GREEN APPROACHES AND SEPARATION TECHNIQUES FOR THE RECOVERY OF PIGMENTS FROM MICROALGAE

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ABSTRACT

Microalgae have provided great exploitation of biofuels and bioproducts production over fossil fuels and other plant-based sources owing to its high accessibility, non-competition, and renewability advantages. The production of natural carotenoids from microalgae are safer than petrochemical feedstock due to the inherent toxicity concerns for direct human consumption. However, the recalcitrant structure of microalgae cell wall restricted the direct recovery and extraction of the carotenoids accumulated inside the biomass which requires an additional pretreatment process. The current conventional technologies (e.g. bead beating, homogenizers, high pressure heating and chemicals) of biomass processing are incompetent and not feasible for the downstream processing of microalgae. This creates an opportunity of developing novel bioprocessing approaches to reduce the overall cost- and time processing and enhance the efficient production of carotenoids.

This thesis presents the development of liquid biphasic- and ionic liquid technologies for the downstream processing of carotenoids such as astaxanthin and fucoxanthin from different microalgae strains. This thesis firstly introduces the application of liquid biphasic flotation system in the extraction and partitioning of astaxanthin from *Haematococcus pluvialis* microalgae. The optimized liquid biphasic flotation system is further employed with ultrasound- and electropermeabilization-assisted technologies for a simultaneous pretreatment and extraction of carotenoids from microalgae. These integrated technologies with liquid biphasic flotation system provide a one-step rapid processing, environmentally friendly, achieved higher yield and separation efficiency. In fact,

the integrated technologies liquid biphasic flotation system was scale-up to investigate the feasibility for large scale and commercializing its usage for industrial proposes. This thesis also presented a greener and sustainable solvent using ionic liquids technology for the cell permeabilization and extraction of astaxanthin and fucoxanthin from microalgae. The green concept of the proposed ionic liquid in this research work utilized carbon dioxide as one of its reactants and they can be easily separated from the extracted bioproduct compared to conventional imidazole- and pyridinium-based ionic liquids. The characterization of the synthesized ionic liquid was comprehensively evaluated in this work. Besides, the cellular and surface morphology after treated with the ionic liquid were investigated. This work also illustrated the recyclability studies of the proposed ionic liquid for subsequent extraction. Moreover, the antioxidant properties of the extracted astaxanthin and fucoxanthin were subjected for antioxidant analysis. The research achievements in these works and future opportunities are highlighted in the last chapter of the thesis.

LIST OF PUBLICATIONS

Journal publications

1. **Kuan Shiong Khoo**, Sze Ying Lee, Chien Wei Ooi, Xiaoting Fu, Xiaoling Miao, Tau Chuan Ling, Pau Loke Show*. (2019) "Recent advances in biorefinery of astaxanthin from Haematococcus pluvialis" *Bioresource Technology*, 288, 121606. DOI: 10.1016/j.biortech.2019.121606 [Accepted: 2 June 2019; IF: 7.539]
2. **Kuan Shiong Khoo**, Kit Wayne Chew, Chien Wei Ooi, Hwai Chyuan Ong, Tau Chuan Ling, and Pau Loke Show*. (2019) "Extraction of natural astaxanthin from Haematococcus pluvialis using liquid biphasic flotation system." *Bioresource Technology*, 290, 121794. DOI: 10.1016/j.biortech.2019.121794 [Accepted: 10 July 2019; IF: 7.539]
3. **Kuan Shiong Khoo**, Kit Wayne Chew, Guo Yong Yew, Sivakumar Manickam, Chien Wei Ooi, and Pau Loke Show*. (2020) "Integrated ultrasound-assisted liquid biphasic flotation for efficient extraction of astaxanthin from Haematococcus pluvialis." *Ultrasonics Sonochemistry* 67, 105052. DOI: 10.1016/j.ultsonch.2020.105052 [Accepted: 1 March 2020; IF: 6.517]
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8. **Kuan Shiong Khoo**, Yen Mun Chong, Wen Sing Chang, Jie Min Yap, Su Chern Foo, Ianatul Khoiroh, Phei Li Lau, Kit Wayne Chew, Chien Wei Ooi, and Pau Loke Show* (2020). "Permeabilization of Chlorella sorokiniana and extraction of lutein by distillable CO2-based alkyl carbamate ionic liquids." *Separation and Purification Technology*, 256, 117471. DOI: 10.1016/j.seppur.2020.117471 [Accepted: 26 July 2020; IF: 5.774]
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10. Su Chern Foo*, **Kuan Shiong Khoo**, Chien Wei Ooi, Pau Loke Show, Nicholas MH Khong, and Fatimah Md Yusoff (2020). Meeting sustainable development goals: Alternative extraction processes for fucoxanthin in algae. *Frontiers in Bioengineering and Biotechnology*, 8. DOI: 10.3389/fbioe.2020.546067 [Accepted: 9 November 2020; IF: 3.644]
11. **Kuan Shiong Khoo**, Chien Wei Ooi, Kit Wayne Chew, Su Chern Foo, and Pau Loke Show* (2021). "Bioprocessing of Chaetoceros calcitrans for the recovery of fucoxanthin using CO2-based alkyl carbamate ionic liquids." *Bioresource Technology*, 322, 124520. DOI: 10.1016/j.biortech.2020.124520 [Accepted: 4 December 2020; IF: 7.539]
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1. **Kuan Shiong Khoo**, Kit Wayne Chew, and Pau Loke Show* (2019), August. POCER 1913: An Alternative Recovery of Astaxanthin from *Haematococcus Pluvialis* Microalgae. In Colloquium for Environmental Research (POCER 2019) 8-9 August 2019 Pulse Grande Hotel, Putrajaya, Malaysia (Vol. 8, p. 33).

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1. **Kuan Shiong Khoo**, Hanna Yusof, Sze Ying Lee, and Pau Loke Show* (2020). Polymer-based liquid biphasic system. *Liquid Biphasic System: Fundamentals and Applications in Bioseparation Technology*, p.17.
2. **Kuan Shiong Khoo**, Kin Loong Tan, Sze Ying Lee, and Pau Loke Show* (2020). Organic solvent-based liquid biphasic system. *Liquid Biphasic System: Fundamentals and Applications in Bioseparation Technology*, p.39.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
AS_R	Astaxanthin recovery
AS_T	Astaxanthin recovery of top phase
AS_B	Astaxanthin recovery of bottom phase
ATPS	Aqueous two-phase system
BBM	Bold's Basal Medium
BG-11	Blue-Green 11
C_A	Concentration of astaxanthin
CDS	Cyclodextrins
CMC	Critical micelle concentration
Chl a	Chlorophyll *a*
Chl b	Chlorophyll *b*
CPC	C-phycocyanin
CGTase	Cyclodextrin glycosyltransferase
DA-6	Diethyl aminoethyl hexanoate
DACARB	Diallyammonium diallycarbamate
DBCARB	Dibutylammonium dibutylcarbamate

DES	Deep-eutectic solvent
DIMCARB	Dimethylammonium dimethylcarbamate
DPCARB	Dipropylammonium dipropylcarbamateVOCs
DPPH	1’1-diphenyl-2-picrylhydrazyl
DSC	Differential scanning calorimetry
EAE	Enzyme-assisted extraction
E_{AS}	Extraction efficiency of astaxanthin
EO	Ethylene oxide
EOPO	Ethylene oxide- propylene oxide
ESEM	Environmental Scanning Electron Microscope
F-C	Folin–Ciocalteu
FESEM	Field Emission Scanning Electron Microscope
FTIR	Fourier-transform infrared spectroscopy
FRAP	Ferric reducing antioxidant properties
GHG	Greenhouse gas
HPH	High-pressure homogenizer
HPLC	High-performance liquid chromatography
IFN	Interferon

ILs	Ionic liquids
K	Partition coefficient
LBS	Liquid biphasic system
LBF	Liquid biphasic flotation
LCA	Life cycle assessment
NMR	Nuclear Magnetic Resonance
MAE	Microwave-assisted extraction
MLT	Melatonin
MRA	Multidimensional risk analysis
OFAT	One-factor-at-a-time
OHM	Optimal Haematococcus Medium
PEG	Polyethylene glycol
PLE	Pressurised liquid extraction
PFT	Purification factor
PO	Propylene oxide
SDGs	Sustainable development goals
SC-CO_2	Supercritical carbon dioxide
SS	Solvent sublation

STL	Slope tie line
SUPRAS	Supramolecular solvent
TEAC	Trolox equivalent antioxidant capacity
T_c	Crystallization temperature
T_d	Thermal decomposition
T_r	Reference temperature
TLL	Tie line length
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
UALBF	Ultrasound-assisted liquid biphasic flotation
UV-Vis	Ultraviolet-visible
VOCs	Volatile organic compounds
V_r	Volume ratio
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon nuclear magnetic resonance

CHAPTER 1 INTRODUCTION

1.1 Research Background

Astaxanthin, or known as 3,3′-dihydroxy-β,β′-carotene-4,4′-dione, is a bright red secondary carotenoid that is widely used in food, cosmetic, aquaculture, nutraceutical and pharmaceutical industries (Shah et al., 2016). It's antioxidant effect is beneficial for preventing cancer, diabetes, cardiovascular disease, ulcer, immune response and inflammation (Yuan et al., 2011). *T*he stereoisomers of astaxanthin include all-*cis* (3S, 3'S), *cis-trans* (3R, 3'S) and all-*trans* (3R, 3'R) (Yang et al., 2013). Astaxanthin is the pigment that gives bright red colour to the flesh and shell of salmonids (e.g., salmons and trout), shrimps and crayfish (Koller et al., 2014). Astaxanthin can be derived from natural sources (e.g. algae, yeast, salmon, trout, krill, shrimp, and crayfish) or produced synthetically. In 2014, commercial market of synthetic astaxanthin valued at $447 million, corresponding to the production of 280 metric tons per year (Koller et al., 2014, Panis and Carreon, 2016).

Synthetic astaxanthin has a lower production cost (approximately $1000/kg), despite of its commercialized quantity being less than 1%, which is relatively low when compared to microalgae-derived astaxanthin (Li et al., 2011a, Pérez-López et al., 2014). The production of synthetic astaxanthin comprises the complex synthesis route using petrochemical resources as raw material. The source of synthetic astaxanthin has been a point of safety concern for consumers due to inherent toxicity of raw material used in the production of astaxanthin (Li et al.,

2011a, Milledge, 2011). To-date, synthetic astaxanthin has yet to be approved for direct human consumption as food supplements, but only be used as additive and pigment for fish feed purposes only (Li et al., 2011a). Therefore, a greener and sustainable approach should be attempted to produce natural astaxanthin for a wider range of applications in food, animal feed, cosmetics and pharmaceuticals industries. In addition, several works have reported that the antioxidant activity of natural astaxanthin is significantly higher than the synthetic astaxanthin and other bio-compounds such as vitamin C, β-carotene, canthaxanthin, zeaxanthin, lutein and α-tocopherol (Miki, 1991, Borowitzka, 2013, Koller et al., 2014, Pérez-López et al., 2014). **Table 1.1** presents a comparison of synthetic astaxanthin and natural astaxanthin with regards to their advantages and disadvantages.

Table 1.1: A comparison summary of synthetic and natural astaxanthin for their advantages and disadvantages.

Type	Advantage	Disadvantage	References
Synthetic astaxanthin	• Stereoselectivity intermediates • Low price of biosynthesis product • Long lifespan of biosynthesis product • Low antioxidant activity • High availability of synthetic astaxanthin	• Use of petrochemical reactants which are highly hazardous • Complex biosynthesis route • High environmental impact • Not sustainable and renewable	(Milledge, 2011, Li et al., 2011a)
Natural astaxanthin	• Safe for human consumption • High antioxidant activity • Low environmental impact • Sustainable and renewable • Environmental-friendly	• Costly biorefinery processes • Spacious and time consuming for complex cultivation of *H. pluvialis* • Short lifespan of bioproduct • Expensive cost of production of natural astaxanthin bioproduct • Low availability of natural astaxanthin	(Koller et al., 2014, Pérez-López et al., 2014)

Microorganisms such as algae (e.g., *Chlorella zofingiensis, Chlorococcum and Haematococcus)* and yeast (e.g., *Phaffia rhodozyma*) are the well-known sources of the natural astaxanthin (Ambati et al., 2014). Amongst these natural

sources, researchers have put tremendous efforts on studying the natural astaxanthin derived from *Haematococcus pluvialis* (*H. pluvialis*). It was reported that the accumulation of astaxanthin in *H. pluvialis* can reach around 3.8 to 5.0 % of dry weight depending on the cultivation conditions and photobioreactor design (Boussiba et al., 1999, Ranga et al., 2009, Wayama et al., 2013). On the other hand, some recent approaches utilized the mutagenesis strategy to genetically improve algae strains for rapid growth rate and high accumulation of astaxanthin (Sharon-Gojman et al., 2015, Hong et al., 2018).

However, a large-scale biosynthesis of natural astaxanthin from *H. pluvialis* still remains a challenge, due to the energy-extensive cultivation and downstream processing. The cultivation of *H. pluvialis* consists of two major phases, namely green motile stage and red non-motile stage. During the cultivation of *H. pluvialis* under nutrient stress, the green vegetative cells will transform into red non-motile haematocyst cells within 12 to 15 days (Fábregas et al., 2001). Once the maturation stage of red non-motile haematocyst cell is reached, the harvesting strategies (e.g., centrifugation and sedimentation) take place to concentrate the biomass from the depleted culture medium (Han et al., 2013). To preserve *H. pluvialis* biomass, drying techniques such as spray drying and freeze drying were often adopted to eliminate moisture in the biomass for preventing the degradation of pigments (Li et al., 2011a, Milledge, 2013, Panis and Carreon, 2016). The dried biomass was typically pretreated with the mechanical disruption (e.g., milling and grinding) (Zhang et al., 2014) to lyse the *H. pluvialis.* The astaxanthin was often recovered via the solvent extraction using solvents such as organic solvents (Zou et al., 2013), concentrated acid and alkali (Mendes-Pinto et al., 2001, Sarada et al., 2006), vegetables oils

(Kang and Sim, 2008, Dong et al., 2014), and pressurised liquids (Jaime et al., 2010), or the extraction assisted with ultrasound (Zou et al., 2011). Recently, alternative solvents were used in the extraction of astaxanthin, including supercritical carbon dioxide (SC-CO_2) (Machmudah et al., 2006, Nobre et al., 2006, Wang et al., 2012, Reyes et al., 2014, Molino et al., 2018, Sanzo et al., 2018), ionic liquids (ILs) (Desai et al., 2016, Praveenkumar et al., 2015, Choi et al., 2019, Liu et al., 2019b), magnetic-assisted extraction (Zhao et al., 2016a, Zhao et al., 2016b) and supramolecular solvent (SUPRAS) extraction (Salatti-Dorado et al., 2019).

Fucoxanthin is a xanthophyll with molecular formulae of $C_{42}H_{58}O_6$, which is a major carotenoid contributing about 10% of total accumulation carotenoids found only in macroalgae and microalgae species (Foo et al., 2017). Fucoxanthin has a unique structural features composed of an allenic bond, a conjugated carbonyl, a 5,6-monoepoxide and an acetyl group, in which these attributes as an antenna pigment carotenoid in the main light-harvesting complexes responsible in the transfer of energy for photosynthesis electron transport chains in both micro- and macroalgae. Besides, the exploration of natural fucoxanthin over synthetic chemical routes was mainly due to its beneficial antioxidant properties to prevent these chronic oxidative stress diseases that includes atherosclerosis, Alzheimer's disease, and cancer. The production of fucoxanthin has a higher quantity in microalgae (e.g., *Phaeodactylum tricornutum*, *Odontella aurita* and *Chaetoceros calcitrans*) compared to the macroalgae (e.g., *Eisenia bicyclis*, *Laminaria digitate*, *Fucus vesiculosus, Sargassum muticum* and *Saccharina latissimi)* (Kim et al., 2012a, Xia et al., 2013, Conde et al., 2015, Shannon and Abu-Ghannam, 2017). In fact, these bioactive compounds extracted from microalgae (i.e., fatty acid, polysaccharide,

phycobilin, carotenoids and phenolic compounds) are favourable feedstock for the development of future food supplement as it has the ability to enhance beneficial towards health effect in terms of anti-inflammatory, anti-diabetic, anti-cancer, anti-malarial, anti-angiogenic and photo-protection activities (Suchern et al., 2020, Mohamadnia et al., 2020, Sathasivam and Ki, 2018). Based on literatures studies, the extraction of fucoxanthin microalgae involves conventional organic solvents using methanol, ethanol, isopropanol, acetone, hexane, tetrahydrofuron, ethyl acetate and dimethyl sulfoxide, respectively and this triggered the consideration in various aspects such as environmental impact, toxicity, high consumption volume and sustainability of these organic solvents (Derwenskus et al., 2019, Suchern et al., 2020, Khoo et al., 2020e).

This research aims to explore the green alternative approaches for the extraction and separation of carotenoids from microalgae. A well-established bioseparation technology namely liquid biphasic system (LBS) technologies has attracted numerous researchers' attentions in the separation and purification of biomolecules (Leong et al., 2019a). It is also known as liquid-liquid extraction technology in the downstream processing. The liquid biphasic extraction technology is comprised of two liquids which is separated by an interfacial layer when the mixture of two incompatible liquids is beyond the critical condition. Generally, the characteristics of the phase-forming components creates a physico-chemical interaction which can easily acclimatize the target biomolecules to be partitioned to either the top or bottom phase depending on the selectivity of the components. Moreover, the implementation of various assisted technologies such as bubbling-, ultrasound- and electropermeabilization-assisted have been incorporated into the

LBS to enhance the effectiveness of biomolecules separation (Phong et al., 2018, Phong et al., 2017a, Lee et al., 2016, Sankaran et al., 2018b).

