments. Cell wall structure has an important effect on the disruption efficiency and the release of intracellular biomolecules (Safi et al., 2014a). Nonetheless, the microalgal cell walls are complex, diverse, not fully understood and this requires further investigation. The thickness, rigidity and composition of cell wall can be varied dramatically within and between species. Apart from that, other factors that contribute to the variation in the cell wall compositions are growth phase, cultivation conditions (Safi, Zebib, Merah, Pontalier, & Vaca-Garcia, 2014c) and the presence of stress factors (Günerken et al., 2015). For example, the nascent cell wall of microalgae is generally thin and fragile, but the thickness is gradually increased in the mature stage (Safi et al., 2014c). Due to the diverse cell wall structure, different species of microalgae have different disruption propensities (Halim et al., 2012). This explained the variation in the protein release rate from different strains of microalgae in this study despite using the same disruption method (Figs. 4.1-4.4).

On the other hand, it has been reported that the intra-species variations in the cell walls of *Chlorella* sp. can be substantial (Gerken, Donohoe, & Knoshaug, 2013). This explained the significant difference of protein yield in each solvent in the case of *C. vulgaris* and *C. sorokiniana* although subjected to the same treatment (Figs. 4.1-4.2). Glucosamine, the common dominant cell wall polymer, was postulated to be the main factor contributing to cell wall rigidity and resistance in *Chlorella* species, namely *C. vulgaris and C. sorokiniana*. However, 30–44% of the cell wall composition are still remain undetermined (Kapaun, Loos, & Reisser, 1992).

4.1.2 The effect of solvents on protein release

Both *Chlorella* species, when subjected to alkaline treatment and combination treatment, respectively, produced the highest protein yields in methanol than the other solvents. As shown in Fig. 4.1, the percentage of protein release from *C. sorokiniana* by alkaline and combination treatment was 6.16±0.22% (w/w) and 7.44±0.16% (w/w), respectively. The protein yield from *C. vulgaris* after the alkaline and combination treatment was 5.45±0.18% (w/w) and 7.01±0.33% (w/w), respectively (Fig. 4.2). Amongst all the solvents, water was observed to be the best solvent in all the treatments for *Chlamydomonas* sp. Tai-03 (Fig. 4.3). For example, when treated with combination treatment, *Chlamydomonas* sp. Tai-03 that was dispersed in water exhibited higher protein release [6.99±0.09% (w/w)] than methanol [4.29±0.08% (w/w)], ethanol [4.98±0.16%) (w/w)] and 1-propanol [5.33±0.06% (w/w)] (Fig. 4.3). On the other hand, *Scenedesmus* sp. Esp-07 that were treated with combination method showed similar percentage of protein release in each solvent of water and methanol, and the percentage of protein release in each solvent was about three-fold higher than that ethanol and 1-propanol (Fig.4.4).

4.1.3 The effect of different treatments on protein release

Among all the disruption treatments tested, combination treatment displayed the highest efficacy in facilitating protein release from microalgae of different strains (Figs. 4.1-4.4). Similar trend was also noted for *Scenedesmus* sp. Esp-07, although its overall protein yield was relatively low (Fig. 4.4). As compared to the other treatments, *Scenedesmus* sp. Esp-07 showed an increase in the percentage of protein release after treated with combination treatment (Fig. 4.4). Likewise, when dispersing *C. sorokiniana* in water, cells treated with combination treatment showed the highest percentage of

protein release [6.15±0.12% (w/w)], compared to the alkaline treatment [5.36±0.11% (w/w)], ultrasonication treatment [4.83±0.03% (w/w)] and control [3.5±0.10% (w/w)] (Fig. 4.1). *Chlamydomonas* sp. Tai-03 that was dispersed in water displayed the highest percentage of protein release [6.99±0.09% (w/w)] under combination treatment, followed by alkaline treatment [5.98±0.08% (w/w)], ultrasonication treatment [5.02±0.08% (w/w)] and control [3.02±0.05% (w/w)] (Fig. 4.3). Based on the results, it can thus be concluded that the combination treatment was the most effective method for cell disruption and protein solubilisation.

4.1.4 The effect of ultrasonication on protein release

Compared to the control of respective solvent, a significant increase in the concentration of protein was observed after exposing each strain of microalgal cells to ultrasonic vibration (Figs 4.1-4.4). Likewise, the combination of both alkaline and ultrasonication treatment improved the percentage of protein release from microalgae compared to the single alkaline treatment. Amongst all the solvents used, microalgae that were dispersed in 1-propanol generally showed higher protein yield after ultrasonication treatment rather than alkaline treatment (Figs 4.1-4.4). As shown in Fig. 4.2, the percentage of protein released from *C. vulgaris* in 1-propanol under ultrasonication treatment was almost three-fold higher than that of the control or alkaline treatment. *C. sorokiniana*, when treated with ultrasonication in ethanol and 1-propanol, respectively, displayed an increase in the protein yield by two-fold compared to their corresponding control (Fig. 4.1).

It has been reported that ultrasonication could generate greater forced penetration of solvent into cellular components within short extraction time, thus enhancing the percentage of protein release from biomass cells (Show et al., 2014). Guldhe et al. (2014) explained that the improved extraction performance of microalgae was attributed

by the cavitational effect of ultrasonication which facilitated the solvent access through cell disruption and mass transfer (Guldhe et al., 2014). The positive effect of ultrasonication on cell disruption for protein release was also agreed by Parimi et al. (2015), who found that ultrasonication resulted in a higher protein recovery in the supernatant than that of the control (Parimi et al., 2015). A study conducted by Araujo et al. also observed that the application of ultrasonication in the extraction process was able to increase the efficiency of the lipid extraction from microalgae (Araujo et al., 2013).

4.1.5 The effect of alkaline treatment on protein release

The effect of alkaline treatment on cell disruption and protein release was illustrated in Figs. 4.1-4.4. All microalgae that were treated with alkaline treatment in different solvents (except 1-propanol), showed a significant increase in the protein concentration compared to the control or ultrasonication treatment. As shown in Figs. 4.1-4.3, this was particularly obvious when dispersing the *C. sorokiniana, C. vulgaris* and *Chlamydomonas* sp. Tai-03 in the binary mixture of KOH and methanol (alkaline treatment), which resulted more than three-fold higher protein release compared to the control (methanol without the addition of KOH). At least a two-fold increase in the protein yield was observed when dispersing the *C. vulgaris, Chlamydomonas* sp. Tai-03 and *Scenedesmus* sp. Esp-07 in the water that contained KOH (alkaline treatment) compared to the corresponding control (Figs. 4.2-4.4). This proved that potassium hydroxide (KOH) was the key chemical aiding in cell wall disruption and protein release.

This finding was in agreement with the previous studies. The alkaline treatment which acted on the microalgal cell envelope was similar to mercerization, as shown by a significant increase in the protein concentration of the extract (Safi et al., 2014b).

Alkaline treatment was found to be effective in disturbing the cell wall of microalgae, particularly those with thick extracellular covering. This treatment could induce the hydrolysis of the hydrogen linkages and ester bonds between non-polysaccharides and polysaccharides on the cell envelope and subsequently stimulate the release of intracellular biomolecules (Chen & Vaidyanathan, 2013; Costa & Plazanet, 2016; Kim et al., 2013). Solvent added with sodium hydroxide was able to weaken the cell wall of *C. vulgaris* and *N. oculata* by penetrating the microcrystalline structure of the cellulose-rich cell walls and dissolving the hemicelluloses attached to cellulose (Safi et al., 2013; Safi et al., 2014b). Consequently, the permeability of cell wall can thus be improved, making the microalgal cells highly susceptible to protein extraction (Safi et al., 2014b). A research performed by Dong et al. also found that alkali might be able to modify the cell wall of *C. sorokiniana*, causing it to become lipid-permeable (Dong et al., 2015).