In recent years, researchers have expended a tremendous amount of effort in exploring ionic liquids (ILs), or the molten salts, as an environmentally friendly alternative to the conventional volatile organic solvents (VOCs). Physico-chemical properties of ILs, such as negligible vapor pressure, high melting point, and high thermal stability, are desirable for many fields of chemistry (Lee et al., 2015). Low vapor pressure of ILs mitigates the issue of air pollution due to the minimal evaporation of ILs during operation, as compared to VOCs. In addition, the high melting point and thermal stability allow ILs to withstand intensive operational conditions (i.e., high temperature and pressure). Since ILs comprises of anions and cations, the interionic interaction forms hydrogen bonding that is important for solvation purpose. The unique combinations of ions in ILs earn them a title of designer solvent, as ILs can be specifically designed to fit a specific condition or application by altering their functional groups and selection of suitable cation and anions (Wilkes, 2004). Despite the advantages brought by ILs in these processes, a good understanding of toxicology and sustainability of these ILs is important for a further effort in commercialization and marketing of these IL-driven technologies.

1.2 Problem Statement

The increase of human population has put tremendous pressure on the market demand for food sources and dietary supplement in which high cost, supplies and sustainability has to be throughout considered. The production of synthetic bioactive compound comprised of complex synthesis route using petrochemical

resources as its raw material, in which has been a point of safety concern due to its inherent toxicity and lack of sustainability to both human and the ecosystem. This has driven the needs of renewable feedstock such as microalgae as the key composition for biofuels, bioproducts and bioactive compounds production. The high-value carotenoids content in microalgae has higher quality than then terrestrial plant sources which can provide human benefitting properties such as excellent antioxidant and anti-inflammatory that can be used to replace synthetic colouring and for direct human supplements. However, the recalcitrant structure of microalgae cell wall hindered the direct recovery and extraction of the carotenoids accumulated inside the biomass which requires an additional pretreatment process. In fact, the current conventional extraction approaches utilizing volatile organic solvents (VOCs) possess toxicity and hazardous properties which would contributes to various environmental impacts in managing the disposal of solvent.

Therefore, the development of novel bioprocessing approaches is much needed to reduce the overall bioprocessing and effectively enhance the production of carotenoids. This research work proposed a green alternative and sustainable extraction process using liquid biphasic system and ionic liquid technology for an efficient extraction and recovery of carotenoids from microalgae. The incorporation of recent advances technologies such as ultrasound- and electropermeabilization-assisted with liquid biphasic flotation system can enhance the extraction process of bioactive compound. The evaluation of the proposed techniques was scaled-up to verify its feasibility in large-scale system for the production of carotenoids. The implementation of ionic liquids technology as alternative green solvent was

proposed for the cell permeabilization and extraction of carotenoids from microalgae. The green concept of the proposed ionic liquid in this research work utilized carbon dioxide as one of its reactants and they can be easily separated and recycled from the extracted bioproduct for subsequent extraction processes. This research would contribute an efficient, rapid extraction, greener and sustainable technologies for the downstream processing of biomolecules from microalgae.

1.3 Research Objectives

This research work was aimed to explore the green alternative extraction approach of astaxanthin and fucoxanthin from microalgae. The objectives of the research were to identify the potential pretreatment approaches and alternative extraction approach for the recovery of high-value carotenoid. The scopes of this research are stated as followed:

1) To evaluate the application of liquid biphasic system technologies for the extraction of astaxanthin and fucoxanthin from microalgae.

2) To achieve a one-step pretreatment and extraction approach for the extraction of astaxanthin and fucoxanthin from microalgae.

3) To perform astaxanthin and fucoxanthin extraction from microalgae using green CO_2-based alkyl carbamate ionic liquids.

1.4 Research Contributions

The research scopes proposed in Section 1.3 were achieved and each scope is described in detail in the following Chapter 3 to Chapter 7. The summary of the research contributions of this thesis are as followed:

1) Extraction of natural astaxanthin from *Haematococcus pluvialis* using liquid biphasic flotation system: Two step-approach in which biomass are mechanically disrupted by grinding using mortar and pestle as pretreatment followed by extraction using liquid biphasic flotation (LBF) system. The optimization of phase component composed of food grade alcohol and salt of the liquid biphasic flotation system were evaluated. Various parameters such as types and concentration of alcohol and salts, volume ratio, addition of neutral salt, flotation period, mass of biomass and scaling-up approach were investigated. The separation efficiency, partition coefficient and recovery yield of extracted astaxanthin were examined.

2) Integrated ultrasonic assisted liquid biphasic flotation for efficient extraction of astaxanthin from *Haematococcus pluvialis*: An efficient one-step extraction process via integrated ultrasonic-assisted liquid biphasic flotation (UALBF) was developed for simultaneous pretreatment and extraction of astaxanthin from *Haematococcus pluvialis* biomass. This work is the continuation of previous optimized liquid biphasic flotation system condition with additional parameters focuses more on the ultrasound-assisted system. Various parameters such as position of ultrasound horn, mode of ultrasonication (pulse and continuous), amplitude of ultrasonication, air flowrate, duration of air flotation, and mass of *H. pluvialis* microalgae were evaluated. The extracted astaxanthin yield, separation

efficiency and partition coefficient from UALBF were evaluated and compared with optimized LBF system.

3) Permeabilization of *Haematococcus pluvialis* and solid-liquid extraction of astaxanthin by CO_2-based alkyl carbamate ionic liquids: Green and sustainable extraction approach using CO_2-based alkyl carbamate ionic liquids (ILs) were proposed as a promising solvent to permeabilize the robust microalgae cell wall for an effective extraction of astaxanthin. Unlike conventional imidazolium and pyridinium-based ILs, this CO_2-based alkyl carbamate ILs can be a potential substituent of these conventional ILs as they be easily distillate from the extracted product under moderate temperature. Various parameters such as type of CO_2-based IL, concentration of IL, extraction time and temperature were evaluated. The DIMCARB-treated microalgae were subjected to the surface morphology analysis by a compound microscope and Field Emission Scanning Electron Microscope (FESEM). Recyclability studies were also conducted to determine the efficiency of the recycled ILs for multiple rounds of extraction. In addition, the antioxidant properties of the extracted astaxanthin were evaluated using Trolox equivalent antioxidant capacity (TEAC) and total phenolics content (TPC).

4) Electropermeabilization-assisted liquid biphasic flotation for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae: Green and non-thermal extraction approach via electropermeabilization-assisted liquid biphasic flotation for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae. Owning to its non-thermal nature unlike ultrasound-assisted technology, electropermeabilization-assisted extraction of carotenoid prevents the degradation

of these temperature-sensitive carotenoids. The optimization of liquid biphasic flotation system and electropermeabilization-assisted liquid biphasic flotation were conducted to compare the effect of electric-field treatment. Various parameters such as type and concentration of alcohol and salts, flotation time, flow rate, period of electropermeabilization, position of copper electrode, voltage applied, and biomass concentration were evaluated. The antioxidant properties of extracted fucoxanthin obtained from optimized LBF system and electropermeabilization-assisted LBF system were also assessed.

5) Bioprocessing of *Chaetoceros calcitrans* for the recovery of fucoxanthin using CO_2-based alkyl carbamate ionic liquids: A rapid cell permeabilization and extraction of fucoxanthin from *Chaetoceros calcitrans* microalgae by CO_2-based alkyl carbamate ILs. The extraction conditions such as type of CO_2-based alkyl carbamate ILs, concentration of IL, incubation period and temperature were investigated. Surface morphology of the permeabilized cells was analysed by light microscope and Field Emission Scanning Electron Microscope (FESEM). Antioxidant properties of the extracted fucoxanthin, including Trolox equivalent antioxidant capacity (TEAC) and total phenolic content (TPC) were assessed. Studies on the recyclability of ILs were conducted to evaluate the performances of ILs for the successive batches of extraction

1.5 Outline of Thesis

The outline of thesis consists of eight (8) chapters and stated as followed. **CHAPTER 1** covers the research backgrounds and the current problem statement faced in the downstream processing of carotenoids from microalgae. The research

objectives and contribution of this research are highlighted as well. **CHAPTER 2** covers the literature reviews on the upstream and downstream of microalgae, conventional and recent advances biorefinery approach for the recovery of astaxanthin and fucoxanthin, liquid biphasic system technologies and sustainability of ionic liquids technology. **CHAPTER 3** outlines the extraction of astaxanthin from *Haematococcus pluvialis* microalgae using liquid biphasic flotation system (manuscript published in *Bioresource Technology*). **CHAPTER 4** depict a one-step extraction approach for the extraction of astaxanthin from Haematococcus pluvialis microalgae with integrated ultrasonic-assisted liquid biphasic system (manuscript published in *Ultrasonics Sonochemistry*). **CHAPTER 5** report a greener and sustainable extraction approach using CO_2-based alkyl carbamate ionic liquids (ILs) were proposed as a promising solvent to permeabilize the robust microalgae cell wall for an effective extraction of astaxanthin (manuscript published in *Chemical Engineering Journal*). **CHAPTER 6** demonstrate a greener and non-thermal extraction approach for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae using electropermeabilization-assisted liquid biphasic flotation system. (submitted to *Separation and Purification Technology*) **CHAPTER 7** unveil a rapid cell permeabilization and extraction of fucoxanthin from *Chaetoceros calcitrans* microalgae by CO_2-based alkyl carbamate ILs (manuscript published in *Bioresource Technology*). Lastly, in **CHAPTER 8** summarized the list of research achievement along with future research works and recommendation of the remaining and consecutive research gaps.

1.6 Flow Diagram of the Research

The flow chart of illustrating the scopes in this research is shown in **Figure 1.1.** The carotenoids from *Haematococcus pluvialis* and *Chaetoceros calcitrans* microalgae were subjected to various pretreatment and extraction processes. Recent advances technologies such as ultrasound- and electropermeabilization-assisted were incorporated into the liquid biphasic flotation system and ionic liquids technologies were implemented to process these valuable carotenoids from microalgae.

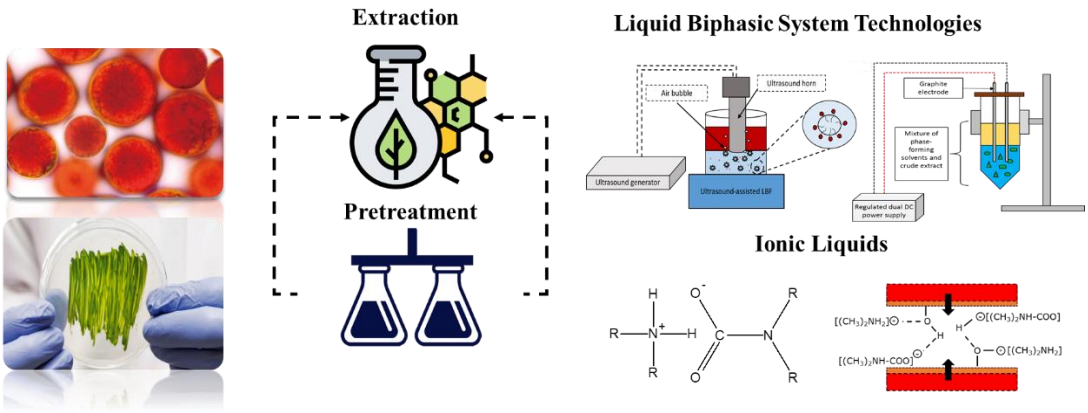


Figure 1.1: Flow diagram of this research work

CHAPTER 2 LITERATURE REVIEW

2.1 Life science, morphologies and life cycle of *Haematococcus pluvialis*

Haematococcus pluvialis (*H. pluvialis*), also known as *Haematococcus lacustris* or *Sphaerella lacustris*, is a biflagellate volvocalean unicellular green algae categorized under the class of Chlorophycea (Wayama et al., 2013). *H. pluvialis* is primarily found in temporary freshwater bodies such as rain pools, natural or artificial ponds and birdbath (Burchardt et al., 2006, Proctor, 1957). *H. pluvialis* was successfully isolated from the sources like the free-standing water container on the rooftop of the Goddard building at the University of Queensland St Lucia campus (Ma et al., 2018), on coastal rock located on the seashore in Russia (Chekanov et al., 2014) and freshwater fishpond in Romania (Dragoş et al., 2010). *H. pluvialis* is capable of withstanding the intense environment and climate (e.g., light, temperature and salt concentration) by turning into red non-motile encysted cell enclosed with a thick membrane (Proctor, 1957).

There are four types of cellular morphologies in the progress of *H. pluvialis* towards maturation, namely macrozooid (zoopores), microzooid, palmella and haematocyst (aplanospores) (Hazen, 1899, Shah et al., 2016). In the green motile stage, the cell appeared in the forms of macrozooid, microzooid and palmella. In the red non-motile stage, the cell is known as haematocyst wherein the astaxanthin is accumulated in the thick membrane. Macrozooid, which appears in irregular spherical or pear shape, is the starting stage of *H. pluvialis*'s life cycle. Biflagellate with an equal length is extended from the anterior end of the cell to allow the cell

movement. Under the favourable culture condition, macrozooid grows rapidly in the vegetative asexual reproduction stage. Macrozooid undergoes a process called "mitosis" which divides the mother cell into 2 to 32 daughter cells forming a fragment of macrozooid (Praveenkumar et al., 2015, Wayama et al., 2013).

In contrast, unfavorable culture condition causes the macrozooid to undergo morphogenesis by losing both its flagella, expanding in size, and forming a spherical green non-motile "palmella" cell. The inner cell consists of amorphous multi-layered extracellular matrix encapsulating the primary wall that protects the cell composition when they become the resting vegetative cell (Hagen et al., 2002). At this intermediate stage, the accumulation of small amount of astaxanthin in the cytoplasm renders a greenish-red intermediate cell. The prolonged unfavorable cultivation stress (e.g., nutrient stress, high light intensity and high salinity) will turn the "pamella" cell into the "haematocyst" cell. At this stage, the red non-motile cell becomes more hindrance to predominate the intense cultivation condition by forming a new rigid trilaminar sheath, secondary and tertiary walls that are composed of sporopollenin-like biopolymer material (algaenan) (Boussiba et al., 1999, Kobayashi and Okada, 2000). Under unfavourable optimal cultivation condition, *H. pluvialis* was able to reach a fully mature haematocyst cell within the time period in 2 weeks (Kobayashi et al., 1997b). A mature haematocyst is capable to accumulate a large amount of astaxanthin up to 5 % of cell dry weight. The astaxanthin was deposited in lipid droplets in the cytoplasm, giving a reddish colour pigment of the cell (Boussiba et al., 1999, Hagen et al., 2002, Wayama et al., 2013).

When the culture condition becomes favourable for red non-motile haematocysts cell, gametogenesis occurs and up to 64 gametes known as microzooids are produced. Microzooid is lesser than 10 µm, which is relatively smaller as compared to macrozooid with size of 20 – 50 µm in diameter (Hazen, 1899, Shah et al., 2016). Furthermore, the germination of haematocyst might occur, resulting in a thinner and weaker cell wall of motile microzooid due to the breakages of trilaminar sheath, secondary and tertiary cell walls. As a result, the biorefinery of astaxanthin is more efficient if *H. pluvialis* cells are harvested after the germination of haematocyst (Praveenkumar et al., 2012).

2.1.1 Biosynthesis of natural astaxanthin from *H. pluvialis* microalgae

Figure 2.1 shows the overview of the processes in biosynthesis of natural astaxanthin from *H. pluvialis*. Many works have highlighted the importance of optimized cultivation conditions for achieving high cell density and accumulation of astaxanthin in *H. pluvialis*; this included type of culture medium, temperature, pH and light intensity (Domınguez-Bocanegra et al., 2004, Gıannelli et al., 2015, Saha et al., 2013). It is also crucial to evaluate the stress condition at a certain period, since there are several reports demonstrating the retardation of cell growth under highly intense stress condition (Su et al., 2014). It has been proven that *H. pluvialis* can be cultivated in photoautotropic, heterotropic and mixotropic conditions depending on the feasibility of the culture system for industrial production of astaxanthin (Liu et al., 2017). Several cultivation techniques utilizing the common photobioreactors like flat type, airlift column, bubbling column, and tubular column

have been developed (Choi et al., 2011, García-Malea et al., 2009, Kang et al., 2010, Wang et al., 2013).

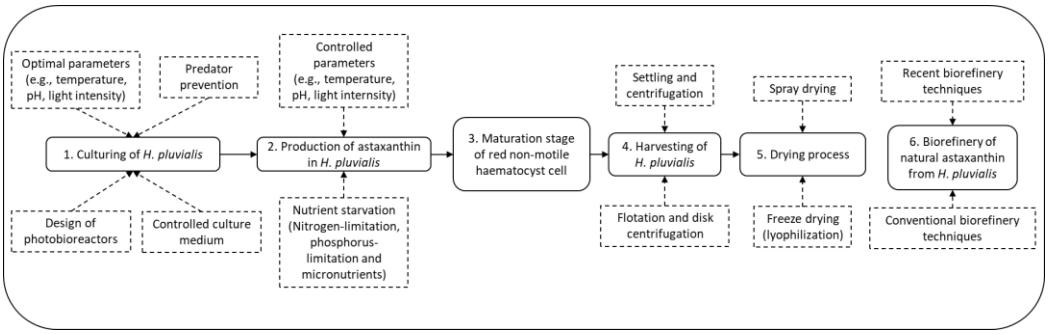


Figure 2.1: Overview of the processes in the production of astaxanthin from *H. pluvialis* covering cultivation and downstream processing.