It was found that alkaline solution served the dual purpose of disrupting cell wall and enhancing protein solubility, where in both processes can be performed simultaneously (Parimi et al., 2015; Safi et al., 2015). Alkaline solution is commonly used in protein extraction due to the favourable solubility of protein at alkaline pH (Boye & Barbana, 2012). Another author also agreed that protein exhibit maximum solubility at alkaline pH due to their acidic nature. Alkaline solution could induce net electrical charges on the amino acid residues which would contribute to the high solubility of protein in alkaline media (Damodaran, 1996).

4.1.6 The effect of combination treatment (alkaline and ultrasonication) on protein release

Each treatment has its own strengths and limitations. For instance, solvent-mediated treatment could provide a more uniform cell disruption (Günerken et al., 2015). Whereas the concept of ultrasonic disruption is based on the creation of cavities in cell

suspension (Safi et al., 2014b). The mechanical effect of ultrasonication could enhance microalgal protein solubilisation (Gerde et al., 2013) by facilitating solvent access through cell disruption and proper mass transfer (Guldhe et al., 2014). Overall, the application of combination treatment in cell disruption enabled the release of highest protein content from all strains of microalgae. This proved that the combination of both alkaline and ultrasonication treatment with complementary strengths, not only compensated for the shortcomings of each other's, but also resulted in a greater efficiency in protein extraction.

It was observed that the finding of this study was consistent with several previous studies. Combining ultrasonication with solvent systems in microalgae extraction resulted in better yield, as it facilitated the contact between intracellular compounds and solvents (Guldhe et al., 2014). Ursu et al (2014) agreed that the optimal treatment for protein solubilisation was the combination of both alkaline and mechanical treatments (Ursu et al., 2014). Research revealed that the exposure of *C. vulgaris* to alkaline treatment alone induced only a partial permeation of the hemicellulose cell wall, which was still insufficient to solubilize and liberate the major part of the intracellular protein. On the contrary, higher protein solubilisation yield was obtained from *C. vulgaris* when the cells were subjected to both alkaline and mechanical treatments (Ursu et al., 2014). Besides that, a study suggested that ultrasonication combined with binary mixture of solvents led to the maximum recovery of valuable compounds from the microalgae of *Nannochloropsis* spp. (Parniakov et al., 2015). Another recent study which involved the extraction of protein from Irish brown seaweed *Ascophyllum nodosum* also demonstrated that the traditional alkaline based extraction method can be enhanced after combined with ultrasonication (Kadam, Álvarez, Tiwari, & O'Donnell, 2016). The combined process was generally more efficient and economical than the individual process (Wang, Li, Hu, Su, & Zhong, 2015) as it could increase the disruption

efficiency without requiring high energy demand (Günerken et al., 2015). Besides that, it was less laborious and could greatly reduce the extraction processing time, compared to the conventional chemical cell disruption techniques (Guldhe et al., 2014).

4.1.7 Industrial consideration

It is essential to develop an appropriate, rapid, cost-effective, simple, scalable and environment friendly method of cell disruption. The use of sophisticated technology for industrial application is not favourable as it usually requires complicated cleaning process, long processing time, expensive skilled labour, high capital and maintenance cost. Günerken et al (2015) advocated that a practical energy-efficient cell disruption technique should be developed to ensure efficient biological products recovery at minimum operating cost. High-energy and costly cell disruption methods and bio-products recovery are the major techno-economic bottlenecks (Günerken et al., 2015). There are numerous factors which can be very influential on the total costs and collectively determine the suitability of a cell disruption process. The energy consumption and labour cost are generally the most predominant consideration in the production of protein for industrial applications. Cost effectiveness of a cell disruption method might also be the resultant of the supplementary chemicals, solvent type, processing time, the ease of scalability as well as the operational and capital cost (Günerken et al., 2015).

4.1.7.1 Selection of solvents

A disruption process usually consumes large volume of solvents, therefore the choice of solvents to be used in cell disruption is another factor to be considered if the process were to be scaled up. Based on the cost, water is the cheapest solvent, followed by ethanol, methanol, 2-propanol and 1-propanol (Table 1). When dispersing both

Chlorella strains in methanol and 1-propanol respectively, the percentage of protein release in these two solvents were generally slightly higher than in water (Figs. 2-3). However, when considering both factors in terms of efficiency and costs for large scale application, water is still an excellent option of solvent in facilitating the cell disruption and protein solubilisation. This is due to its economic advantage and ubiquitous availability, making the upscaling process much cheaper and simpler than using alcohols as the solvent which must be purchased or obtained separately.

Table 4.1: Estimated cost of different types of solvent per litre in Malaysia.

Types of solvent	Source	Cost per litre (RM)
Methanol	Sigma Aldrich	RM 226
Ethanol	Merck	RM 200
1-propanol	Sigma Aldrich	RM 355
2-propanol	Sigma Aldrich	RM 269
Water	Based on water tariff in Malaysia (commercial usage) http://www.syabas.com.my/consumer/water-bill-water-tariff	RM 0.00207

4.1.7.2 Selection of cell disruption treatment

Solvents can be used to weaken cell membranes and reduce the heat or electricity required in the disruption process (Kim et al., 2013). In this case, alkalis induced swelling of the cell wall and thus reduced its strength. This would eventually increase the tendency of cell wall to be disrupted upon exposure to mechanical disruption (Lee et al., 2012). Therefore, combining both alkaline and ultrasonication treatment is the ideal method for industrial application, as the combination treatment demonstrated the most optimum protein yield from all strains of microalgae. The ultrasonication-assisted alkaline treatment has a combined advantage of cell disruption provided by the ultrasonic cavitation as well as the solvent solubility power. It was found that at the same exposure time, the protein yield of microalgae treated with combination method

was generally higher than that of the single treatment of either alkaline or ultrasonication (Figs. 2-5).

The specific energy consumption consumed by different disruption methods has been investigated. Researchers observed that the energy consumption of a disruption method is the result of varying design and operational parameters as well as microalgal species, thus an universal comparison between the energy consumption of different cell disruption techniques can be ambiguous and unjust (Günerken et al., 2015; Naveena, Armshaw, & Pembroke, 2015). In view of this, disruption methods that were operated under similar conditions should only be comparable. Previous studies reported that high pressure homogenization consumed much higher energy than ultrasonication under similar conditions such as using the same strain of microalgae (*Chlorococcum* sp.) at the same concentration of dry cell weight (Günerken et al., 2015; Halim et al., 2012; Lee et al., 2012). The energy consumption for high pressure homogenization was equivalent to 529 MJ kg^{-1} of the biomass. Whereas ultrasonication required only a quarter of the energy input at 132 MJ kg^{-1} of the biomass (Günerken et al., 2015; Halim et al., 2012; Lee et al., 2012). As such, high pressure homogenization was not chosen for the development of disruption process in this study.

Apart from estimating the specific energy consumption of a disruption method, several factors like labor cost, overall energy demand, operational and capital expenditures should also be taken into account when choosing a practical disruption method (Günerken et al., 2015). In general, the ultrasonication-assisted treatment offers clean extraction by releasing the intracellular contents from biomass without shattering the cells into very fine pieces. On the contrary, cell disruption using high pressure homogenization and bead milling would lead to the generation of very fine cell debris which could increase the difficulty of separation in the subsequent purification step (Günerken et al., 2015). Such methods would demand higher downstream processing

costs and may negatively impact the scalability of the process for large scale application.