The most commonly used culture media for the cultivation of *H. pluvialis* are Bold's Basal Medium (BBM) (Bischoff & Bold, 1963), Blue-Green Medium (BG-11) (Rippka et al., 1979), KM1-basal (i.e., a modified variant of BBM) (Kobayashi et al., 1997a) and Optimal Haematococcus Medium (OHM) (Fábregas et al., 2001). Recently, the common culture media was reformulated with additives, such as melatonin (MLT) and diethyl aminoethyl hexanoate (DA-6), to trigger the stress-specific effect and the expression of hormones in *H. pluvialis* for increasing the cell density and accumulation of astaxanthin (Ding et al., 2019, Ding et al., 2018, Sun et al., 2015). The induction of carotenogenesis happens when the cells are exposed to stress conditions derived from nutrient (e.g., nitrogen and phosphorus) starvation, high salinity, and the combination or multiple of stress factors which

induce the astaxanthin accumulation. It was reported that the concentration of astaxanthin (3.5%, w/w) in *H. pluvialis* cultivated under nitrogen limitation was two-fold as compared to that in *H. pluvialis* cultivated under phosphorus limitation (Niizawa et al., 2018). Nitrogen limitation during cultivation causes the cellular rupture due to the breakdown of chlorophyll in the cell. In addition, only 8 days were required for the accumulation of astaxanthin up to 4.0% (w/w) during the algae cultivation under nitrogen limitation, whereas the same concentration of astaxanthin was accumulated only offers 14 days of algae cultivation under phosphorus limitation (Boussiba et al., 1999). Nonetheless, the biosynthesis of astaxanthin in the "haematocyst" cell could be increased by adding micronutrients such as Fe^{3+} (ferrous sulfate) and acetate. Several studies reported that the addition of approximately 0.45 to 0.5 mM Fe^{3+} was sufficient for the formation of hydroxyl radicals that promote the biosynthesis of astaxanthin in haematocyst cell (Hong et al., 2016, Kobayashi et al., 1997a).

Besides, it is also important to maintain the cultivation temperature within 20 – 28°C for the optimum cell growth and astaxanthin accumulation in *H. pluvialis* (Giannelli et al., 2015, Kang et al., 2010, Wan et al., 2014). By increasing the temperature greater than 30°C, the vegetative green motile cells might turn into red non-motile haematocyst cells; the high temperature condition increased the metabolic activity in the cell, giving the maximum growth rate during cultivation stage as compared to optimum condition (Goldman and Carpenter, 1974). Tjahjono et al. (1994) reported that the biosynthesis of astaxanthin at 30 – 33 °C was 2.5 to 3 times higher than that of in the optimum condition (Tjahjono et al., 1994).

Furthermore, the authors reported that the green motile cells rapidly changed to the large dark red haematocyst cells within two days of cultivation. An increase in temperature enhanced the biosynthesis of astaxanthin in the haematocyst cell due to the presence of reactivity oxygen radicals triggering their reactivity in the cell (Halliwell and Gutteridge, 2015).

An optimal pH condition is essential for the maximum growth of cell density and the biosynthesis of astaxanthin. Several researchers have found that the pH range of 7 – 7.85 was ideal for stress-induced astaxanthin biosynthesis (Hata et al., 2001, Sarada et al., 2002b, Sarada et al., 2002a). However, the cultivation of *H. pluvialis* strain under a neutral pH condition might encounter challenges such as contamination caused by fungal parasites, zooplanktonic, cyanobacteria and various microalgae (Gutman et al., 2009, Han et al., 2013, Shah et al., 2016). A recent study demonstrated that *H. pluvialis* had a rapid growth rate of more than 99% within ten days under acidic culture condition (pH 3 – 4) could prevent the contamination by a lethal fungus, *Paraphysoderma sedebokerensis* (Hwang et al., 2019). This was due to the successful prevention of contamination by *Paraphysoderma sedebokerensis*, which is a lethal fungus having an optimum growth in neutral or alkaline condition. Therefore, the strategy of acidic cultivation condition emerges as an alternative solution to overcome the contamination problem, although some modifications of other cultivation factors such as light intensity and nitrogen-related limitation might be required (Kim et al., 2009, Yeh and Chang, 2011).

Light intensity is the most prominent factor that influences the cell cycle, morphological changes, and carotenogenesis induction of *H. pluvialis*. Since *H.*

pluvialis will undergo two stages of growth, namely green motile stage and red non-motile stage, different optimal light intensity is required to achieve best yield of cell and astaxanthin. For the cultivation of green motile cells, the optimum light intensity ranged between 20 to 90 $\mu mol \cdot m^{-2}s^{-1}$, depending on the different modification and approaches (Kim et al., 2009, Ma et al., 2018, Park et al., 2014). When the cells reached the exponential growth phase, the culture will be transferred into another controlled medium and exposed to a higher light intensity ranging around 100 – 480 $\mu mol \cdot m^{-2}s^{-1}$ for the transformation of cells into red non-motile haematocysts (Chekanov et al., 2014, Domınguez-Bocanegra et al., 2004, Ma et al., 2018, Saha et al., 2013, Zhang et al., 2014). Recent studies showed that the exposure of culture to the weak incident of blue light (wavelength of 480 nm) along with a low intensity of white light could increase the biosynthesis of astaxanthin in red non-motile haematocyst (Lee et al., 2018, Ma et al., 2018). The decrease in the light intensity could be beneficial for reducing the cost of cultivation, thereby making the cultivation process more feasible for commercial production.

The feasibility of implementing electricity treatment has shown a rapid growth rate of *H. pluvialis* (Kim et al., 2018). Additionally, the present of electricity treatment enhance the astaxanthin accumulation up to 32.6 mg/L in red non-motile haematocyst cell. On the other hand, it is crucial to increase the cell density of *H. pluvialis* during the green vegetative stage as this would promote a higher astaxanthin production in the red non-motile haematocyst cell. The application of direct current at 60 mA and 100 mA to the culture gave an increase in cell density up to 20% difference when compared to the cultivation without electrical treatment.

However, at a high current (≥ 120 mA) condition, the cell growth was unfavorable and the cell density could decrease.

2.1.1.1 Harvesting of *H. pluvialis*

In most of the biorefineries, the harvesting step remains as a bottleneck for the large-scale application. The harvesting of *H. pluvialis* for recovery of astaxanthin commences once the red non-motile haematocyst cells reach maturation. The gravitational settling and centrifugation are among the conventional harvesting techniques used. These processes allow the red non-motile haematocyst cells to be pre-concentrated and fully separated from culture medium (Han et al., 2013, Pérez-López et al., 2014). Panis et al. (2016) reported a 95% of the recovery of biomass using floatation and disk stack centrifugation (Panis and Carreon, 2016). Despite the high efficiency of biomass recovery, the operations of both techniques are often limited by their high cost and the narrow suitability for the selected species. A recent study reported an electrocoagulation floatation technique for harvesting microalgae, yielding 89 ± 2 % of biomass concentration (Landels et al., 2019). The approach utilized charged particles which is fast-acting and environmentally friendly. Besides, bioflocculation of microalgae-bacteria was recently applied for enhancing microalgae harvesting and nutrient removal from wastewater effluent (Nguyen et al., 2019).

2.1.1.2 Drying of *H. pluvialis*

After the pretreatment of algae biomass, a drying process is required to prevent the degradation of pigment and to extend the shelf life (Mata et al., 2010). One of the most effective and frequently used techniques is spray drying (Li et al.,

2011a, Milledge, 2013, Panis and Carreon, 2016, Raposo et al., 2012). Spray drying reduced the moisture content in red non-motile haematocyst cell to as low as 5% (Pérez-López et al., 2014). Despite that, spray drying requires a high operation cost due the requirement of high working temperature, i.e., 180 – 220°C (Grima et al., 2003, Raposo et al., 2012). Freeze drying, or known as lyophilization, is another drying method that involves the freezing of algae biomass usually at -20°C and the temperature is maintained at 4°C (Choi et al., 2019). This technique is milder than high-temperature spray drying; however, it is more expensive to be applied in the industrial scale (Milledge, 2013).

2.1.1.3 Pretreatment of *H. pluvialis*

It is vital for the red non-motile haematocyst cells to undergo a pretreatment step for an effective recovery of astaxanthin from the rigid and recalcitrance structure of haematocyst cell. The conventional pretreatments involve mechanical methods such as expeller pressing, bead milling and grinding (Mercer and Armenta, 2011, Razon and Tan, 2011). However, high energy consumption is a factor to be considered for large-scale bioprocessing of microalgae (Aguirre and Bassi, 2014). The expeller pressing subjects the *H. pluvialis* cell under compression at high pressure for the disruption of sporopollenin wall. The technique has successfully recovered 75% of algae oil in a one step process (Mohan et al., 2014, Topare et al., 2011). On the other hand, bead milling involves the agitation of tiny glass ceramic or steel beads at a high speed. The effectiveness of cell disruption is dependant of the time interval in the processing (Greenwell et al., 2010). Bead milling is still the most effective technique of cell disruption when compared to the enzymatic

treatment, spray drying and chemical pretreatment (Mendes-Pinto et al., 2001). Once the cell walls are disrupted, it is recommended that the astaxanthin must be rapidly recovered within few hours, and the lysed cells must be minimally exposed to the light condition to prevent the degradation of light-sensitive astaxanthin pigment.

2.1.1.4 Recovery of astaxanthin from *H. pluvialis*

The conventional solvent extraction techniques utilized aliphatic alcohols (e.g., methanol, ethanol) or concentrated acids and alkalis [e.g., potassium hydroxide, dimethyl sulfoxide (DMSO)] for the recovery of astaxanthin. However, the type of solvent used is highly dependent on the industrial application; for example, astaxanthin applied in food or pharmaceutical industries requires a milder recovery method (Ni et al., 2007). The use of concentrated acid is not favourable due to its hazardous and toxicity that cause adverse effect towards human health. Furthermore, conventional solvent extraction techniques might not be as effective as other advanced technologies, including SC-CO_2 extraction (Mendes-Pinto et al., 2001, Fujii, 2012, Pan et al., 2012, Reyes et al., 2014, Sanzo et al., 2018, Yen et al., 2015). SC-CO_2 extraction provides a short extraction time (within 20 to 120 mins), a better quality of bioproduct, and a low-cost process. Besides, SC-CO_2 is a sustainable and green solvent as compared to the conventional solvents (Guedes et al., 2011, Machmudah et al., 2006).

2.1.2 Biorefinery of natural astaxanthin

Various conventional and advanced extraction techniques for natural astaxanthin were summarised and are reported in **Table 2.1** and **Table 2.2**,

respectively. Ongoing researches are putting effort to evaluate the impact of type of solvents used for extraction, pretreatment techniques (e.g., size of glass bead, acid or alkali treatment), direct extraction using vegetables oils and microwave- or ultrasound-assisted solvent extraction for efficient recovery of natural astaxanthin from *H. pluvialis*.

Table 2.1: Conventional biorefinery techniques of astaxanthin from *H. pluvialis*.

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Autoclave	Acetone (30 min)	Acetone (2 mL, 16 h, 20 °C)	*H. pluvialis* CCAP 34/7 (dried): Bold's Basal, 21 ± 1 °C, 80 µmol photon m^{-2} s^{-1} for 2 weeks	18.0	> 85	(Mendes-Pinto et al., 2001)
Acid pretreatment + cell homogenisation	Acetone	Acetone (2 mL, 16 h, 20 °C)	*H. pluvialis* CCAP 34/7 (dried): Bold's Basal, 21 ± 1 °C, 80 µmol photon m^{-2} s^{-1} for 2 weeks	19.0	> 85	(Mendes-Pinto et al., 2001)
Acid pretreatment	HCl (30 min)	Acetone (2 mL, 16 h, 20 °C)	*H. pluvialis* CCAP 34/7 (dried): Bold's Basal, 21 ± 1 °C, 80 µmol photon m^{-2} s^{-1} for 2 weeks	9.0	≈ 35	(Mendes-Pinto et al., 2001)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Alkali pretreatment	NaOH (30 min)	Acetone (2 mL, 16 h, 20 °C)	*H. pluvialis* CCAP 34/7 (dried): Bold's Basal, 21 ± 1 °C, 80 µmol photon m^{-2} s^{-1} for 2 weeks	6.0	≈ 30	(Mendes-Pinto et al., 2001)
Acid pretreatment + homogenisation using mortar and pestle with presence of neutral glass powder	HCl (2 N, 5 – 10 min heating at 70°C)	Acetone (10 mL, centrifuged at 5000 rpm at 4°C for 10 min)	*H. pluvialis* SAG 19-a (wet): Autotrophic medium, 25 ± 1 °C, 3.0 klux, 0.2% NaCl and 4.4 mM sodium acetate for 2 weeks	-	96 ± 3	(Sarada et al., 2006)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Acid pretreatment + homogenisation using mortar and pestle with presence of neutral glass powder	DMSO (5 – 10 min heating at 70 °C)	Acetone (10 mL, centrifuged at 5000 rpm at 4 °C for 10 min)	*H. pluvialis* SAG 19-a (wet): Autotrophic medium, 25 ± 1 °C, 3.0 klux, 0.2% NaCl and 4.4 mM sodium acetate for 2 weeks	-	66.64 ± 0.78	(Sarada et al., 2006)
Acid pretreatment + homogenisation using mortar and pestle with presence of neutral glass powder	Citric acid (5 – 10 min heating at 70 °C)	Acetone (10 mL, centrifuged at 5000 rpm at 4°C for 10 min)	*H. pluvialis* SAG 19-a (wet): Autotrophic medium, 25 ± 1 °C, 3.0 klux, 0.2% NaCl and 4.4 mM sodium acetate for 2 weeks	-	3.44 ± 0.11%	(Sarada et al., 2006)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
No pretreatment	-	Vegetable oil direct extraction using soybean, corn, grapeseed and olive oil (30 mL, vigorously stirring at 70°C)	*H. pluvialis* NIES-144 (wet): 23°C, 5% CO_2, 200 μmol photon m^{-2} s^{-1} of light for 1 week	-	Soybean: 91.7 Corn: 89.3 Grapeseed: 87.5 Olive: 93.9	(Kang and Sim, 2008)
No pretreatment	-	Soybean oil direct extraction (20 mL, 2 h agitation at 20°C)	*H. pluvialis* (dried): Yunnan Yunlin Biological Technology Co. Ltd. (Yunnan, China)	0.9 ± 0.1	-	(Dong et al., 2014)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Grinding	Repeating process of freezing with liquid nitrogen and mashing in ceramic mortar	Pressurized liquid ethanol (10.3 MPa)	*H. pluvialis* BNA 10/024 (dried): Bold's Basal, 25 °C, 200 μmol m^{-2} s^{-1} for 6 days	21.0	-	(Jaime et al., 2010)
Grinding	Repeating process of freezing with liquid nitrogen and mashing in ceramic mortar	Methanol:dichloromethane [3:1 (v/v)]	*H. pluvialis* SAG 34-1b (dried), University of Göttingen, Germany, 25 ± 1 °C, 30 μmol m^{-2} s^{-1}, 1.5% (v/v) CO_2 for 10 days.	23.0	-	(Zhang et al., 2014)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Bead milling	Glass bead (0.5 mm)	Acetone	*H. pluvialis* INETI 33 (dried): 1.8% (w/w), 25 °C, 1,000 μE m^{-2} s^{-1}, nitrogen starvation and 2% of NaCl	18.0	-	(Nobre et al., 2006)
Bead milling	Glass bead (2.5 mm) for 4 min	Sucrose (0.2 M, centrifuged for 10 min)	*H. pluvialis* Flotow 1844: nitrate-free mBG-11 medium, 350 μmol photon m^{-2} s^{-1} for 1 week	168-200 μg/mL oil	-	(Peled et al., 2011)
Bead milling	Duplicate process of glass bead (1 mm) for 10 min	Acetone	*H. pluvialis* (dried): Biogenic Co., Ltd, Tokyo, Japan	7.80	-	(Boonnoun et al., 2014)
Ultrasound-assisted solvent extraction	Ultrasound irradiation of 200 W for 16 min with ethanol and ethyl acetate	Ethanol: ethyl acetate [1:1, (v/v)]	*H. pluvialis* (dried): Jingzhou natural Astaxanthin Inc. (Hubei, China)	27.58 ± 0.40	-	(Zou et al., 2013)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
No pretreatment	-	Ethanol:ethyl acetate (1:1 (v/v), 2 h)	*H. pluvialis* (dried): Jingzhou natural Astaxanthin Inc. (Hubei, China)	17.34 ± 0.85	-	(Zou et al., 2013)
Ultrasound-assisted acid pretreatment (20 min, ice bath)	Hydrochloric acid (1 mL, 4 M, 2 min, 70°C)	Acetone (1 mL)	*H. pluvialis* (dried): Yunnan Yunlin Biological Technology Co. Ltd. (Yunnan, China)	19.8 ± 1.1	-	(Dong et al., 2014)
Ultrasound-assisted solvent extraction	Ultrasound-assistant (20 min, ice bath)	Hexane: isopropanol (centrifuged at 3500 rpm at 4°C for 5 min)	*H. pluvialis* (dried): Yunnan Yunlin Biological Technology Co. Ltd. (Yunnan, China)	9.7 ± 0.6	-	(Dong et al., 2014)

Table 2.1 (Continue)

Pretreatment technique	Pretreatment condition	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
Ultrasound-assisted solvent extraction	Ultrasound-assistant (5 min, ice bath)	Two-step extraction methanol/acetone (centrifuged at 3500 rpm at 4°C for 5 min)	*H. pluvialis* (dried): Yunnan Yunlin Biological Technology Co. Ltd. (Yunnan, China)	13.8 ± 0.4	-	(Dong et al., 2014)

2.1.2.1 Conventional biorefinery technique of natural astaxanthin from *H. pluvialis*

The conventional biorefinery of *H. pluvialis* utilizes organic solvents, concentrated acids and ultrasound-assisted solvent extraction approaches. Sarada et al. (2006) conducted an experiment for extraction of astaxanthin from *H. pluvialis* SAG 19-a using different solvents (acetone, methanol, DMSO) and acids (hydrochloric acid, tartaric acid, citric acid, acetic acid and formic acid) (Sarada et al., 2006). Up to 96 ± 3% of astaxanthin was successfully recovered from the treatment of *H. pluvialis* SAG 19-a using 1 – 2 N hydrochloric acid within 5 – 10 min under heating at 70°C, followed by acetone extraction. In the same study, the second highest recovery (66.64 ± 0.78%) was achieved by using DMSO, while the treatment of cells with citric acid gave the lowest recovery of astaxanthin of (3.44 ± 0.11%). A similar study conducted by Mendes-Pinto et al. (2001) showed that the physical pretreatment (e.g., autoclave and mechanical disruption) prior to acetone extraction improved the recovery of astaxanthin greater than 85% (Mendes-Pinto et al., 2001). This might be due to the cleavage of some bonds in the cell wall during physical disruption process, causing an effective leaching of carotenoids due to the extended disruption of the cell wall. Although these methods gave a higher recovery, however, it is not favourable in the application of astaxanthin for food, additives and pharmaceutical purposes. A greener recovery approach using vegetable oils (e.g., soybean, corn, grapeseed and olive) gave an effective recovery up to 93.9% (Kang and Sim, 2008). Although this approach is an alternative green extraction process for astaxanthin used in the food industry, the long processing time (> 48 h) is often required for a higher recovery of astaxanthin. On the other hand, it is more favorable

to extract astaxanthin using an oil-based solvent due to the lipophilic characteristics of astaxanthin. A similar solvent extraction approach conducted by Dong et al. (2014) using soybean oil reported that only 0.9 ± 0.1 mg/g of astaxanthin was obtained; this could be due to less effective disruption of thick cell wall of red non-motile haematocyst cell under stirring condition at room temperature (≈ 20°C) (Dong et al., 2014).

A study by Jaime et al. (2010) investigated the recovery of astaxanthin from the dried *H. pluvialis* BNA 10/024 utilizing continuous grinding as pretreatment (Jaime et al., 2010). Then, followed by pressurized liquid extraction (≈ 10.3 MPa) with ethanol and hexane. The recovery yields of astaxanthin were 21.0 mg/g and 35.0 mg/g for which ethanol and hexane were used, respectively (Jaime et al., 2010). The continuous grinding step enhanced the astaxanthin recovery due to the more effective rupture of haematocyst cell. By using a similar approach without the pressurized liquid, Zhang et al. (2014) reported that the yield of astaxanthin recovered from the dried *H. pluvialis* SAD 34-1b using a mixture of methanol and dichloromethane (with a volume ratio of 3:1) was 23.0 mg/g (Zhang et al., 2014). On the other hand, the yield of astaxanthin from the dried *H. pluvialis* INETI 33 with bead milling (glass bead with size of 0.5 mm) coupled with acetone extraction was only 18.0 mg/g (Nobre et al., 2006). A similar approach of extraction with modification, namely a two-step bead milling with glass bead (1 mm) gave an even lower yield of astaxanthin (7.8 mg/g) (Boonnoun et al., 2014). Thus, the use of different sizes of glass beads significantly affects the cell disruption of red non-motile haematocyst cell.

Zou et al. (2013) examined the role of ultrasonic wave in an ultrasonic-assisted solvent extraction approach where the yield of astaxanthin was 27.58 ± 0.40 mg/g under ultrasound irradiation of 200 W for 16 min in ethanol and ethyl acetate solutions (Zou et al., 2013). With the aid of ultrasonic wave, the cell disruption was enhanced by the effect of acoustic cavitation produced in the solvent (Ghafoor et al., 2009, Zou et al., 2011). This allowed a greater penetration and diffusion of liquid phase into the rigid cell wall (Rostagno et al., 2003). In comparison, the solvent extraction technique utilising a mixture of ethanol and ethyl acetate (with a volume ratio of 1:1) gave a lower yield of astaxanthin (17.34 ± 0.85 mg/g) and required a longer processing time (≤ 2 h). Despite the advantages of ultrasonic-assisted solvent extraction, the selection of solvents used is critical for an effective mass transfer and an optimal recovery of astaxanthin. Dong et al. (2014) applied ultrasonication in both steps of acid pretreatment and solvent extraction for the recovery of astaxanthin (Dong et al., 2014). The yields of astaxanthin were 19.8 ± 1.1, 9.7 ± 0.6 and 13.8 ± 0.4 mg/g for the solvent extractions using acetone, hexane/isopropanol and methanol/acetone mixtures solvents, respectively. To prevent the degradation of astaxanthin in the end products, it is recommended to control and monitor the temperature along with the time period of irradiation (Kaczor and Baranska, 2011).

2.1.2.2 Recent advanced biorefinery techniques

2.1.2.2.1 Supramolecular solvent extraction

SUPRAS are made of nanostructured liquids of amphiphiles (e.g., carboxylic acids, alkanols, alkyl sulfates and alkyl phenols). A recent study adopting SUPRAS and NLCs (called SUPRAS-NLCs) showed an efficient extraction of

astaxanthin from *H. pluvialis* as compared to supercritical fluid extraction (SFE) (Salatti-Dorado et al., 2019). The yield of astaxanthin recovered using SUPRAS-NLCs was 71 ± 4%. Moreover, these SUPRAS-NLCs solvents were capable to preserve the antioxidant activity of astaxanthin with a stability period up to 180 days at 4°C. However, this approach required an optimal selection of SUPRAS amphiphiles that fulfil the requirements in extraction, encapsulation and stabilization of astaxanthin.

2.1.2.2.2 Supercritical carbon dioxide extraction

Among the recovery methods used, SC-CO_2 is the most efficient, sustainable, and widely used method for the biorefinery of astaxanthin from *H. pluvialis*. Ethanol is often used as a polar co-solvent, mainly due to the high solubility of astaxanthin in a mixture of SC-CO_2 and ethanol that promotes a high selectivity of astaxanthin in the solvent (Reyes et al., 2014). Moreover, Cheng et al. (2018) conducted an extraction study using SC-CO_2 and 20% (v/v) ethanol under the low-pressure condition (8 MPa) at 55°C for 15 min; the recovery of astaxanthin was 98.3% (Cheng et al., 2018). In another study, the extraction of astaxanthin under high pressure (40 MPa) of SC-CO_2 in the presence and absence of ethanol and without ethanol yielded 292.70 mg/g and 277.1 mg/g of astaxanthin, respectively (Molino et al., 2018). The results showed that the recovery of astaxanthin could be enhanced by increasing the pressure rather than utilizing co-solvent and additional pretreatment techniques that might increase the cost of the process. On the other hand, an increase in flow rate of CO_2 has a slight effect on the efficiency of astaxanthin extraction (Machmudah et al., 2006). Till date, there is no study

reporting an optimised condition of SC-CO_2 extraction for the biorefinery of astaxanthin.

2.1.2.2.3 Magnetic-assisted extraction

Magnetic-assisted extraction has become the emerging technology for the industrial biorefinery of microalgae (Zielińska-Dawidziak et al., 2012). The presence of magnetic susceptible additives in the liquid generates a magnetic field that attracts the target biomolecules and draw them towards the magnet. This approach provides a quick and rapid phase separation in a shorter time period (Wikström et al., 1987). However, the magnetic-assisted extraction approach is rarely used in the extraction of astaxanthin from *H. pluvialis*. A recent study by Zhao et al. (2016a) demonstrated the effectiveness of magnetic-assisted extraction of astaxanthin from *H. pluvialis* using acetic ether as solvent; the recovery of astaxanthin under the sonication with 5 mT of magnetic intensity for 50 min was 62.72% (Zhao et al., 2016a). It was reported that the increase in magnetic intensity from 0 to 15 mT led to a noticeable increase in astaxanthin concentration (from 45.2 to 52.7%). However, when a higher magnetic intensity was applied (> 15 mT), the recovery of astaxanthin decreased. It was found that the acetic ether acted as a diamagnetic substance that produces a low magnetic field in the surrounding when the magnetic induction intensity increased (Yongli et al., 2007). Another study reported that the magnetic-assisted extraction achieved the highest yield of astaxanthin (40.27%) as compared to other extraction techniques such as maceration, ultrasound-, microwave- and magnetic-assisted extractions wherein the yields of astaxanthin were in the range of 36.5 – 36.88% (Zhao et al., 2016a). The

report highlighted that magnetic-assisted approach is an effective alternative for the biorefinery of astaxanthin. Apart from that, the selection of appropriate solvent that is compatible with astaxanthin in magnetic-assisted extraction has yet to be explored.

2.1.2.2.4 Ionic liquid-based extraction

ILs have emerged as environmentally friendly solvents for use in microalgae biorefinery, owing to their distinctive characteristics including low vapour pressure, low melting point, high thermal stability and recyclability (Plechkova and Seddon, 2008). ILs have also been utilized for the pretreatment of thick and rigid cell wall of microalgae, which further ease the extraction of intercellular valuable components by organic solvent (Kim et al., 2012b). A pretreatment study conducted by Desai et al. (2016) used [Emim][DBP] as a permeabilising agent on the resistant cell wall of *H. pluvialis* for the extraction of astaxanthin (Desai et al., 2016). The study showed that the recovery of astaxanthin was up to 70%. Another similar study of extraction of astaxanthin from wet *H. pluvialis* NIES-144 using [Emim][$EtSO_4$] reported that 32.5 pg/ g.cell of astaxanthin was recovered under the maximal 12 h of cell germination of 12 h and 24 h of extraction time (Praveenkumar et al., 2015). Besides, a recent study reported that imidazolium-based ILs with HSO_4^-, $CH_3SO_3^-$ and $(CF_3SO_2)_2^-$ anions have a higher efficiency for pretreatment of *H. pluvialis*, wherein 99% of astaxanthin were successfully recovered under the optimized conditions, i.e., 6.7 % (v/v), 30 °C, 60 min (Choi et al., 2019). The study proved that imidazolium-based ILs have a greater potential as compared to ammonium- and pyridinium-based ILs for the pretreatment of thick cell wall of microalgae. Owing

to the expensiveness of ILs, a study of reusability and recyclability of ILs has showed that the recovered ILs were able to be reused for three times without any regeneration or treatment required (Liu et al., 2019b). The reused [Bmim][Cl] and [Emim][Cl] yielded 82.75% and 59.87% of astaxanthin, respectively. A further comparative study on various pretreatment techniques (e.g., pulsed electric field, ultrasound, high-pressure microfludisation and ILs) concluded that the pretreatment of cells using ILs is more promising for astaxanthin extraction due to their mild condition under ambient temperature, atmospheric pressure and low energy consumption (Liu et al., 2018). Despite the benefits offered by ILs, it is recommended to the elucidate the interaction between ILs and cell wall of *H. pluvialis* to understand the extraction principle.

Table 2.2: Recent biorefinery techniques of astaxanthin from *H. pluvialis*

Extraction media + any pretreatment	Pretreatment technique	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
SUPRAS-NLCs	SUPRAS (Centrifuged at 1000 rpm, 10 min)	SUPRAS (1 g biomass: 5 mL of 10 mM HCL: 2 mL of SUPRAS)	*H. pluvialis* (dried): 3% (w/w), Pigmentos Naturales	-	71 ± 4%	(Salatti-Dorado et al., 2019)
SC-CO_2	SC-CO_2 (7 MPa, 45°C, 120 min)	SC-CO_2 and ethanol co-solvent (50%, v/v)	*H. pluvialis* (dried): 3% (w/w), Atacama Bio Natural Products Inc	22.0	124.0	(Reyes et al., 2014)
SC-CO_2	Hydrothermal	SC-CO_2 and olive oil, co-solvent (20%, v/v)	*H. pluvialis* (wet): 25°C, 60 µmol m^{-2} s^{-1}, Iconthin Biotech Corp	-	98.6	(Cheng et al., 2018)

Table 2.2 (Continue)

Extraction media + any pretreatment	Pretreatment technique	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
SC-CO_2	Hydrothermal	SC-CO_2 and ethanol, co-solvent (20%, v/v)	*H. pluvialis* (wet): 25°C, 60 µmol m^{-2} s^{-1}, Iconthin Biotech Corp	-	98.3	(Cheng et al., 2018)
SC-CO_2 + milling	SC-CO_2 (55 MPa, 65°C, 20 min)	SC-CO_2 direct extraction	*H. pluvialis* (dried): 2% (w/w)	277.1	-	(Molino et al., 2018)
SC-CO_2 + milling	SC-CO_2 (55 MPa, 65°C, 20 min)	SC-CO_2 and ethanol co-solvent (12.5%, v/v)	*H. pluvialis* (dried): 2% (w/w)	292.70	-	(Molino et al., 2018)

Table 2.2 (Continue)

Extraction media + any pretreatment	Pretreatment technique	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
SC-CO_2	SC-CO_2 (55 MPa, 50 °C, 120 min)	SC-CO_2 direct extraction	*H. pluvialis* (dried): 20mg/g Micoperi Blue Growth	-	98.6	(Sanzo et al., 2018)
Magnetic-assisted extraction	Sonication with magnetic intensity (5 mT, 50 min)	Acetic ether (77:1, v/w)	*H. pluvialis* (dried): 3% (w/w), Jingzhou Natural Astaxanthin Inc. China	-	62.72	(Zhao et al., 2016a)
Magnetic-assisted extraction	Magnetic intensity (20 mT, 60 min)	Ethyl acetate	*H. pluvialis* (dried): *H. pluvialis* (dried): 3% (w/w), Jingzhou Natural Astaxanthin Inc. China	11.12 ± 0.01	40.27	(Zhao et al., 2016b)

Table 2.2 (Continue)

Extraction media + any pretreatment	Pretreatment technique	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
ILs	Permeabilisation (45°C ,90 min)	1-ethyl-3-methylimidazolium di-butylphosphate, ([Emim][DBP])	*H. pluvialis* (dried): 3.2% Feyecon (Weesp, The Netherlands)	-	≥ 70.0	(Desai et al., 2016)
ILs	Germination (12 h)	1-ethyl-3-methylimidazolium ethylsulfate, [(Emim)($EtSO_4$)], (1 min)	*H. pluvialis* NIES-144 (wet): NIES-N medium, 25 °C, 5 (%, v/v) CO_2, 69 μmol m^{-2} s^{-1} for 15 days	19.5 pg/cell	-	(Praveenkumar et al., 2015)
ILs	Germination (12 h)	1-ethyl-3-methylimidazolium ethylsulfate, [(Emim)($EtSO_4$)], (24 h)	*H. pluvialis* NIES-144 (wet): NIES-N medium, 25 °C, 5 (%, v/v) CO_2, 69 μmol m^{-2} s^{-1} for 15 days	32.5 pg/cell	-	(Praveenkumar et al., 2015)

Table 2.2 (Continue)

Extraction media + any pretreatment	Pretreatment technique	Biorefinery of astaxanthin	Strains	Astaxanthin yield (mg/g)	Astaxanthin recovery (%)	Reference
ILs + milling	1-ethyl-3-methylimidazolium-based ILs with HSO_4^-, $CH_3SO_3^-$, $(CF_3SO_2)_2^-$ anions [6.7% (v/v), 30°C, 60 min]	Hexane	*H. pluvialis* (lyophilized): industrial flue gas CO_2 3.5% (v/v), 23 ± 2 °C, pH 7.5, 300–500 μmol photon m^{-2} s^{-1}	-	> 99.0	(Choi et al., 2019)
ILs	1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) [40% (v/v), 60°C, 60 min]	Methanol	*H. pluvialis* (dried): 3.5% (w/w), Kunming Biogenic Co. Ltd. (Yunnan, China)	-	85.42	(Liu et al., 2019b)
ILs	1-ethyl-3-methylimidazolium chloride ([Emim][Cl]) [40% (v/v), 60°C, 60 min]	Methanol	*H. pluvialis* (dried): 3.5% (w/w), Kunming Biogenic Co. Ltd. (Yunnan, China)	-	65.29	(Liu et al., 2019b)

2.2 Potential of fucoxanthin sources in microalgae

Fucoxanthin ($C_{42}H_{58}O_6$) is a major carotenoid of microalgae found in the diatoms from the class Bacillariophyceae such as *Paeodactylum tricornutum* and *Chaetoceros calcitrans* and brown algae from class Prymnesiophyceae (Foo et al., 2017). The presence of an allenic bond, a conjugated carbonyl, a 5,6-monoepoxide and an acetyl group functional group contains in the fucoxanthin structure functions as the main light-harvesting complexes responsible in the transfer of energy for photosynthesis electron transport chains in both micro- and macroalgae. These carotenoids act as a scavengers by trapping radicals and add electrons to their conjugated double-bond yielding a ground-state oxygen and a triplet-state carotenoid (Stahl and Sies, 2012). Similarly, the excess energy that excited the carotenoid structure dissipate the energy into the surrounding environment by returning to its ground state (Takashima et al., 2012). It has been proven that it exhibit anti-proliferative activities against cancer cells and ability to enhance beneficial towards health effect in terms of anti-inflammatory, anti-diabetic, anti-cancer, anti-malarial, anti-angiogenic and photo-protection activities (Suchern et al., 2020, Mohamadnia et al., 2020, Sathasivam and Ki, 2018). The main purpose for the exploitation of natural fucoxanthin over synthetic chemical routes was mainly due to its prevalent antioxidant properties to prevent these chronic oxidative stress diseases that includes atherosclerosis, Alzheimer's disease, and cancer. Besides, the incorporated fucoxanthin into conventional food such as rice, milk, and pasta as bioactive ingredient has creates a huge market of opportunity in promoting nutritional value and sensory qualities to replace synthetic colouring in food and

nutricosmetics industry (Abu-Ghannam and Shannon, 2017, Prabhasankar et al., 2009).