The setup parameters of a disruption method play a key role in the efficiency of cell disruption. The disruption efficiency of a bead milling depends on the shape, diameter and composition of the beads, agitation speed, the design of the agitator and milling chamber, residence time as well as characteristics of the feed such as temperature, viscosity and concentration (Lee et al., 2012; Safi et al., 2015). These parameters need to be optimized in order to reduce energy consumption (Safi et al., 2015). However, the process intensification requires an extremely skilled person for operation and thus may not be cost-effective because of the high labour costs. As such, Lee et al. postulated that the application of a bead-beating method at large scale production is not easy (Lee et al., 2012). On the other hand, ultrasonic devices is easily scalable from batch to continuous processing (Gerde et al., 2012; Naveena et al., 2015). The setup parameters of ultrasonication are less complicated and less sophisticated which does not require intensive technical training for operation, much easier to operate with lower maintenance cost and modest power requirements (Naveena et al., 2015). Another advantage of using ultrasonication in disruption was its ability to rupture cell walls without the addition or separation of beads as required by bead milling (Middelberg, 1995).

4.2 Extractive disruption integrated process

Extractive disruption integrated process involved the integration and simultaneous operation of both cell disruption and aqueous two-phase extraction. Based on the finding from the first experiment, the combination of both alkaline and ultrasonication disruption treatment was demonstrated to be the most effective in rupturing the cell wall for the release of protein from microalgae. As such, the combination disruption

treatment was chosen as the disruption method in this experiment. On the other hand, the protein-rich strain of *C. sorokiniana* and methanol/potassium salt ATPS were selected for evaluation in this integrated process. Potassium salts was able to serve two different functions in the integrated process. Besides being the main component for the formation of ATPS, the addition of potassium salts into the system could create an alkaline condition which is favorable for microalgal cell disruption and protein solubilisation.

4.2.1 Phase diagram of ATPS

ATPS is a liquid-liquid fractionation method (Raja et al., 2011) which composed of two liquid phases of structurally different components that are immiscible when the limiting concentrations are exceeded (Hatti-Kaul, 2000). Reliable phase diagram are beneficial to the selection of an appropriate ratio of the phase composition when designing an ATPS. Information about the concentration of phase forming components required for two phases formation, the salting-out and phase-separation abilities as well as the ratio of phase volumes can be extrapolated from the phase diagram (Wang, Wang, Han, Hu, & Yan, 2010b). The distinct boundary of the phase separation presented on the diagrams (Figs. 4.5-4.6) is known as a binodal curve. It divides a region of component concentrations that will form two immiscible aqueous phases (beyond the curve) from those that will form one phase (at and below the curve) (Hatti-Kaul, 2000). Exclusion of hydrophilic solvents or salts crystallization are two common phenomena resulted from the mutual competition between hydrophilic solvent and salt for water molecules (Wang et al., 2010b).

The two systems considered in this study were two aqueous phases of different natures: a predominant hydrophobic top phase constituted mainly of methanol and a more hydrophilic salt-rich bottom phase. By referring to the phase diagrams as

illustrated in Figs. 4.5-4.6, the appropriate concentrations of methanol and potassium salt (above the binodal curve) that can induce phase separation to yield two phases were chosen for the optimization of protein recovery from microalgae in the present study. The influences of potassium salt types, salt concentrations, methanol concentrations, NaCl addition, the possibility of upscaling and the effectiveness of reusing the recycled phase components were investigated in this study.

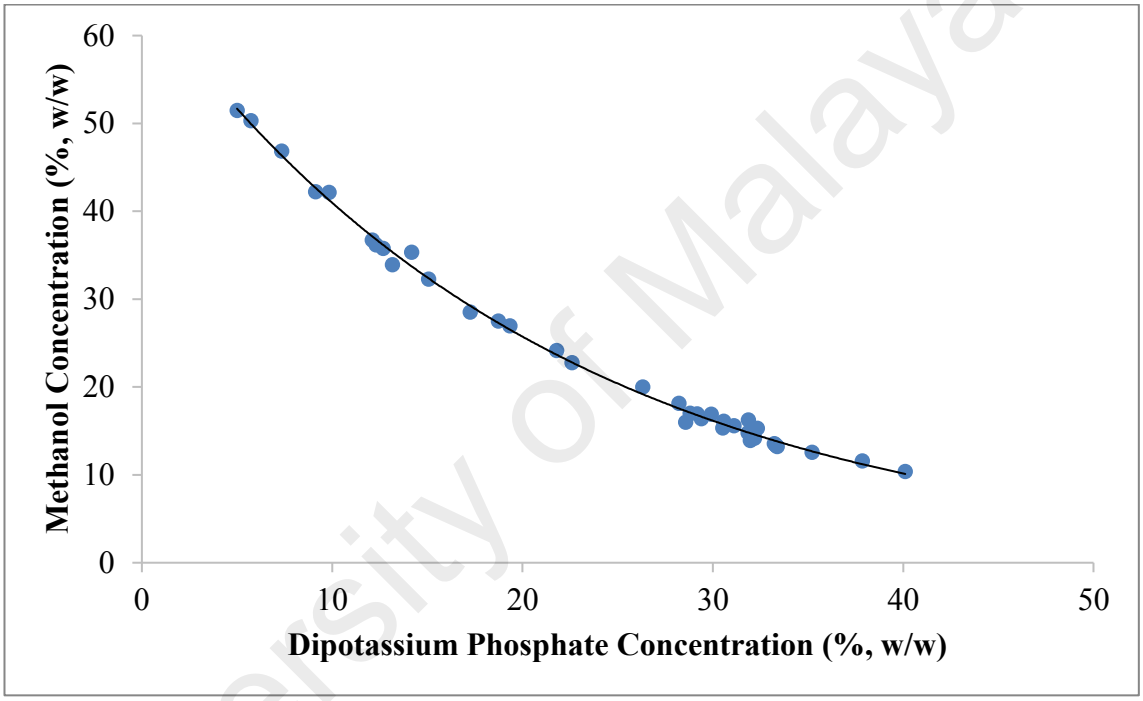

Figure 4.5: Phase diagram of methanol/dipotassium phosphate ATPS.

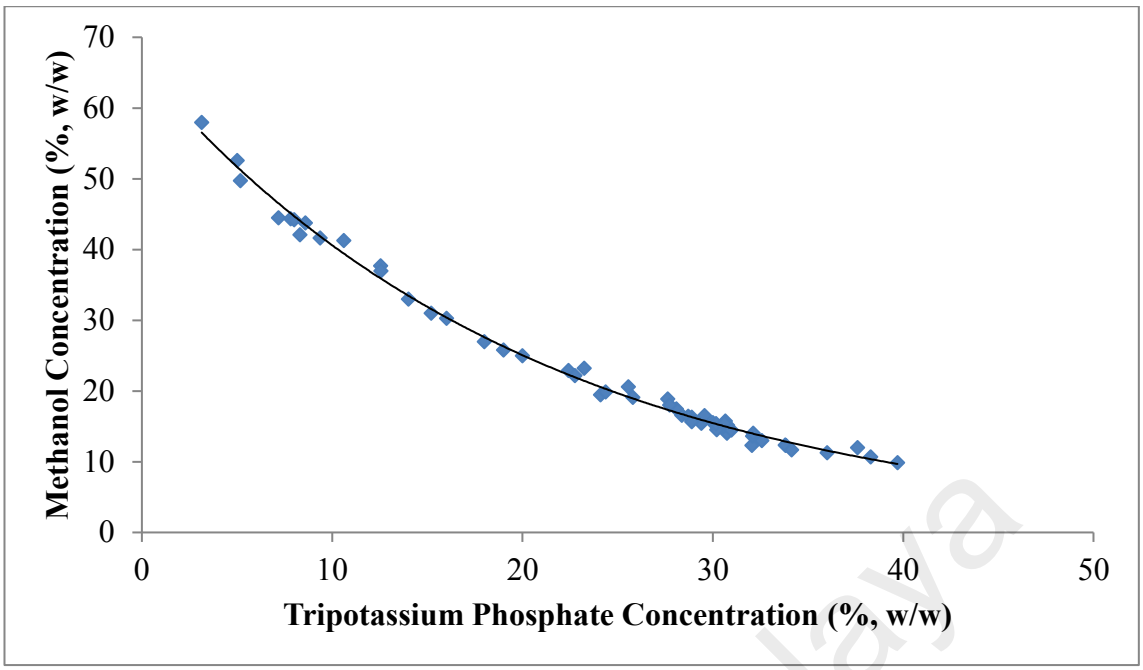

Figure 4.6: Phase diagram of methanol/tripotassium phosphate ATPS.

4.2.1.1 The effect of salt types

Water-miscible methanol has the ability to form ATPS with the kosmotropics salts namely K_2HPO_4 and K_3PO_4 (Wang, Han, Xu, Hu, & Yan, 2010a). The multivalent anions $PO4^{3-}$ and $HPO4^{2-}$ have a great influence in inducing phase separation. These strong salting-out inducing anions tend to create ion-hydration complexes by excluding water from the alcohol-rich phase, hence favouring the formation of ATPS (Golubitskii, Budko, Basova, Kostarnoi, & Ivanov, 2007). The selection of the best phase-separation salt with a high salting-out ability is the primary step in designing ATPS for efficient protein recovery from microalgae. Figs. 4.7-4.8 showed that the protein partitioning behaviour in ATPS was influenced by the type of potassium salts, resulting in varying partition coefficients and yields. The influence of different salt types on protein partitioning can be explained by the nature of the salt ions and the system's net charges (Nagaraja & Iyyaswami, 2014). The non-uniform distribution of the salt ions between two phases and the difference in the electric potential were reported to have great

impacts on the movement of protein to the other phase through electrostatic repulsion or attraction (Nagaraja & Iyyaswami, 2014).

Sample with higher partition coefficient value suggested that majority of the proteins were concentrated in the methanol-rich top phase. Overall, it was found that better protein partitioning was achieved with ATPS composed of K_3PO_4, whereas the protein partition efficiency was lower in the system with K_2HPO_4 salt (Figs. 4.7-4.8). For example, when comparing both salts at the same concentration of 25% (w/w), the partition coefficient of K_3PO_4 (5.47) was noticeably higher than K_2HPO_4 (4.46) (Figs. 4.7-4.8). This phenomenon can be explained by the Hofmeister series which describes the classification of ions based on their salting-out ability and phase separation influence in aqueous media. The relative effectiveness of salt types in promoting phase separation in this study was seen to follow the Hofmeister series: K_3PO_4 > K_2HPO_4 (Ananthapadmanabhan & Goddard, 1986). As such, ATPS formed by K_3PO_4 was chosen for the subsequent studies.

4.2.1.2 The effect of salt concentrations

Besides the salt types, salt concentrations also have strong effect in controlling the distribution of protein in ATPS. Fig. 4.8 showed that the partition coefficient was enhanced significantly from 4.34 to 6.05 ($p < 0.05$) when the salt concentrations of K_3PO_4 were increased from 20% (w/w) to 30% (w/w), while the methanol concentration remained constant at 30% (w/w). The increase in partition coefficient value could be due to the hydrophobic interaction and net charge effect which may have strong contribution to the preferential migration of protein in ATPS (Andrews, Schmidt, & Asenjo, 2005). However, when the K_3PO_4 concentration of the system was increased above 35% (w/w), the bottom phase of the system was found to have incompatible issue with BCA protein assay (Fig. 4.8). The formation of insoluble white complex was

clearly seen upon the addition of BCA working reagent into the sample. The same incompatible issue was also observed in the ATPS containing 38% (w/w) K_2HPO_4 and above (Fig. 4.7). Based on the results illustrated in Fig. 4.8, ATPS formed by 30% (w/w) of K_3PO_4 and 30% (w/w) of methanol showed the highest partition coefficient of 6.05 ($p < 0.05$) and was thus selected for the subsequent studies.

A similar trend had also been reported in an experiment to partition fish protein from fish processing industrial effluent using ATPS formed by 15% of PEG2000 (w/w) with increasing salt concentrations. It was explained that at higher salt concentration, the ions decreased the solubility of protein in the salt rich bottom phase (salting-out effect), with decreasing free water available in the bottom phase for the protein dissolution. The salting-out effect of the salt over protein increased the hydrophobic interaction between the protein and polymer phase, leading to the partitioning of the protein to top phase (Nagaraja & Iyyaswami, 2014).

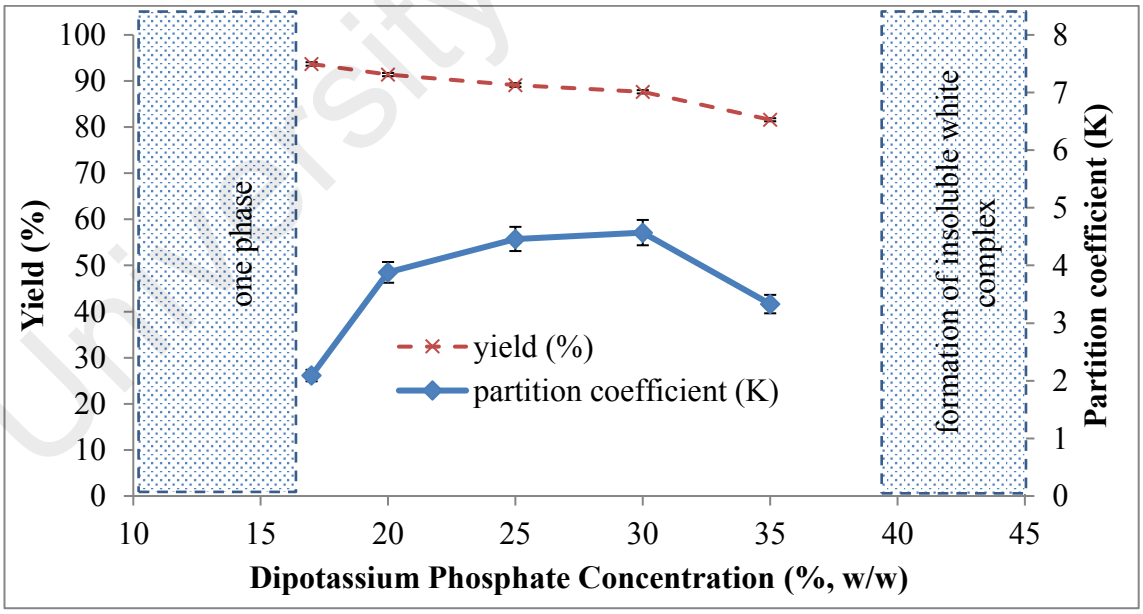

Figure 4.7: The effect of dipotassium phosphate concentrations on partitioning and yield of protein. The optimization of the system was performed by varying the potassium salt concentrations. Above dipotassium phosphate concentration of 37% (w/w), white insoluble complex was formed upon the addition of BCA working reagent into the sample and blocked absorbance measurement. Partition coefficient and yield were calculated using equations (2) and (3), (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