2.2.1 Fucoxanthin extraction processes

The isolation of fucoxanthin from microalgae feedstock can be attained by various extraction methods. The selection of extraction techniques is driven by the cost of operation, complexity of feedstock, demand for the quality and the yield of final bioproducts. For example, to commercialize fucoxanthin in the fields of pharmaceutical, cosmetics, food or analytical testing, the bioactivity and purity of fucoxanthin must be well preserved. The characteristics of microalgae biomass possess a challenge to the extraction of fucoxanthin and one of them is the type of cell wall (e.g. cellulosic or siliceous) (Shannon and Abu-Ghannam, 2018). Hence, the extraction parameters influencing the performance of extraction need to be identified and optimized for maximizing the product yield while minimizing the operation time, chemical consumption, utility cost and waste generation. In the past, fucoxanthin extraction from microalgae feedstock was achieved by organic-solvent-based extraction with the aid of maceration or Soxhlet extraction. To date, alternative extraction techniques have been adopted as an environmentally friendly route to extract fucoxanthin. In the following sections, the conventional and alternative methods for extraction of fucoxanthin were reviewed and compared in the aspects of working principle, extraction performance, strength, and weakness of the method.

2.2.1.1 Conventional solvent extraction of fucoxanthin

The common techniques for extraction of carotenoids include maceration (soaking or direct organic-solvent extraction), Soxhlet extraction or steam/hydro distillation (Khoo et al., 2019a, Kadam et al., 2013). In general, the selection of organic solvent and the operation cost must be taken into consideration (Zarekarizi et al., 2019) when treating different types of macroalgae or microalgae for carotenoid extraction. In addition, the operation involving organic solvents must be handled with care because of the highly volatile and flammable characteristics of these solvents. Examples of organic solvents used in the solvent extraction of fucoxanthin are acetone, methanol, ethanol, n-hexane, dimethyl sulphoxide, dichloromethane, tetrahydrofuran and ethyl acetate. The properties of solvent systems, including dielectric constant and polarity index, affect the extraction yield of carotenoids. Fucoxanthin can be dissolved in mid-polar solvent systems because of the semi-polar characteristic of fucoxanthin and the oxygen molecule in the fucoxanthin structure, but water was found to be ineffective in solubilizing fucoxanthin (Guler et al., 2020). Although acetone was commonly used in the direct solvent extraction of fucoxanthin (Shannon and Abu-Ghannam, 2017), the yield of extraction was typically lesser than that by ethanol due to the lack of hydroxyl functional group for a better hydrophilic interaction. Tetrahydrofuran was found to be less efficient in extracting fucoxanthin because it generates peroxides that degrade fucoxanthin (Guler et al., 2020). A previous study showed that the ethanolic extraction of fucoxanthin from diatom *Phaeodactylum tricornutum* yielded 15.71 mg/g dried weight (Kim et al., 2012a). Similarly, a recent study found that the extraction of fucoxanthin from *Phaeodactylum tricornutum* was governed by the

type of solvent used; the selectivity of solvent for fucoxanthin was in the descending order of d-limonene > ethyl acetate > ethyl lactate > ethanol (del Pilar Sánchez-Camargo et al., 2017).

Moreover, the extraction of fucoxanthin from *Phaeodactylum tricornutum* can be improved by the application of hot soaking process with acetone (Pasquet et al., 2011). However, this approach caused the degradation of chlorophyll *a* from diatom *Cylindrotheca closterium*, while the cold soaking process rendered the chlorophyll *a* to be partially decomposed after 60 min. Similarly, the extraction efficiency of fucoxanthin from *Phaeodactylum tricornutum* was improved when the temperature of ethanol (50%) increased from 30^{o}C to 70^{o}C (Kim et al., 2012a). Nonetheless, an extremely high temperature condition of solvent extraction (e.g. above the boiling point of solvent) could cause the localized overheating effects on the fucoxanthin that render its degradation and low recovery.

Soxhlet extraction is a solid-liquid extraction approach that involves continuous mass transfer of non-volatile target compounds via reflux of organic solvents. Efficiency of extraction depends on the selectivity of solvents for the compounds, the diffusion rate of solvents, and the solubility of target compounds in the solvents (Kim et al., 2012a). Although Soxhlet extraction is a simple diffusion process without applying shear stress to the biomass, it is unsuitable for the extraction of temperature-sensitive carotenoids as their bioactivity will be degraded during the heating cycles (Kim et al., 2012a).

Although the maximum yield of product remains a priority, the selection of solvents should thoroughly consider other criteria such as environmental impact,

toxicity and sustainability of the selected solvent (**Table 2.3**). For instance, in the extraction of fucoxanthin from *Phaeodactylum tricornutum*, the extraction efficiency of methanol was higher than that of ethanol (Guler et al., 2020). However, by considering the toxicity of solvents, methanol is relatively hazardous to both environment and human use. Other alternative organic solvents such as petroleum ether and n-hexane are typically incompatible to the extraction of fucoxanthin because of their hydrophobic properties. Moreover, solvent extraction often suffers from the large consumption of organic solvent (Khoo et al., 2019a). Nonetheless, the feasibility of recycling solvents via distillation and evaporation under vacuum could mitigate the chemical consumption and waste generation. More importantly, the extracted fucoxanthin must be depleted of organic solvents used in the solvent extraction process if the final product is used as a functional ingredient in food or supplements

Table 2.3: Conventional extraction methods and fucoxanthin yield from microalgae and macroalgae

Extraction methods	Type of solvent	Species	Class	Temperature	Fucoxanthin yield	Reference(s)
Solvent extraction or maceration	Ethanol	*Phaeodactylum tricornutum*	Bacillariophyceae	30°C	15.71 mg/g	(Kim et al., 2012a)
	Ethanol	*Odontella aurita*	Bacillariophyceae	45°C	17.20 mg/g	(Xia et al., 2013)
Solvent extraction or maceration	Ethanol (96%)	*Sargassum muticum*	Phaeophyceae	40°C	0.55 mg/g	(Conde et al., 2015)
Solvent extraction or maceration	Methanol	*Chaetoceros calcitrans*	Bacillariophyceae	25°C	22.71%	(Foo et al., 2015)
Solvent extraction or maceration	Acetone	*Phaeodactylum tricornutum*	Bacillariophyceae	25°C	4.60 mg/g	(Kim et al., 2012a)
	Ethyl acetate	*Phaeodactylum tricornutum*	Bacillariophyceae	25°C	2.26 mg/g	(Kim et al., 2012a)

Table 2.3 (Continue)

Extraction methods	Type of solvent	Species	Class	Temperature	Fucoxanthin yield	Reference(s)
Solvent extraction or maceration	Dimethyl sulfoxide	*Laminaria japonica*	Phaeophyceae	25°C	122.10 µg/g	(Wang et al., 2005)
Solvent extraction or maceration	Acetone	*Fucus vesiculosus*	Phaeophyceae	30°C	0.70 mg/g	(Shannon and Abu-Ghannam, 2017)
Solvent extraction or maceration	Tetrahydrofuran	*Phaeodactylum tricornutum*	Bacillariophyceae	35°C	1.28 mg/g	(Guler et al., 2020)
Solvent extraction or maceration	Dichloromethane	*Phaeodactylum tricornutum*	Bacillariophyceae	35°C	1.28 mg/g	(Guler et al., 2020)
Solvent extraction or maceration	Methanol	*Phaeodactylum tricornutum*	Bacillariophyceae	35°C	0.57 mg/g	(Guler et al., 2020)
Solvent extraction or maceration	Acetone + methanol (1:1, v/v)	*Saccharina japonica*	Phaeophyceae	25°C	0.48 mg/g	(Sivagnanam et al., 2015)

Table 2.3 (Continue)

Extraction methods	Type of solvent	Species	Class	Temperature	Fucoxanthin yield	Reference(s)
Solvent extraction or maceration	Acetone + methanol (1:1, v/v)	*Sargassum horneri*	Phaeophyceae	25°C	0.71 mg/g	(Sivagnanam et al., 2015)
Solvent extraction or maceration	Hexane	*Saccharina japonica*	Phaeophyceae	25°C	0.16 mg/g	(Sivagnanam et al., 2015)
Solvent extraction or maceration	Hexane	*Sargassum horneri*	Phaeophyceae	25°C	0.05 mg/g	(Sivagnanam et al., 2015)
Solvent extraction or maceration	Ethanol	*Saccharina japonica*	Phaeophyceae	25°C	0.12 mg/g	(Sivagnanam et al., 2015)
Solvent extraction or maceration	Ethanol	*Sargassum horneri*	Phaeophyceae	25°C	0.08 mg/g	(Sivagnanam et al., 2015)
Solvent extraction or maceration	Cold acetone-methanol (7:3 v/v)	*Sargassum binderi*	Phaeophyceae	25°C	0.73 mg/g	(Noviendri et al., 2011)

Table 2.3 (Continue)

Extraction methods	Type of solvent	Species	Class	Temperature	Fucoxanthin yield	Reference(s)
Solvent extraction or maceration	Cold acetone-methanol (7:3 v/v)	*Sargassum duplicatum*	Phaeophyceae	25°C	1.01 mg/g	(Noviendri et al., 2011)
Solvent extraction or maceration	Acetone, 120 min	*Cylindrotheca closterium*	Bacillariophyceae	20°C	5.34 µg/mg	(Pasquet et al., 2011)
Solvent extraction or maceration	Acetone, 60 min	*Cylindrotheca closterium*	Bacillariophyceae	56°C	5.23 µg/mg	(Pasquet et al., 2011)
Soxhlet extraction	Ethanol (80%)	*Phaeodactylum tricornutum*	Bacillariophyceae	80°C	15.42 mg/g	(Kim et al., 2012a)
Soxhlet extraction	Ethanol	*Laminaria japonica*	Phaeophyceae	40°C	191 µg/g	(Kanazawa et al., 2008)
Soxhlet extraction	Ethanol	*Undaria pinnatifida*	Phaeophyceae	78°C	50 µg/g	(Kanda et al., 2014)

2.2.1.2 Alternative fucoxanthin extraction methods

The importance of Sustainable Development Goals (SDG) no. 12 (i.e. responsible consumption and production) has become more prominent as the world faces challenges in coping with pollution problems and food demand. The sustainability in food supply chain and the low-carbon footprint of the commercial food products should begin with the growth of sustainable crops as well as the greener extraction methods in processing of bioproducts. The emergence of alternative extraction methods has opened new avenue to the sustainable extraction of fucoxanthin from algal sources (**Table 2.4**).

2.2.1.2.1 Supercritical fluid extraction

Supercritical fluid extraction (SFE) utilizes carbon dioxide (CO_2) at high pressure and constant temperature to extract bioactive components from feedstock (Kanda et al., 2014). Supercritical fluids has a better transport performance than liquid because of its low viscosity and high diffusivity (Kadam et al., 2013). Moreover, the dissolving power of supercritical fluid is dependent on its density, which is regulated by temperature and pressure (Kadam et al., 2013). Physical appearance of the final product is typically in oily and concentrated forms. This extraction method is deemed to be a sustainable processing method as it aligns to SDGs via the usage of environmentally benign solvents (Ramsey et al., 2009). The utilization of CO_2 in its supercritical fluid state (SC-CO_2) as an extraction solvent reduces the reliance on organic solvents and minimizes the generation of hazardous waste during processing. The low viscosity of SC-CO_2 ensures a more efficient mass transfer for rapid penetration of solid matrices and extraction of compounds

(Ramsey et al., 2009). More importantly, the solvating strength and polarity of SC-CO_2 can be manipulated by controlling the density of SC-CO_2, which can be regulated by the temperature and pressure (Kadam et al., 2013). The low critical temperature of CO_2 (31^oC) allows the extraction of carotenoid at a relatively lower temperature as compared to other traditional extraction methods involving high temperature (Ramsey et al., 2009).

A SFE study performed by Kanda et al. (2014) showed that the extraction of fucoxanthin from *Undaria pinnatifida* by SC-CO_2 increased at least 16-fold in the presence of ethanol as an entrainer (3.23%). Similarly, the addition of entrainer (15% ethanol) was effective in the extraction of fucoxanthin from *Sargassum muticum* and the yield of fucoxanthin was improved marginally (Conde et al., 2015). Ethanol was commonly used as an entrainer in SC-CO_2 extraction to increase the polarity of CO_2; this effect is beneficial to the performance of fucoxanthin extraction. A recent work by Guler et al. (2020) showed that the yield of fucoxanthin extracted from *Phaeodactylum tricornutum* using SC-CO_2 was 0.69 mg/g, which was comparable to the yield of fucoxanthin (0.57 mg/g) obtained by the conventional solvent extraction with methanol.

Yet, there are limitations in SFE of fucoxanthin because the optimal extraction conditions are dependent on the characteristics of algae species and fucoxanthin. For example, the vapour pressure of fucoxanthin is an important factor influencing the extraction efficiency; the high vapour pressure of fucoxanthin at a higher temperature enhances its diffusion from the solid matrices (Quitain et al., 2013). In addition, the algae biomass subjected to SFE must undergo an energy-

intensive drying step because the water layers on wet biomass obstruct the penetration of SC-CO_2 (Derwenskus et al., 2019). Therefore, implementation of SFE for industrial application may face some challenges. For example, the requirement of pressurized gas and the expensive equipment may impose the greater operational and investment costs. Nonetheless, CO_2 could be easily recycled by separating the gas stream during the process depressurization. The application of entrainer or co-solvents can improve the extraction efficiency of SFE but an additional step of solvent separation from the extract is required (Quitain et al., 2013). Moreover, polar impurities such as pigments may be co-extracted because the entrainer tends to enhance the polarity of SC-CO_2.

2.2.1.2.2 Pressurised liquid extraction

Pressurised liquid extraction (PLE) is an extraction technique utilizing high temperature (50 –200^{o}C) and pressure (3.5 – 20 MPa) to improve solubility and diffusion rate of biomolecules from complex crude extracts to the solvent phase (Derwenskus et al., 2019, Kadam et al., 2013). In PLE, the high-pressure condition increases the fluid density and maintains the solvent in the liquid (subcritical) state above their boiling point, while the high-temperature condition accelerates the penetration of solvents by lowering the viscosity and surface tension of solvents (Gilbert-López et al., 2017). The major advantages of PLE over the direct solvent extraction and Soxhlet extraction techniques include the rapid extraction process and the lower consumption of solvent. Ethanol was commonly used in the extraction of fucoxanthin via PLE. Although the high-temperature condition of PLE enhances the solubility and diffusivity characteristics of compounds, it was not favourable for

the extraction of fucoxanthin from *Eisenia bicyclis* because the yield of fucoxanthin was only about 0.42 mg/g (Shang et al., 2011). The similar observation was also reported by Gilbert-López et al. (2017), who discovered that the yield of fucoxanthin dropped by 25% when the operating temperature of PLE increased from 50^{o}C to 170^{o}C. Fucoxanthin, which is a temperature-sensitive bioactive compound, can undergo oxidation process at high-temperature condition and result in the poor yield of extraction. Therefore, it is envisaged that an optimal operating temperature for the extraction of fucoxanthin is to be used to preserve the extracted fucoxanthin and subsequently its bioactivities. Furthermore, the safety of PLE operation must be considered because of the high pressure used. The operation period needs to be optimized for ensuring a sufficient contact time between fucoxanthin and solvents until the concentration gradient of fucoxanthin between solvent phase and plant matrix reached a balance. However, a prolonged period of PLE was not encouraged because fucoxanthin could undergo isomerization under the extreme physical conditions of PLE (Guler et al., 2020).

2.2.1.2.3 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) has proven useful in overcoming the bottlenecks of conventional solvent extraction process such as extraction duration and solvent consumption (Papadaki et al., 2017). This approach has been widely used for the extraction of various carotenoids and high-value bioactive compounds (e.g. lutein, astaxanthin, canthaxanthin, β-carotenes, docosahexaenoic acid, eicosapentaenoic acid) from complex feedstock (Goula et al., 2017, Dey and Rathod, 2013, Cravotto et al., 2008, Taghi Gharibzahedi et al., 2015, Sankaran et

al., 2018a, Chew et al., 2018). The ultrasound technology induces cavitation bubbles that collapse and produce heat energy along with ultrasonic wave (Chemat et al., 2017b). This generated mechanical shear forces are responsible for disrupting the cell wall of algae, thereby releasing the target compounds into the solvent phase. The advantage of UAE lies on the disruptive-extractive forces that facilitate the extraction of target compound from complex feedstock (e.g. algae with thick cell wall) in a single-step approach within a shorter period of extraction. Moreover, the mixing effect caused by the acoustic streaming enhances the contact between solvents and target compounds. In conjunction with SDGs, ultrasound technology has been a potential extraction method for the green and sustainable processing of bioactive compounds from natural resources (Tiwari, 2015).