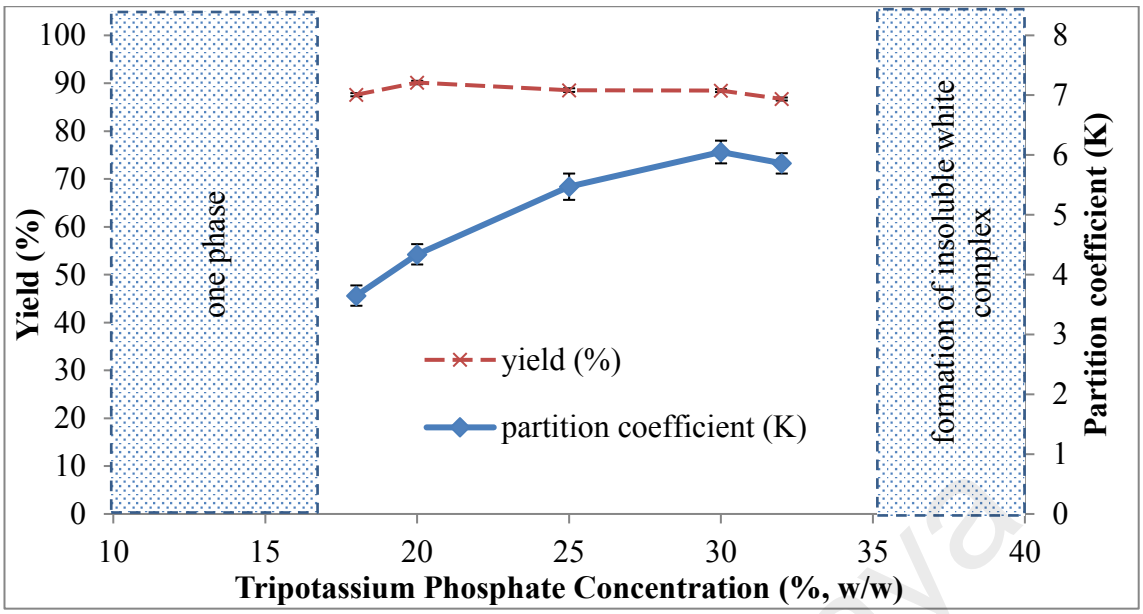

Figure 4.8: The effect of tripotassium phosphate concentrations on partitioning and yield of protein. The optimization of the system was performed by varying the potassium salt concentrations. Above tripotassium phosphate concentration of 35% (w/w), white insoluble complex was formed upon the addition of BCA working reagent into the sample and blocked absorbance measurement. Partition coefficient and yield were calculated using equations (2) and (3), (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

4.2.1.3 The effect of methanol concentrations

The influence of methanol concentrations on protein partitioning was carried out with varying concentrations, while maintaining the concentration of K_3PO_4 at 30% (w/w). It should be noted that according to the phase diagram of K_3PO_4, the minimum limit of methanol capable of forming two phases should be above 16% (w/w). Therefore, the concentrations of the methanol selected in this study were from 20% (w/w) to 40% (w/w). However, the bottom phase of the system containing 30% (w/w) K_3PO_4 and 40% (w/w) methanol formed insoluble white complex when reacted with the BCA working reagent.

Contrary to what was observed with the potassium salt concentration effect, the decrease in the concentration of methanol led to higher partition coefficient of protein (Fig. 4.9). This was a good phenomenon because the decrease in the amount of methanol can efficiently save the cost of separation (Li, Teng, & Xiu, 2011) and protein

which preferentially partitioned to the methanol-rich top phase can be concentrated with lower volume ratio. Similar observations were reported in the previous studies using alcohol/salt system to recover lipase (Ooi et al., 2009) and human interferon alpha-2b (Lin et al., 2013), respectively. The decrease in the partition efficiency was due to the gradual dehydration of the bottom phase when the alcohol concentration was increased in the top phase, causing an imbalance of partitioning which was not conducive for the retention of target biomolecules in the alcohol-rich top phase (Lin et al., 2013; Ooi et al., 2009). It was found that ATPS formed by 30% (w/w) K_3PO_4 and 20% (w/w) methanol exhibited the maximum partitioning coefficient of 6.72 ($p < 0.05$) (Fig. 4.9). Hence, this system was chosen for the subsequent optimization studies.

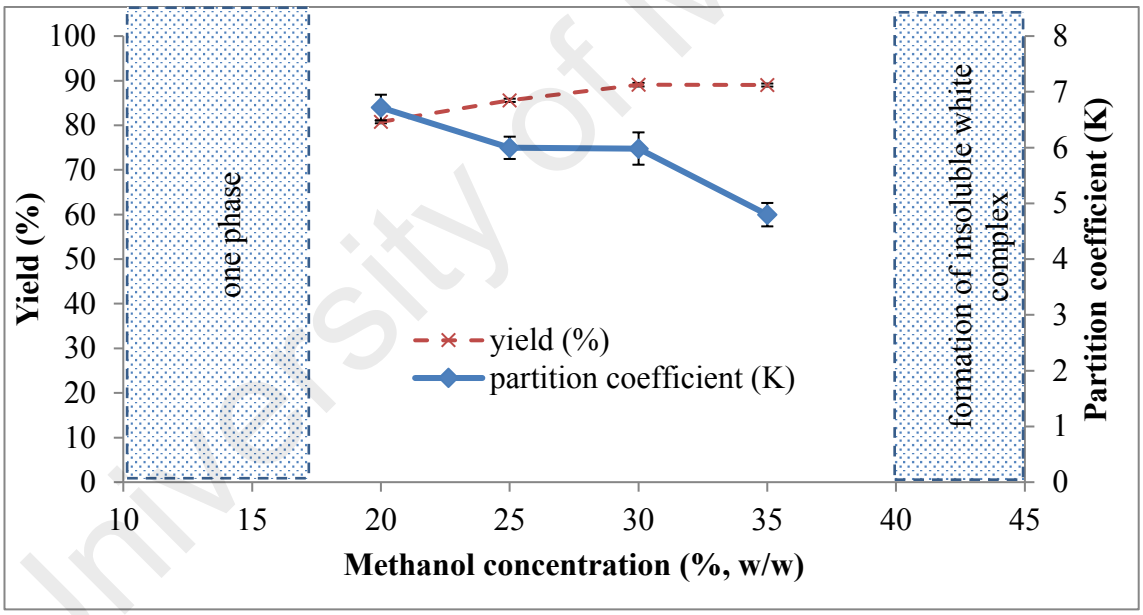

Figure 4.9: The effect of methanol concentrations on partitioning and yield of protein. The optimization of the methanol/tripotassium phosphate system was performed by varying the methanol concentration from 20-40% (w/w), while the concentration of K_3PO_4 was maintained at 30% (w/w). Above methanol concentration of 40% (w/w), white insoluble complex was formed upon the addition of BCA working reagent into the sample and blocked absorbance measurement. Partition coefficient and yield were calculated using equations (2) and (3), (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

4.2.1.4 The effect of NaCl addition

The addition of NaCl into the system could alter the protein partitioning. In this study, the ATPS formed by 30% (w/w) of K_3PO_4 and 20% (w/w) of methanol was used to investigate the effect of different NaCl concentrations ranging from 0 to 5% (w/w) on the partitioning of protein. The effect of NaCl salt on protein partitioning expressed in partition coefficient and protein yield is shown in Fig. 4.10. Based on the results, the partition coefficient of the ATPS was generally improved with increasing NaCl concentrations. However, further addition of NaCl salt from 3.5% (w/w) onwards would lead to the salt precipitation at the bottom phase. This was because the solution has reached its saturation point and can no longer dissolve any more NaCl salt. As illustrated in Fig. 4.10, system that contained 3% (w/w) of NaCl addition showed the highest partition coefficient (7.28) and yield (84.23%), which suggested that most of the hydrophobic proteins were migrated to the methanol-rich top phase. Even though ANOVA analysis showed that NaCl was not statistically significant in influencing the partition behaviour of the integrated system, it was observed in this experiment that the speed of phase separation of ATPS was found to be accelerated upon the addition of NaCl and was particularly obvious at 3% (w/w). This phenomenon is favourable in the large scale process which implied that phase separation of the system is possible to be achieved without requiring the aid of centrifugation which is relatively energy-intensive. By considering these two factors, system that contained 3% (w/w) of NaCl addition was chosen for the next experimental step.

These findings were in agreement with several previously published literatures. Researchers agreed that ATPS added with NaCl could speed up the phase separation by affecting the phase potential and improve the hydrophobic resolution of the system due to the generation of difference in electrical potential between the two phases (Gu & Glatz, 2007; Hatti-Kaul, 2000; Leong, Koroh, Show, Chi-Wei, & Loh, 2015). In

addition, specific interaction between salt and protein was believed to be responsible for protein partitioning efficiency (Settu, Velmurugan, Jonnalagadda, & Nair, 2015). In the ATPS composed of PEG and poly(acrylic acid) (PAA), the partition coefficient of BSA increased from 2.75 to 6.4 with increasing NaCl concentrations (from 0 to 1M), indicating that the addition of NaCl promoted the migration of BSA to the PEG-rich top phase (Settu et al., 2015). This can be explained by the electrostatic interaction and repulsion between two charged phases generated by the non-uniform distribution of chloride ions (Settu et al., 2015). Another research team also reported about the preferred migration of protein to the PEG-rich top phase in ATPS containing PEG and potassium citrate (Ramyadevi, Subathira, & Saravanan, 2013).

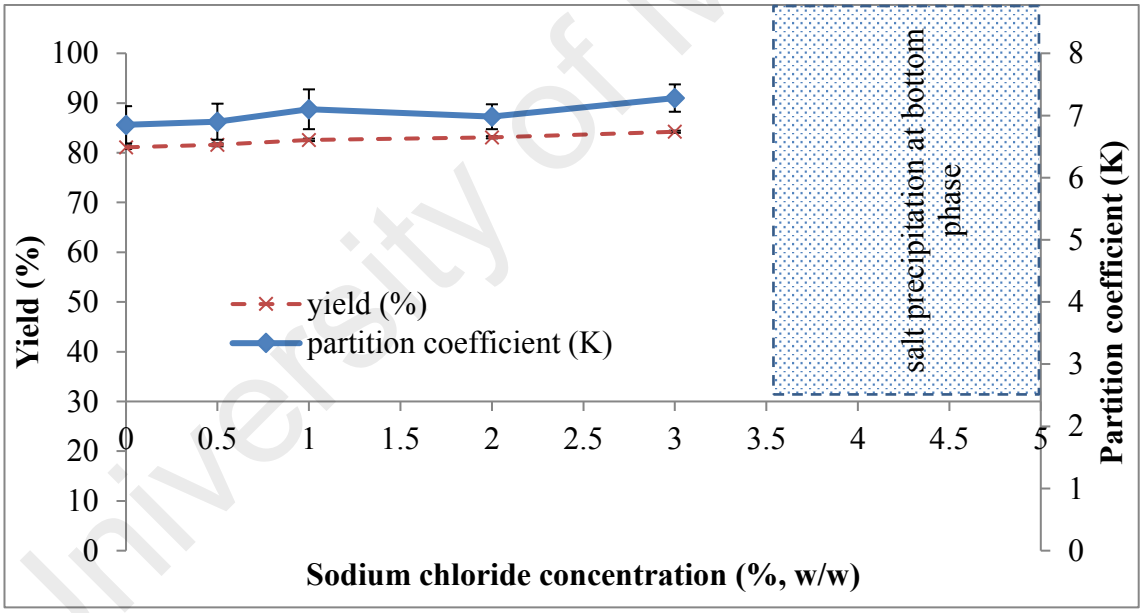

Figure 4.10: The effect of sodium chloride (NaCl) concentrations on partitioning and yield of protein. The optimization of the methanol/tripotassium phosphate system was performed by varying the NaCl concentrations from 0-5% (w/w). However, precipitation at the bottom phase was observed due to salt saturation when the concentration of NaCl was added above 3.5% (w/w). Partition coefficient and yield were calculated using equations (2) and (3), (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

4.2.2 Scale up of integrated process

The ATPS integrated process could be an attractive alternative to the traditional discrete process attributed to several advantages such as simple, rapid and cost-effective (Buyel et al., 2015; Goja et al., 2013; Rito-Palomares & Lyddiatt, 2002; Zhao et al., 2014). The integrated process allowed the operation of cell disruption and protein recovery to be carried simultaneously. Therefore, the possibility of scaling up the integrated process is undoubtedly one of the most exciting topics among researchers to determine its feasibility and applicability at commercial level. In this study, a 100-fold increment in the total weight of ATPS was upscaled to determine the effectiveness of implementing the integrated process at larger scale. The difference between the small scale and big scale of the integrated system was analysed statistically using t-Test. Based on the results, the values of the test statistic for both partition coefficient ($-3.182 < -2.637 < 3.182$) and yield ($-2.777 < 0.0502 < 2.777$) did not fall in the rejection region. This implied that there were no significant differences in terms of partition coefficient and yield compared to the small scale (Table 4.2). When the system was scaled up to 100-fold, the partition coefficient and yield obtained from the large scale integrated process were 6.88 and 84.26%, respectively. These findings suggested that the efficiency of the integrated process can be extrapolated from the small scale system without compromising the efficiency of protein partition and yield. Application of this process in downstream processing is thus possible.

In addition to partition coefficient, phase volume ratio is another important factor in determining the effectiveness of extraction (Anandharamakrishnan, Raghavendra, Barhate, Hanumesh, & Raghavarao, 2005). ATPS was found to be capable of concentrating target protein into one of the phases (Zhao et al., 2014). Based on the result obtained from the present study, the top phase volume of the chosen system was relatively lower than the bottom phase volume, with volume ratio not more than 0.80.

Lower phase volume ratio is favourable because it could reduce the volume to be handled in subsequent purification steps (Anandharamakrishnan et al., 2005). It has been reported that large scale of biomass separation using centrifugation and cross-flow membrane filtration involves high energy demand, high capital investment and operating costs (Wilk & Chojnacka, 2015). This technical difficulty can be overcome by ATPS integrated process, attributed to its rapid phase separation characteristic. In this study, it was observed that cell debris with higher density was trapped in the lower phase whereas protein was partitioned into the top phase, exhibited the high partition coefficient value. Similar observation was also reported previously in the attempt to extract animal protein via ATPS (Boland, 2002).

Table 4.2: ATPS integrated system containing 30% of K_3PO_4, 20% methanol with 3% of NaCl addition was scaled up to a final total weight of 1000g. Partition coefficient, volume ratio and yield were calculated using equations (2), (3) and (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

Integrated system	Small scale	Big scale
Final total weight (g)	10	1000
Partition coefficient (K)	7.28±0.22	6.88±0.13
Yield (%)	84.23±0.22	84.26±0.30

4.2.3 Recycling of phase components

Phase-forming chemicals can be a considerable proportion of the cost of the ATPS integrated process. Waste disposal of those materials would also incur some costs. In the recent decades, study on the recycling of phase-forming materials has caught the attention of researchers. This is because an effective recycling of the phase components could lower the production costs, minimize waste generation and environmental pollution (Carlsson, Berggren, Linse, Veide, & Tjerneld, 1996; Michalak & Chojnacka, 2014).

The possibility of recycling both top and bottom phases were investigated in this study. As stated in the methodology, the methanol from the top phase was evaporated to be recycled back and reused in the secondary system. Methanol is the cheapest and has low boiling point compare to other alcohols with longer hydrocarbon chain. This can be explained by the fact that the boiling point of alcohols increases as the length of hydrocarbon chain increases. Methanol cannot form azeotrope with water, thus it can be separated via evaporation easily for recycling in the integrated process. As a result, the energy demand and cost for methanol recycling were relatively low compare to other longer chain alcohols (Li et al., 2011).