The UAE can be achieved with either an ultrasound bath or an ultrasound probe. It is recommended to adopt probe instrument due to its effectiveness in cell disruption and energy efficiency. However, the drawback of the ultrasound probe is the overheating of the tip of ultrasound probe that could damage the heat-labile compounds. To overcome the overheating issue, the sample is usually chilled in an ice bath prior to the ultrasonic treatment. In general, UAE with an ultrasound probe gave a higher yield of fucoxanthin (15.96 mg/g), as compared to the ultrasound bath yielding only 0.75 – 0.97 mg/g of extracted fucoxanthin (Kim et al., 2012a, Papadaki et al., 2017, Raguraman et al., 2018). Till date, there is still insufficient literature reporting the application of UAE of fucoxanthin from diatom species.

2.2.1.2.4 Microwave-assisted extraction

Microwave-assisted extraction (MAE) is a rapid and efficient extraction process for the recovery of bioactive compounds. The heating of sample by microwave can be typically done in less than a minute, and the homogenous heating of the sample by microwave irradiation ensures no hot spots or limitation in heat transfer (Pasquet et al., 2011). Microwave irradiation induces heat energy through molecular interaction between solid and liquid (Chew et al., 2019b). The heating effect generated from the incident electromagnetic waves promotes the rapid dissolution of photosynthetic membranes by a selective heating of the more polar part of cellulose (Banik et al., 2003). This heating effect is useful for releasing fucoxanthin from the fucoxanthin-chl *a*/*c*-protein complexes (Xia et al., 2013, Halim et al., 2012). Besides, the microwave facilitates an efficient release of intracellular bioactive compounds by improving the penetration of solvent into the matrix (Kadam et al., 2013). In aligning to the principles of green chemistry, microwave technology is favoured for a green and clean process of extraction without the need for high-pressure condition.

Ethanol has been commonly chosen as the solvent for MAE of fucoxanthin. This bio-based solvent favourably interacts with membrane-related lipid complexes. MAE of fucoxanthin in ethanol has been applied to macroalgae such as *Laminaria japonica, Undaria pinnatifida* and *Sargassum fusiforme* under the operating conditions of 300 W, 60^{o}C and 10 min. Among the tested macroalgae strains, the yield of fucoxanthin obtained from brown seaweed *Sargassum fusiforme* was the least, which might be due to the rigidity of the cellular wall structure of algae. On

the other hand, there are two separate studies demonstrating the rapid extraction of fucoxanthin from microalgae *Phaeodactylum tricornutum* via MAE in ethanol, which could be completed within 1 – 2 min (del Pilar Sánchez-Camargo et al., 2017, Zhang et al., 2018). The higher yield of fucoxanthin extracted from microalgae was attributed to the fact that cell wall of microalgae is less recalcitrant than that of macroalgae. To date, the scaling-up of MAE operation remains a challenge and the operating parameters such as temperature and duration of treatment must be optimized systematically. Preferably, the MAE of carotenoid should not exceed 60^{o}C (Pasquet et al., 2011). The pulsed microwave processing or the continuous interval microwave processing with a short period of treatment time could circumvent the over-heating of sample, which effectively reduces the rate of fucoxanthin degradation during the MAE process.

2.2.1.2.5 Enzymatic-assisted extraction

Enzymatic-assisted extraction (EAE) involves the use of hydrolytic enzymes such as pectinase and cellulase to hydrolyse algal cell walls. Algal cell walls mainly consist of cellulose (Domozych, 2001). The enzymatic treatment of algae is effective in hydrolysing the cell wall to release the intracellular components into the extraction medium. Furthermore, EAE can be considered as a relatively low-cost technology if common food-grade enzymes including amylase, cellulose, pectinase or β-galactosidase are used. In comparison to other alternative extraction techniques, EAE does not depend on energy-intensive equipment and it can be applied for large-scale extraction of algal bioactive compounds. However, EAE can be inadequate for large-scale application due to the main drawbacks such as long enzymatic process,

low selectivity, and poor yield. Apart from the duration of enzymatic treatment, the temperature condition of EAE must be optimized to maximize the extraction yield.

A recent work by Shannon and Abu-Ghannam (2018) demonstrated the applicability of EAE of fucoxanthin in brown seaweeds pre-treated at low temperature followed by drying and mechanical blending. A commercial enzyme cocktail, Viscozyme, was found to be effective not only in hydrolyzing the cellulose in cell wall of seaweeds but also in reducing the viscosity of mixture. However, the efficiency of EAE was dependent on the physical texture and the target part (e.g. blade, stipe or holdfast) of seaweed. EAE could also be used as a pre-treatment step to improve the yield of fucoxanthin obtained from solvent extraction (dimethyl ether and ethanol); a 9.3% increase in fucoxanthin yield was obtained from *Undaria pinnatifida* biomass that was pre-treated by alginate lyase (Billakanti et al., 2013). EAE of fucoxanthin from microalgae was feasible, although the yield was lower as compared to that from the conventional extraction approaches.

Table 2.4: Emerging methods for extraction of fucoxanthin from macro- and microalgae.

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Supercritical CO_2	SC-CO_2	70ºC, 40 MPa, 3 hr	*Undaria pinnatifida* (seaweed)	59.51 μg/g	(Kanda et al., 2014)
Supercritical CO_2	Entrainer (3.23%)	60ºC, 40 MPa, 3 hr	*Undaria pinnatifida* (seaweed)	994.53 μg/g	(Kanda et al., 2014)
Supercritical CO_2	SC-CO_2	40ºC, 10 MPa, 1 hr	*Sargassum muticum* (seaweed)	1.50 mg/100g	(Conde et al., 2015)
Supercritical CO_2	Ethanol (15%)	40ºC, 10 MPa, 40 min	*Sargassum muticum* (seaweed)	135.00 mg/100g	(Conde et al., 2015)

Table 2.4 (Continue)

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Supercritical CO_2	SC-CO_2	40ºC, 40MPa, 3 hr	*Undaria pinnatifida* (seaweed)	1.22 ± 0.04 g/100g	(Quitain et al., 2013)
Supercritical CO_2	SC-CO_2	60ºC ,40MPa ,150 min	*Undaria pinnatifida* (seaweed)	58.00 μg/g	(Goto et al., 2015)
Supercritical CO_2	SC-CO_2	40ºC, 30MPa, 3 hr	Saccharina japonica (seaweed)	2.08 ± 0.06 mg/g	(Getachew et al., 2018)
Supercritical CO_2	Methanol	35ºC ,20 MPa, 1 hr	*Phaeodactylum tricornutum* (diatom)	0.69 mg/g	(Guler et al., 2019)
Pressurised liquid extraction (PLE)	Ethanol:water	(9:1), 110ºC, 10.3MPa	*Eisenia bicyclis* (brown algae)	0.42 mg/g	(Shang et al., 2011)

Table 2.4 (Continue)

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Pressurised liquid extraction (PLE)	Ethanol	100ºC,10.3MPa	*Phaeodactylum tricornutum* (diatom)	16.51 mg/g	(Kim et al., 2012a)
Pressurised liquid extraction (PLE)	Dimethyl ether	25ºC, 10.3MPa	*Undaria pinnatifida* (seaweed)	390.00 µg/g	(Kanda et al., 2014)
Pressurised liquid extraction (PLE)	Ethanol:water	(9:1), 110ºC, 10.3MPa	*Eisenia bicyclis* (brown algae)	0.42 mg/g	(Shang et al., 2011)
Pressurised liquid extraction (PLE)	Ethanol	100ºC, 10.3MPa	*Phaeodactylum tricornutum* (diatom)	7.73 mg/g	(Gilbert-López et al., 2017)
Pressurised liquid extraction (PLE)	Ethanol	170ºC, 10.3MPa	*Phaeodactylum tricornutum* (diatom)	5.81 mg/g	(Gilbert-López et al., 2017)

Table 2.4 (Continue)

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Pressurised liquid extraction (PLE)	Ethanol	100ºC, 10.3MPa	*Phaeodactylum tricornutum* (diatom)	26.1 ± 2.0 mg/g	(Derwenskus et al., 2019)
Ultrasound-assisted	Ethanol	25ºC, 70 KHz, 30 min	*Phaeodactylum tricornutum* (diatom)	15.96 mg/g	(Kim et al., 2012a)
	Acetone	10 W, 10 min, cold soaking process.	*Cylindrotheca Closterium* (diatom)	4.49 ± 0.08 μg mg	(Pasquet et al., 2011)
Ultrasound-assisted	Coconut oil	450 W, 25 KHz, 15 min (bath)	*Phaeodactylum tricornutum* (diatom)	0.97 mg/mL	(Papadaki et al., 2017)

Table 2.4 (Continue)

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Ultrasound-assisted	80% of ethanol	230 W, 50 Hz, 30 min (bath)	*Padina tetrastromatica* (seaweed)	0.75 mg/g	(Raguraman et al., 2018)
Microwave-assisted	Acetone	50 W, 5 min	*Cylindrotheca closterium* (diatom)	4.24 ± 0.09 μg/mg	(Pasquet et al., 2011)
Microwave-assisted	50% of ethanol	300 W, 60ºC,10 min	Laminaria japonica (seaweed)	5.13 mg/100 g	(Xiao et al., 2012)
Microwave-assisted	50% of ethanol	300 W, 60ºC,10 min	*Undaria pinnatifida* (seaweed)	109.30 mg/100 g	(Xiao et al., 2012)
Microwave-assisted	50% of ethanol	300 W, 60ºC,10 min	Sargassum fusiforme (seaweed)	2.12 mg/100 g	(Xiao et al., 2012)
Microwave-assisted	50% of ethanol	300 W, 60ºC, 5 mins	*Undaria pinnatifida* (seaweed)	0.73 mg/g	(Xiao et al., 2012)

Table 2.4 (Continue)

Novel extraction techniques	Extractive solvents	Operating condition	Algae strains	Extracted fucoxanthin	References
Microwave-assisted	Ethanol	850 W, 2455 MHz, 2 min	*Phaeodactylum tricornutum* (diatom)	4.59 mg/g	(Gilbert-López et al., 2017)
Microwave-assisted	Ethanol	700 W, 2450 MHz, 1 min	*Phaeodactylum tricornutum* (diatom)	58.07 ± 0.73 %	(Zhang et al., 2018)
Enzymatic-assisted	Sodium buffer, Cellulase:pectinase	50°C, 80 min	Laminaria japonica (seaweed)	18.30 mg/100 g	(Qin et al., 2013)
Enzymatic-assisted	Sodium acetate buffer (0.1M), 100 fungal β-glucanase units /g	50°C, 100 rpm, 12 hr	*Fucus vesiculosus* (seaweed)	0.657 mg/g	(Shannon and Abu-Ghannam, 2018)

2.3 Liquid Biphasic System

Liquid biphasic system (LBS) or commonly known as aqueous two-phase system (ATPS) has been long introduced for the separation, recovery and purification of biomolecules, and it is the current research trend adopted in the separation and purification technology. It was started back in 1896 when Martinus Willem Beijerinck accidentally mixed an aqueous starch solution with gelatin and found that an immersible layer was formed between both the aqueous solutions (Grilo et al., 2016, van Berlo et al., 1998). This idea of LBS as an analytical separation technique was sparked by Per-Åke Albertsson in 1960s who discovered the phenomenon by mixing two different polymers (e.g., polyethylene glycol and dextran) resulting in an aqueous medium containing two separable phases (Albertsson, 1961, van Berlo et al., 1998). This application was then extended to several generations of scientists and engineers who have been working in the industrial biotechnology field. **Figure 2.2** shows a schematic diagram of the principles of LBS.

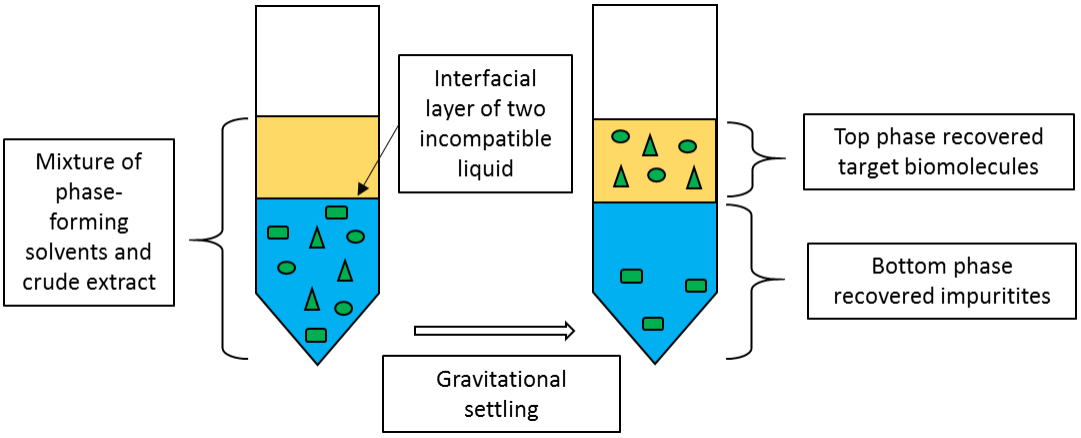


Figure 2.2: Schematic diagram of the principle in the liquid biphasic system

LBS is well-known for the extraction of different biotechnological materials such as proteins, lipids and carotenoids (Asenjo and Andrews, 2012, Khoo et al., 2019b, Ooi et al., 2009). The specialty of LBS compared to traditional organic solvent extraction technique is the composition of the phase-forming components which contains large amount of water while maintaining a low interfacial layer that separates both phases. It can be either used to separate proteins from cellular debris or to purify targeted proteins from contaminated proteins. Likewise, LBS has the capability of directing the target biomolecules by partitioning them to the top phase for extraction (Zhao et al., 2014). Conventional polymer-based LBS which possess a low ionic system is generally used for the separation and purification of biomolecules which are sensitive toward ionic condition (Albertsson, 1961). Nevertheless, polymer-based LBS was neglected due to lack of compatibility between high ionic strength biomolecules, expensive phase-forming components and its high viscosity system. Further development in LBS using different phase-

forming components such as alcohol-, ionic liquids-, deep-eutectic solvent- and surfactant-based was utilized to replace the conventional polymer-based LBS.

The selective partitioning of the LBS allows the extraction of biomolecules to be operated in a single-step process compared to traditional extraction techniques which require multiple operation steps. LBS possess an environmental-friendly, inexpensive, ease of scaling-up, rapid and efficient techniques for recovery and purification of biomolecules. During the planning stage, it is crucial to understand the complexity of the physical and chemical interaction reaction throughout the partitioning process in the LBS (Rosa et al., 2010). The selection of various parameters which are compatible to the system properties are important to achieve an optimal extraction, recovery and purification condition. It is also important to evaluate the interactions during the selection of various parameters (e.g., salt precipitation, crystallization and absence of biphasic system) as it may affect the findings. Lastly, is to assess the effect of each process parameters on the product recovery and purity (Rosa et al., 2010).

Fundamental principles for the formation of LBS requires a phase diagram or also called the binodal curve where these provide a set of information regarding the two-phase formation and their required concentration in the top and bottom phases (Raja et al., 2011). A detailed study has been evaluated previously by Iqbal et al. (2016) on the tie line length (TLL) and slope tie line (STL) for the construction of phase diagrams (Iqbal et al., 2016). Binodal curves can be constructed using three methods namely, turbidometric titration, cloud point and node determination method for predetermined phase diagram (Hatti-Kaul, 2000, Raja and Murty, 2012,

Raja et al., 2011). Moreover, the partition coefficient (K) LBS is to evaluate the equilibrium relationship between the top and bottom phase in the LBS. However, there is still lack of studies reporting on the theory and chemistry of these phase forming mixtures in the LBS which is a gap to-be-filled. Apart from that, factors that affect the partition coefficient can be manipulated using electrical, hydrophobicity-phase forming components, bio-specific affinity, molecular size and surface area to understand the physicochemical properties of the partitioning mechanism in the LBS.

2.2.1 Phase component of liquid biphasic system

2.2.1.1 Polymer-based liquid biphasic system

The conventional polymer-based LBS is typically made up of two polymers (e.g., polyethylene glycol (PEG) and dextran) and PEG-salt combinations (e.g., phosphate-, sulphate- and citrate-based) as the phase-forming components. The purpose of using polymer-based LBS is that the chemical composition of a non-ionic characteristics toward an ionic environment is compatible towards biomolecules having low ionic strength (Albertsson, 1961). Aside from that, the phase forming component from polymer-based has the ability to be recycled and reused for subsequent extraction process and this reduces the cost of polymers phase-forming component (Johansson et al., 2011). Polymer-based LBS are commonly used for protein extraction due to its poor hydrophilic and hydrophobic interaction in polymer/salt-based LBS (Chew et al., 2019a). However, it is important to maintain concentration of salt solution as high salt concentration may denature and damage the fragile protein in the system.

In most work, conventional polymer-based LBS has been replaced by using thermo-separating polymers as the phase-forming component to overwhelm the limitation of polymer-based LBS such as high viscosity and difficulties in recycling process (Hou et al., 2014, Tan et al., 2017). Thermo-separating polymers are random, di-block and tri-block co-polymers of ethylene oxide (EO) and propylene oxide (PO) (Leong et al., 2016). Thermo-separating polymers have a low cloud point temperature (≤ 47 °C) which is suitable to achieve temperature-induced phase separation where a target protein can be recovered from the polymer (Show et al., 2012). Generally, a back-extraction process such as ultrafiltration, diafiltration and crystallization is needed to separate the target protein from the polymer. However, an in-depth understanding on the mechanism by the polymer phase-forming component for the recovery of biomolecules is still poorly understood. This shows a gap for future researchers to further explore the fundamental principles of this LBS extraction technique.

Several studies have been conducted involving cyclodextrin glycosyltransferase (CGTase) from Bacillus cereus. Ng et al. (2012) reported that the TLL of 41.2 % (w/w), volume ratio (VR) of 1.25, pH 7 and crude loading (w/w) of 20 % were the optimal conditions to recover cyclodextrins using polymer-based LBS with ethylene oxide–propylene oxide (EOPO) 3900 and two phosphate salts (Ng et al., 2012). This experiment showed that the highest CGTase was purified up to 13.1-fold with a yield of 87 % recovered in the EOPO-rich top phase. However, this experiment did not discuss the time period in cyclodextrins recovery. Another research carried out by Lin et al. (2015) with modified method using flotation

technique and the combination of PEG 8000 and potassium phosphate salt. The optimum conditions in cyclodextrins (CDs) recovery was optimized at 18 % (w/w) PEG 8000 and 7.0 % (w/w) potassium phosphate with TLL of 27.2 % (w/w), VR of 3.0, pH 7 and crude loading (w/w) of 20 % (Lin et al., 2015). The experiment showed that the recovery of CDs was affected by alternating each of the parameters such as TLL, VR, and pH where the purification factor (PFT) which corresponded to the highest CGTase purity up to 21.8 with a yield of 97.1 % was recovered in the PEG-rich top phase within a short period (Lin et al., 2015).

A similar approach utilizing polymer-based LBS was employed for the recovery of lignin peroxidase from Amauroderma rugosum (Blume & T. Nees) (Jong et al., 2017). However, this experiment used a lower molecular weight (PEG 600) for a high purification of lignin peroxidase. Generally, this approach showed that a higher molecular weight polymer reduces the purification factor of lignin peroxidase due to the interaction of PEG and hydrophobic enzyme. An optimal condition in lignin peroxidase recovery was optimized at 15 % (w/w) PEG 600 and 16 % (w/w) dipotassium phosphate with highest purification factor of 1.33 ± 0.62 and recovery yield of 72.18 ± 8.50 %.

2.2.1.2 Organic solvent-based system

Organic solvent-based LBS consists of various water-miscible alcohols (e.g., methanol, ethanol, 1-propanol and 2-propanol) and inorganic salts. This form of LBS has been utilized to overcome the limitation of polymer-based LBS to improve the recovery of biomolecules from the phase-forming component (Khoo et al., 2019a). The use of alcohol as the phase-forming components can easily recover

the biomolecules by evaporating the alcohol from the top phase. A recent study also showed a greener approach using food grade alcohol such as ethanol and 2-propanol compared to the conventional polymer-based LBS for the extraction and recovery of carotenoids from microalgae (Khoo et al., 2019a). Additionally, the phase-forming component can reduce the cost of the process by recycling and reusing the alcohol using rotary evaporator for the next extraction process. Despite its advantages, the drawbacks of using alcohol especially methanol as the phase-forming component is the toxicity and hazardous effects towards the environment.

Ooi et al. (2009) reported a study on purification of lipase from *Burkholderia pseudomallei* using alcohol/salt-based LBS (Ooi et al., 2009). The best lipase recovery was achieved in LBS composed of 16 % (w/w) of 2-propanol, 16 % (w/w) of potassium phosphate and 4.5 % (w/v) sodium chloride with a purification factor of 13.5 along with the yield of 99 %. The presence of alcohol component in LBS also did not inhibit the enzymatic activity of purified lipase. The effect of NaCl on lipase partitioning was found to generate an electrical potential difference in the LBS (Jong et al., 2017). An increase in the salt concentration could generate an electrostatic potential that strongly expelled the negatively charge biomolecules toward the water-miscible alcohol in top phase, thus resulting in a high recovery yield.

Lin et al. (2013) conducted a study using alcohol/salt-based LBS to recover the intracellular human recombinant interferon-α2b (IFN-α2b) from *Escherichia coli* (Lin et al., 2013). A different variety of combinations between alcohol-based top phase (ethanol, 1-propanol and 2-propanol) and salt phase (ammonium sulphate,

dipotassium hydrogen phosphate and monosodium citrate) were conducted. LBS composed of 18 % (w/w) of propanol and 22 % (w/w) ammonium sulphate in 1 % (w/w) sodium chloride was reported to be the optimal conditions for the purification of IFN-α2b achieving a purification factor of 16.2 with the yield of 74.6 %. Ammonium sulphate salt was selected due to its high level of pH in the system which provided a high purification factor of IFN recovery. As the pH environment in LBS increased, the contaminant protein and IFN protein were partitioned toward water-miscible alcohol top phase. This is mainly due to the negatively charge protein which tends to partition to the top phase and repels from the salt-rich bottom phase (Lin et al., 2013).

A recent study conducted on a recyclability test utilizing 1-propanol and ammonium sulphate system for the phlorotannin recovery from *Padina australis* and *Sargassum binderi* (Lin et al., 2013). The highest recovery of phlorotannin were 76.1 % and 91.67 % with purification factor of 2.49 and 1.59 from *Padina australis* and *Sargassum binderi*, respectively. A consistent recovery of phlorotannin was obtained after conducting two cycles of the system. This showed a feasible and eco-friendly approach of utilizing the alcohol-based LBS for biomolecules extraction.

2.2.1.3 Ionic liquid-based liquid biphasic system

A new trend of research by using ionic liquids (ILs) have been an alternative organic compound and non-volatile green solvent in the downstream processes. Their remarkable properties such as negligible vapor pressure, low melting point and high thermal stability have received numerous attention from researchers (Lee et al., 2015, Smiglak et al., 2007). ILs are composed with tuneable physico-chemical

properties of cationic and anionic ions (Zhang et al., 2006). The cationic part of ILs usually consists of choline cation, ammonium cation, quaternary ammonium or phosphonium and guanidium cation. As for the anionic part, it consists of environmentally friendly sources such as carboxylic acid, amino acid and biological buffers. Thus, replacing ILs as the phase-forming component in LBS would be beneficial for the extraction and purification of specific target biomolecules from complex crude extract (Lee et al., 2017a). Additionally, ILs has also been employed for various applications such as electrolytes (e.g., fuel cells, batteries, and sensors), CO_2 capture, lubricants, and fuel additives. The cost of reactant for the synthesis of ILs are expensive. Therefore, it is important for ILs to be recycle- and reuse-able to ensure that ILs-based LBS are more feasible and applicable in the bioprocessing industries for the next extraction processes. A review by Ostadjoo et al. (2017) revealed the green and environmentally friendly, 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) for its potential features in the field of lignocellulose biomass dissolution and biopolymer processing (Ostadjoo et al., 2018, Sun et al., 2009, Swatloski et al., 2002). Yet, there is still insufficient studies related to their toxicity and eco-friendliness on scaling up these ILs, especially imidazole- and pyridinium-based ILs. Here we recommended that these ILs need to be further fabricated by replacing environmentally friendly anionic part such as carboxylic acid, amino acid and biological buffers in order to minimize their toxicity in various application.

Gutowski et al. (2003) reported that by mixing imidazole-based ILs and a kosmotropic salt (i.e., K_3PO_4) would lead to the formation of a biphasic system

(Gutowski et al., 2003). This research had gained interest investigating the phase separation behaviour of IL-based LBS. The study on protein extraction using IL-based LBS in a single step was conducted by Du et al. (2007). The researchers had successfully extracted the protein from human urine into the IL-rich top phase with a distribution of 10 and enrichment factor of 5 (Du et al., 2007). Apart from that, Ng et al. (2014) investigated the purification of CGTase from Bacillus cereus fermentation broth in IL/salt LBS, composing of 35 % (w/w) of (Emim)BF_4 and 18 % (w/w) of sodium carbonate with the addition of 3% (w/w) of NaCl (Ng et al., 2014). The optimized operating conditions showed that the IL-based LBS was a promising approach for the purification and recovery of CGTase in a single step operation attaining a high purification factor of 13.86 and yield of 96.23 %. Ng et al. (2014) also reported that it was crucial in the selection of salt such as citrate and carbonate ions as they played an important role in LBS formation and was able to attract water molecules toward them by forming strong intermolecular interaction (Ng et al., 2014).

Chang et al. (2018) used a series of alkyl bromide imidazole for the extraction of C-phycocyanin (CPC) from Spirulina platensis and found that the longer the alkyl chain, C_8MIM-Br enhanced the extraction efficiency of CPC (Chang et al., 2018). The results indicated that by using C_8MIM-Br/salt LBS the maximum extraction efficiency, partition coefficient and separation factor of CPC were 99.0 %, 36.6 and 5.8, respectively. ILs-based LBS demonstrated an efficient and feasible separation technique for the extraction of various biomolecules from complex crude extract. This was supported by a recent study that evaluated the

protein partitioning in ILs-based LBS composed of Iolilyte 221 PG and citrate salts was found to be feasible but complex depending on various factors such as concentration of phase-forming component, pH, temperature, ionic strength and chemical nature of the target biomolecules (Garcia et al., 2018). Proteins are negatively charged particles therefore it favours a system pH (≥ 6.50) higher than the isoelectric point of protein. Moreover, the partition coefficient for tie-line length within 38 – 76 % were reference points for specific protein (e.g., bovine serum albumin and rubisco) to be partitioned at the top phase.

2.2.1.4 Deep-eutectic-solvent-based liquid biphasic system

Deep-eutectic-solvents (DESs) are defined as subclass from ILs because of their similarity in physical and chemical properties of ILs (Abbott et al., 2004). The behaviour exhibited from DESs are contributed from hydrogen bonding, whereas ILs are dominated by ionic interactions (Shishov et al., 2017). DESs are more environmentally friendly as compared to ILs (e.g., imidazole- and pyridinium-based ILs) which are toxic and non-biodegradable. The synthesis of DESs is by combining hydrogen bond acceptors (e.g., quaternary ammonium and phosphonium salts) and hydrogen bond donors (e.g., alcohols, carboxylic acid and amide). A major advantage from DESs are their charge delocalization properties which are responsible for the decrease in melting point of mixture relative to the raw material (Paiva et al., 2014). The bottleneck from using ILs such as high cost and complex synthesis route have been solved by these DESs. By having the similar characteristic as ILs and exhibiting some distinguishing features, including ease of synthesis, low

cost and valuable for industrial application, DESs have gained interest in many fields especially in LBS (Dai et al., 2013).

Choline chloride (ChCl) is a convention quaternary salt used to synthesize DESs. ChCl-based DESs have the same advantages with ILs besides showing excellent biodegradability and low toxicity (Pang et al., 2017). Zeng et al. (2014) had performed the extraction of bovine serum albumin (BSA) using four different kind of DESs, namely, choline chloride (ChCl)-urea, tetramethylammonium chloride (TMACl)-urea, tetrapropylammonium bromide (TPMBr)-urea and ChCl-methylurea (Zeng et al., 2014). The extraction efficiency of BSA under the optimum LBS conditions composed of 0.7 g mL^{-1} ChCl-urea and 2.0 mL dipotassium phosphate, K_2HPO_4 could reach up to 100.5 % that collectively highlighted the advantages of the DES-based LBS for the extraction of protein. Unfortunately, this work was unable to back-extract the target protein free from the DES-LBS because of the hydrophilicity characteristic of DES in the aqueous solution.

A similar work with different DESs was investigated by Pang et al. (2017) using DES-based LBS which composed of choline chloride-polyethylene glycol (ChCl-PEG or DES) and sodium carbonate were applied for the extraction of specific protein (i.e., BSA and papain) (Pang et al., 2017). ChCl-based DES was prepared by mixing two compounds, 0.68 g mL^{-1} ChCl and 0.1 g mL^{-1} PEG 2000 at the molar ratio of 20:1, stirring up to 100 °C until a homogenous colourless liquid was formed. The result showed that the DES-$NasCO_3$ LBS under the optimum condition had successfully obtained a high extraction efficiency of BSA (95.16 %) and papain (90.95 %). Moreover, the back-extraction of target protein was

performed by extracting 1 mL DES top phase followed by the addition of ammonium sulfate $(NH_4)_2SO_4$ and 0.45 mL ethanol to form a new LBS. However, it was found that by increasing the concentration in the salt-rich bottom concentration would lower the efficiency of the back extraction.

A modified DES-based LBS using ultrasonic-assisted were employed for the extraction of ursolic acid from *Cynomorium songaricum* Rupr (Zhang et al., 2019). This approach was compared to the convention ultrasonic-extraction method. The recovery yield of ursolic acid was comparable. However, the presence of LBS promotes a higher purification of ursolic acid. The recovery yield of ursolic acid was 22.10 ± 0.44 mg/g with purification factor of 42.41 ± 0.84 % as compared to conventional ultrasonic-extraction method where the recovery yield was only 20.9 ± 0.79 mg/g with a low purification factor of 20.17 ± 0.77 %.

2.2.1.5 Surfactant/detergent-based liquid biphasic system

Surfactant-based LBS is the transformation of phase-forming component from conventional polymer-based LBS. The surfactant-based LBS is formed when both cationic and anionic surfactants are separated into two immiscible liquid phases which consist of a high concentration than critical micelle concentration (CMC) and at certain molar ratio of cationic and anionic surfactant composition. This novel approach of surfactant-LBS has gained interest mainly due to the combination phase which exist in many different forms (i.e., spherical micelles, rod-like micelles or vesicles) by simply alternating different composition and concentration of surfactants (Weschayanwiwat et al., 2008). The principle of surfactant-based LBS used the cloud point extraction (CPE) system in which the non-ionic surfactant is

heated above the cloud point temperature, causing dehydration of detergent for the phenomenon of phase separation to occur (Selber et al., 2004). The surfactant-LBS consists of one surfactant-rich phase and the other is the surfactant-dilute phase. The organic contaminant will partition into the surfactant-rich phase and will then aggregate and concentrate at that phase. The presence of small amount of remediated water in the contaminant will remain in the surfactant-dilute phase. Surfactant-based LBS is commonly used to separate hydrophobic and amphiphilic molecules by solubilisation and partitioning of membrane-bound substances.

Surfactant-based LBS composed of 24 % (w/w) Triton X-100 and 20 % (w/w) xylitol was used for the purification of lipase from pumpkin seeds (Amid et al., 2015). The results showed that the surfactant-based LBS had the ability to partition the lipase into the top surfactant-rich phase and leave the impurities at the bottom xylitol-rich phase. The proposed optimized method had successfully recovered the enzyme with purification factor of 16.4 and yield of 97 %. This study also demonstrated that the recovery phase component could be recycled up to five runs with a high percentage of recovery of 97 %. However, it was noted that there was a significant decrease in recovery of the phase component after the fifth cycle in which could be mainly due to the accumulation of impurities present in the phase component.

An example of surfactant-based LBS extraction was conducted by Sankaran et al. (2018) using surfactant and xylitol under the optimum operation condition of 25 % w/w of xylitol concentration, 15 % (w/w) Triton X-100, 80 % w/w of crude lipase, 4 mL of top phase, 35 mL of bottom phase, pH 7 and 15 min of flotation time

showed the maximum lipase extraction and efficiency of 3.63 and 86.46 % (Sankaran et al., 2018d). In addition, the recyclability of both components in surfactant-LBS extraction makes this an excellent process, as this innovative method was practical and feasible to be applied in the biotechnology industry for extraction of other biomolecules. **Table 2.5** summarizes the extraction of biomolecules using various types of phase-forming component in LBS.

Table 2.5: Extraction of biomolecules using various types of phase-forming component in liquid biphasic system.