The recycling of the bottom phase was expected to increase the process yield by minimizing protein loss in which some of the proteins that were retained in the bottom phase could be partitioned back into the top phase in the next recycling process. This observation was supported by the experimental evidence that the protein concentration in the top phase were generally showing a slight upward trend compared to the primary system (Fig. 4.12). However, Fig. 4.11 showed that the partition coefficient and yield were decreasing gradually with increasing numbers of recycling cycle. This may be due to the increasingly saturation of cell debris and other contaminants in the bottom phase from each previous recycling process, resulting in a gradual decrease of the salting out effect by potassium salt after being recycled repetitively. Nonetheless, the partition coefficient and yield of the protein obtained from the 5^{th} recycling cycle were relatively satisfactory, with the value of 5.19 and 76.45%, respectively.

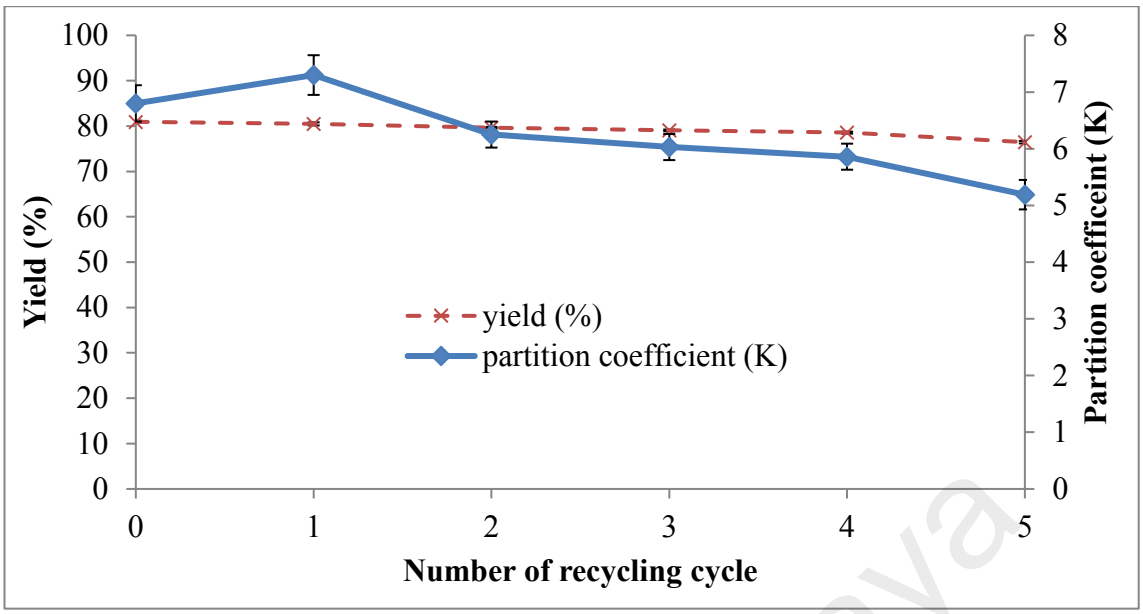

Figure 4.11: The partitioning efficiency and yield of protein using recycled phase components were investigated. The recycling processes were repeated for up to 5 times. Partition coefficient and yield were calculated using equations (2) and (3), (4), respectively. The results were expressed as the means of triplicate readings (mean ± SD).

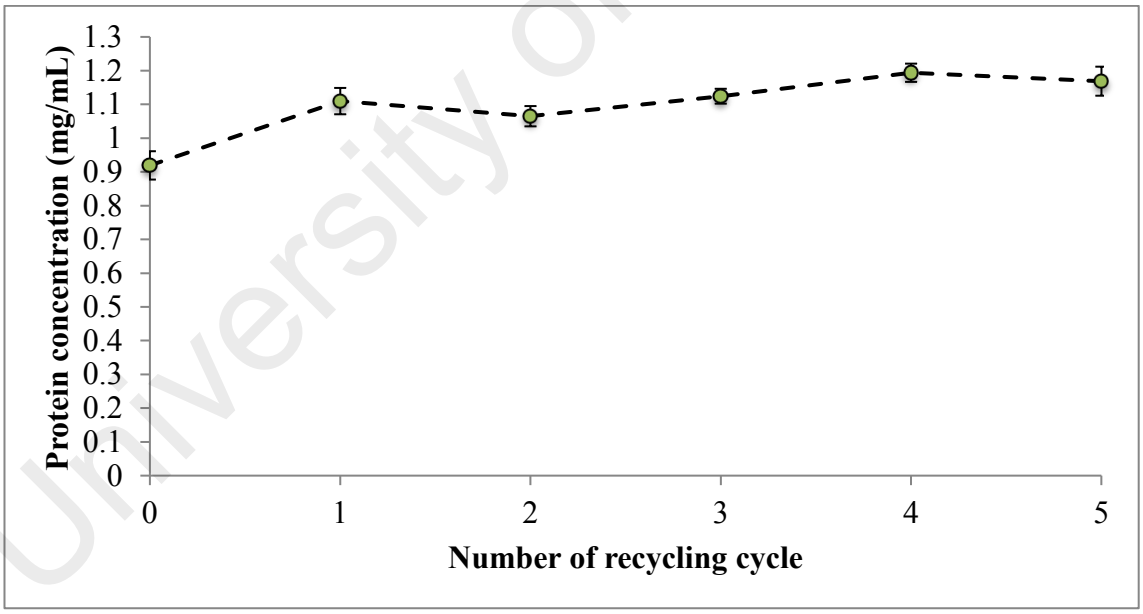

Figure 4.12: The influence of number of recycling cycle on the concentration of protein in the top phase. The protein concentrations were estimated by BCA protein assay. The results were expressed as the means of triplicate readings (mean ± SD).

4.2.4 The effect of integrated process on cell disruption

Cell disruption treatment is essential prior to protein recovery (Ursu et al., 2014) as the release of protein from microalgae are restricted by the multiple layers of recalcitrant cell wall (González-Fernández et al., 2012; Günerken et al., 2015). To enable the extraction of cell content from inside the cells, the complex structure of microalgal cell walls need to be either broken or made permeable. The addition of both potassium salts created the alkaline medium (Reis et al., 2014) which favoured the cell disruption process by inducing the hydrolysis of cell envelope and protein solubilisation process (Kim et al., 2013; Yen et al., 2013). The concept of ultrasonic disruption is based on the creation of cavities in cell suspension (Safi et al., 2014b). To further enhance the protein release and solubility, the system was exposed to ultrasonic vibration which could result in the greater penetration of solvent into cellular components (Show et al., 2014).

4.2.4.1 Microscopy observation

In this experiment, a comparison of microscopy images of untreated and treated cells was evaluated to identify the effect of the integrated process on cell disruption for protein release (Figs. 4.13-4.14). The microscopy image showed that untreated cells were spherical in shape with densely packed cytoplasmic content (Fig. 4.13). In contrast, cells treated with integrated process appeared less dense (Fig. 4.14), suggesting that some alteration of the cell structure had occurred and subsequently led to the considerable loss of cytoplasmic content. This was probably due to the formation of some small cavities in the cell wall that could allow the penetration of the solvent into the inner cells and solubilize the protein that was embedded inside the cell out from the cells into the medium. This study also suggested that the integrated process was capable of disrupting the microalgal cell walls without fragmentising them into very fine pieces.

This phenomenon is favourable in the large scale process whereby the cell debris can be removed easily in the subsequent purification process.

In addition, it was also observed that the aqueous solution turned to intense greenish color following the treatment. This indicated that the chloroplasts of the microalgae were damaged after the integrated process, leading to the release of green pigments (chlorophyll) from the inner cells (Safi et al., 2014b). In this case, proteins that were located in the organelles such as cytoplasm and chloroplast (Safi et al., 2015) would have been released into the aqueous medium altogether, resulting in an increase in protein yield as compared to the control.

Similar phenomenon was also observed in the previous studies which involved the extraction of oil from soybean flakes (Li et al., 2004) and polyphenols from tea leaves (Both et al., 2014) using ultrasound-assisted extraction. From the study, the treated cells indicated the development of micro-fractures and micro-fissures on the cell walls. Shock waves from the ultrasonication could facilitate swelling and hydration of biomass causing the formation of pores on the matrix surfaces (Vinatoru, 2001). The development of pores in the cell wall increased the permeation of solvent to the internal structure and mass transfer, therefore facilitating the release of target compound via the micro-channels (Both et al., 2014; Li et al., 2004; Xie, Huang, Zhang, You, & Zhang, 2015).

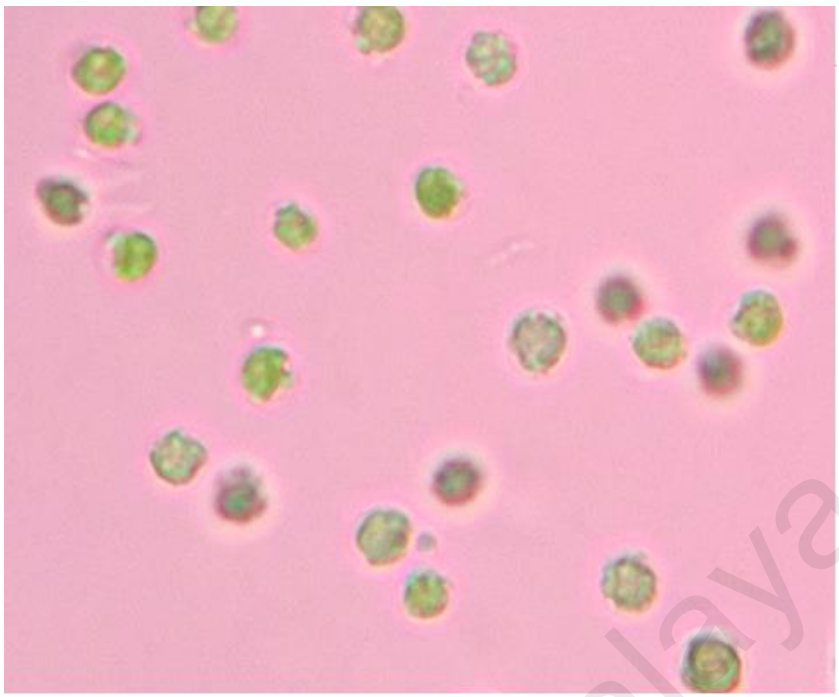

Figure 4.13: Light microscopy image of untreated *C. sorokiniana* (control) (1000x).

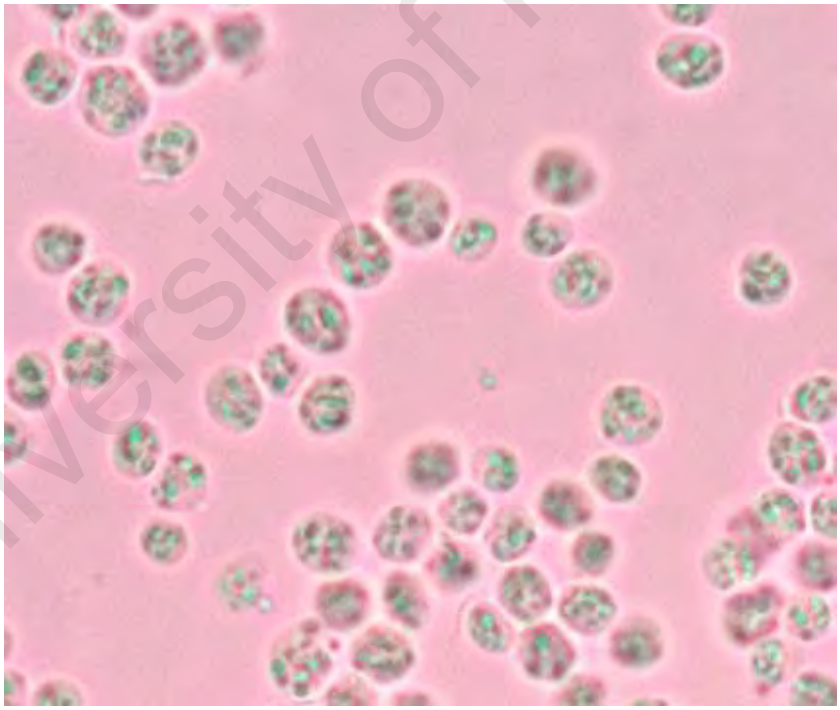

Figure 4.14: Light microscopy image of *C. sorokiniana* after treated with integrated process (1000x).

CHAPTER 5: CONCLUSION

The first experiment provided a preliminary investigation on the effect of solvent types and different treatments on protein extraction from microalgae. It was found that alkaline treatment played a key role in cell disruption and protein solubilisation. Water is the preferable solvent as compared to alcohols for simultaneous microalgal cell disruption and protein solubilisation at the industrial scale with the advantages of low cost, ubiquitous availability, minimal safety risks, and ease of upscaling. The application of combination treatment (alkaline + ultrasonication treatment) to the four microalgal strains was found to be the most effective for protein solubilisation and extraction. Overall, the findings from this experiment could form a basis for further study towards developing a more efficient and versatile disruption and extraction process.

The protein-rich strain of *C. sorokiniana* was selected for subsequent study in the second experiment. In the second experiment, a novel integrated method using methanol/potassium salt ATPS to simultaneously disrupt cell wall and recover protein from *C. sorokiniana* was adopted. The partitioning behaviour of protein in the process can be manipulated by a few influential parameters such as types of salt, the concentrations of methanol and potassium salt as well as the addition of NaCl salt. It was concluded that ATPS composed of 30% (w/w) K_3PO_4 and 20% (w/w) methanol with the addition of 3% (w/w) NaCl showed the best protein recovery capability. In this system, the partition coefficient and protein yield was 7.28 and 84.23%, respectively. There were no significant differences in terms of the partition coefficient and protein yield when the proposed integrated process was scaled up to 100-fold. In addition, it is also less environmentally polluting whereby the phase components can still be recycled and reused effectively at the 5^{th} cycle. Taken together, the results suggested that the

ATPS-based integrated process was simple, rapid and environmental friendly. Undoubtedly, this is a logical approach towards reducing overall cost by simplifying several downstream processing steps such as disruption, isolation, extraction and concentration and thus opens promising perspective for the application of this method at large scale. For that reason, assessing its general applicability to recover a wide range of intracellular compounds such as carbohydrate, lipid, and chlorophyll from microalgae could be an interesting topic for future study.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Published papers

Phong, W.N., Le, C.F., Show, P.L., Chang, J.S., & Ling, T.C. (2016). Extractive disruption process integration using ultrasonication and aqueous two-phase system for protein recovery from *Chlorella sorokiniana*. *Engineering in life sciences, 00,* 1-13.

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Published book chapters

Chang, J.S., Show, P.L., Ling, T.C., Chen, C.Y., Ho, S.H., Tan, C.H., Nagarajan, D., & **Phong, W.N.** (2017). 11 – Photobioreactors. In *Current Developments in Biotechnology and Bioengineering* (pp. 313–352). Elsevier.

Phong, W.N., Show, P.L., & Ling, T.C. (2015). Fermentation in food processing. In *Processing of Foods, Vegetables and Fruits: Recent advances* (pp. 117–138), *ISPDFVF*.

Pending publication

Phong, W.N., Show, P.L., Ling, T.C., Juan, J.C., Ng, E.-P., & Chang, J.S. Mild cell disruption methods for bio-functional proteins recovery from microalgae. *Algal research*, Elsevier.