Type of LBS	**Composition of LBS**	**Type of feedstock**	**Biomolecule**	**Selectivity**	**Partition coefficient, K**	**Purification factor, P_{FT}**	**Recovery yield (%)**	**Reference**
Polymer/salt-based	EOPO 3900 and two phosphate salts	*Bacillus cereus cyclodextrin glycosyltransferase*	Cyclodextringlycosyltransferase (CGTase)	3.19	17.54	5.30	87.0	(Ng et al., 2012)
Polymer/salt-based	18 % (w/w) PEG 8000 and 7.0 % (w/w) potassium phosphate salts	*Bacillus cereus cyclodextrin glycosyltransferase*	Cyclodextringlycosyltransferase (CGTase)	-	-	21.8	97.1	(Lin et al., 2015)
Polymer/salt-based	15 % (w/w) PEG 600 and 16 % (w/w) dipotassium phosphate	*Amauroderma rugosum*	Lignin peroxidase	-	-	1.33 ± 0.62	2.18 ± 8.50	(Jong et al., 2017)

Table 2.5 (Continue)

Type of LBS	Composition of LBS	Type of feedstock	Biomolecule	Selectivity	Partition coefficient, K	Purification factor, P_{FT}	Recovery yield (%)	Reference
Alcohol-based	18 % (w/w) 2-propanol and 22 % (w/w) ammonium sulphate, $(NH_4)_2SO_4$	*Escherichia coli*	Interferon (IFN)/ Glycoproteins	-	0.82	16.24	74.64	(Lin et al., 2013)
Alcohol-based	16 % (w/w) 2-propanol and 16 % (w/w) potassium phosphate	*Burkholderia pseudomallei*	Lipase	287.5	-	13.5	99.3	(Ooi et al., 2009)
Alcohol-based	33.5 % (w/w) of 2-propanol and 10 % (w/w) ammonium sulphate	*Padina australis*	Phlorotannin	-	-	2.49	76.1	(Chia et al., 2018)

Table 2.5 (Continue)

Type of LBS	Composition of LBS	Type of feedstock	Biomolecule	Selectivity	Partition coefficient, K	Purification factor, P_{FT}	Recovery yield (%)	Reference
Alcohol-based	25 % (w/w) of 2-propanol and 12.5 % (w/w) ammonium sulphate	*Sargassum binderi*	Phlorotannin	-	-	1.59	91.67	(Chia et al., 2018)
Ionic-liquid based	35 % (w/w) of (Emim)BF_4 and 18 % (w/w) of sodium carbonate Na_2CO_3	Fermentation broth	*Bacillus cereus* cyclodextrin glycosyltransferase (CGTase)	9.66	-	51.0	96.00	(Ng et al., 2014)
Ionic-liquid based	C_8MIM-Br and tri-potassium phosphate	*Spirulina platensis*	C-phycocyanin (CPC)	5.8	36.6	-	99.00	(Chang et al., 2018)

Table 2.5 (Continue)

Type of LBS	Composition of LBS	Type of feedstock	Biomolecule	Selectivity	Partition coefficient, K	Purification factor, P_{FT}	Recovery yield (%)	Reference
Deep-eutectic solvent based	0.7 g mL^{-1} ChCl-urea and 2.0 mL dipotassium phosphate, K_2HPO_4	Protein	Bovine serum albumin (BSA)	-	-	-	99.6 99.7 and 100.0 BSA	(Zeng et al., 2014)
Deep-eutectic solvent based	Choline chloride and PEG 2000, molar ratio of 20:1	Protein	Bovine serum albumin and papain	-	-	-	Bovine serum albumin (95.16), papain (90.95)	(Pang et al., 2017)
Deep-eutectic solvent based	36 % (w/w) ChCl-glucose and 25 % (w/w) dipotassium phosphate, K_2HPO_4	Ursolic acid	*Cynomorium songaricum* Rupr.	-	-	42.41 ± 0.84	22.10 ± 0.44 mg/g	(Zeng et al., 2014)

Table 2.5 (Continue)

Type of LBS	Composition of LBS	Type of feedstock	Biomolecule	Selectivity	Partition coefficient, K	Purification factor, P_{FT}	Recovery yield (%)	Reference
Surfactant/detergent based	24 % (w/w) Triton X-100 and 20 % (w/w) xylitol	*Cucurbita moschata*	Lipase	-	-	16.4	97.0	(Amid et al., 2015)
Surfactant/detergent based	25 % w/w of xylitol concentration, 15 % (w/w) Triton X-100	*Burkholderia cepacia*	Lipase	2.62	-	2.56	86.46	(Sankaran et al., 2018d)

2.2.2 Advance technologies integrated with liquid biphasic system

2.2.2.1 Bubble-assisted liquid biphasic system

Bubble-assisted LBS or known as liquid biphasic flotation (LBF) is the combination of LBS and solvent sublation (SS), in which the biphasic medium composed of organic solvent and aqueous salt solution is aerated by air bubbles (e.g., nitrogen and oxygen) in promoting the adsorption of target biomolecules during the separation process (Leong et al., 2018). SS is an adsorptive bubble separation technique introduced by Sebba who suggested that the use of an immiscible thin organic solvent layer overlaid on top of the liquid bulk as a modification of ion flotation (Sebba, 1962). LBF has accommodated the ease for extraction of high value biomolecules such as protein, lipase, astaxanthin and betacyanin (Khoo et al., 2019a, Leong et al., 2018, Phong et al., 2017b, Sankaran et al., 2018c). The theory of LBF system is the phenomenon of surface-active biomolecules having a sorption mechanism between the air bubbles surfaces. The bubbles then arise and dissolve in an organic solvent phase on top of the aqueous solution in the system (Lee et al., 2016). With the presence of bubble-assistance in LBS, this could intensively strengthen the adsorption mechanism produced by the bubble transportation; thus, this system is feasible for separation and extraction of biomolecules. **Figure 2.3** illustrated the set-up of bubble-assisted LBS.

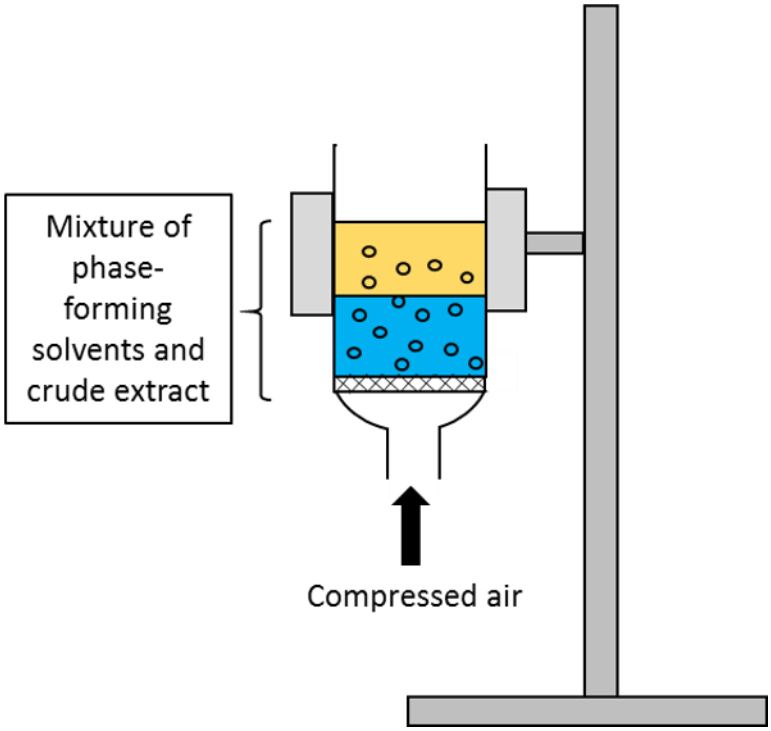


Figure 2.3: Schematic diagram of bubble-assisted LBS

A pilot-scale LBF consist of 0.9 L of 50 % (w/w) of 1-propanol and 1.5 L of 250 g/L ammonium sulfate salt, $(NH_4)_2SO_4$ had been developed for direct recovery of lipase derived from *Burkholderia cepacian* (Mathiazakan et al., 2016). The purpose of this study was to conduct a comparison between the recovery of lipase on pilot-scale and small-scale LBF processes. Mathiazakan et al. (2016) had reported that the pilot-scale alcohol/salt LBF system acquired a purification factor of 12.2, efficiency of 88 % and a recovery yield of 93.27 % which was feasible for purification of lipase to be implemented into the industrial scale processes (Mathiazakan et al., 2016).

Leong et al. (2018) utilized LBF which composed of 10 mL of 100 % ethanol, 20 mL of 200 g/L K2HPO4 salt solution, 1 g FE (peel or flesh of red-purple pitaya) and 15 mins flotation time for betacyanins extraction (Leong et al., 2018). The results under the optimum conditions of LBF revealed that the betacyanins extractions from 1 g FE of peel in alcohol-rich top phase was 95.989 ± 1.708 % with separation efficiency and partition coefficient of 88.361 ± 1.708 % and 24.168 ± 2.949 %, respectively. The recovery from 1 g FE of flesh was 95.488 ± 0.213 % with separation efficiency and partition coefficient of 94.886 ± 0.060 % and 21.195 ± 1.030 %, respectively (Leong et al., 2018). The objective of this work showed that the LBF has a great potential in bioseparation technology as compared with other extraction techniques such as diffusion extraction, ultrafiltration and reverse osmosis in which only able to recover 70 – 75 % of betacyanins (Bi et al., 2010b).

Rather than using alcohol-based LBS, a recent study had showed the extraction of α-Lactalbumin from whey used a different phase forming component (i.e., PEG 1000 and citrate salts) along with bubble-assisted technologies showed a separation efficiency and purification fold of 87.54 % and 5.33 (Jiang et al., 2020). The advantages of this study had showed the feasibility of bubble-assisted technology compared to conventional liquid-liquid extraction providing a low processing cost, rapid and good separation yield. However, a further study is required to fulfil the gaps in the bubble-assisted technology. This is to ensure a better understanding regarding the mass transfer and the development of kinetics model of LBF in the separation of biomolecules.

2.2.2.2 Ultrasound-assisted liquid biphasic system

In the biotechnology processes, cell disruption is considered as the most important process for higher extraction and recovery yield. Ultrasound-assisted LBS is an integrated technique which has been extensively acknowledged by researchers due to its effective properties of cell disruption (Wang et al., 2014, Wu et al., 2011). The advantages of ultrasound-assisted LBS include low operating cost, less energy consumption and short period of time requirement (Gerde et al., 2012) (Gerde et al., 2012). The fundamental of ultrasound irradiation is the high shear forces produced from cavitation bubbles of ultrasonic waves and mechanical shears which enhanced the cell disruption for effective biomolecules extraction (Chemat et al., 2017b). **Figure 2.4** shows a schematic set-up of ultrasound-assisted LBS.

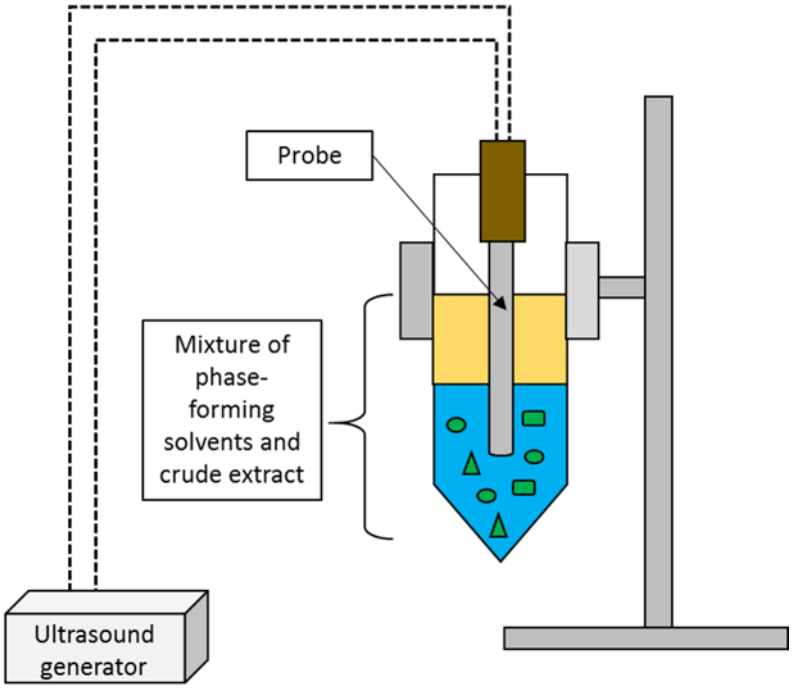


Figure 2.4: Schematic diagram of ultrasound-assisted LBS

A recent study conducted by Sankaran et al. (2018) utilized the application of ultrasound-assisted LBS for extraction of protein from Chlorella vulgaris FSP-E microalgae (Sankaran et al., 2018a). The authors found that the ultrasound-assisted LBS had the ability to break down the rigid cell wall, followed by the release of protein for extraction. The maximum efficiency and yield of protein were 75 % and 65.4 %, respectively (Sankaran et al., 2018a). An integrated system of ultrasound and LBF was used to compare the effectiveness recovery of the release protein into the solution for extraction (Pakhale et al., 2013). It was reported that the ultrasound-assisted LBF had better advantages over the ultrasound-assisted LBS, driven by its higher concentration coefficient and a better separation efficiency. This was mainly due to the presence of air bubbles which enabled the adsorption of surface-active proteins from the bottom phase to the top phase. As a result, this led to a higher separation efficiency and recovery yield. This integrated sugaring-out ultrasound-assisted LBF under the optimum conditions composed of 100 % (w/w) acetonitrile, 200 g/L glucose concentration, biomass concentration of 0.6 % with 5 min of 5 s ON/10 s OFF pulse mode and at a flow rate of 100 cc/min had given rise to the protein separation efficiency and recovery yield of 86.38 % and 93.33 %, respectively.

Aside from that, ultrasound-assisted extraction has also been widely employed for the cell disruption of lignocellulose biomass from plants (Rahim et al., 2019). The extraction of phenylethanoid glycosides (e.g., echinacoside and acteoside) from *Cistanche deserticola* stems using ultrasonic-assisted LBS successfully recovered 27.56 and 30.23 mg/g, respectively (Dong et al., 2015). This

approach showed that ultrasonic-assisted LBS were efficient, eco-friendly and cheap method for extracting and enriching biomolecules from lignocellulose biomass. However, it is crucial to monitor the process temperature when dealing with ultrasonic irradiation. The high shear forces produced from the cavitation bubbles of sonic wave would generate a high temperature process which will degrade or deform the target biomolecules resulting in an unfavourable low extraction yield. Another supporting research of using the application of ultrasound-assisted LBS was the extraction and separation of antioxidants such as xylooligosaccharides (sugar) and phenolic compound from wheat. In ultrasound-assisted LBS composed of 23.8 % (w/w) ammonium sulphate, 24.3 % (w/w) ethanol, 1.2 % (w/w) biomass loading with ultrasound wave (30Hz, 500 W, 10 mins), extraction yielded the highest recovery of sugar and phenols were 16 mg/g and 2.67 mg/g dry material (Đorđević and Antov, 2017). This showed that implementation of ultrasound improved the efficiency of extraction of wheat chaff in LBS yielding 1.3 – 2 times higher, respectively than those without ultrasound.

2.2.2.3 Electricity-assisted liquid biphasic system

Electricity-assisted LBS (see **Figure 2.5**) is a promising mild cell disintegration extraction technique for recovery of biomolecules. For instance, the electricity treatment such as pulsed electric field (PEF) demonstrates the conceptualization of the initiation of short electrical pulses in the order of magnitude of ms or μs subjecting the charge in the cell membrane which is sufficient to perform a rearrangement or disruption of membrane and lead to the pore formation. This process is also known as electroporation. However, an optimum condition is

required as PEF is dependent on the intensity of the treatment and cell characteristics in which pore formation is reversible or irreversible (Eing et al., 2009, Kotnik et al., 2015, Luengo et al., 2014). PEF treatment also increased the mass transfer energy of the system. By combining both PEF and LBS would be an advantage for an efficient extraction of treated sample. This combination is known as an electropermeabilization where the presence of electric and extractive solvent improves the release of intracellular compound from treated sample (Leong et al., 2019a). Moreover, electricity treatment not only provides higher extraction efficiency of biomolecules but also a greener approach in the biotechnology industries.

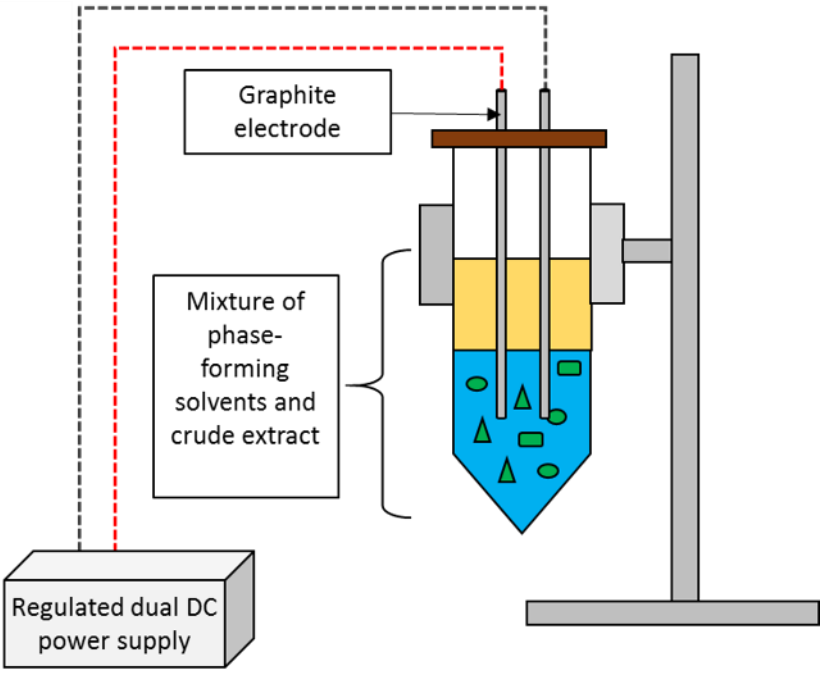


Figure 2.5: Schematic diagram of electricity-assisted LBS

Lam et al. (2017) investigated the operating condition required to release selective proteins from the cell wall of *Chlamydomonas reinhardtii* (cc-124) strain and the cell wall deficient mutant strain (cc-400) using PEF treatment without presence of LBS ('t Lam et al., 2017). The results showed that after PEF treatment with operating condition of 5 – 7.5 kV/cm, 1 – 10 pulses and a pulse length of 0.05 – 0.2 ms on the cell wall deficient mutant (cc-400) was on average three times higher than cell wall strain (cc-124) with average protein yield of 31 ± 6 % protein and 11 ± 3 % protein. Additional experiments utilizing PEF treatment with low energy input (range between 0.01 and 0.5 kWh/kgDW) were also conducted on cell wall deficient mutant strain (cc-400) with a maximum recovery of 30 % at 0.04 kWh/kgDW. Furthermore, the results obtained from PEF treatment with low energy input was compared with bead beating which only obtain an average of 34 ± 4.2 % proteins.

A recent work conducted by Leong et al. (2019) on betacyanins extraction from peel and flesh of red-purple pitaya using the liquid biphasic electric flotation (LBEF) (Leong et al., 2019a), had been reported that this new integration process of electricity supplied in LBF system could cause an electropermeabilization of red-purple pitaya membrane structure and improve the betacyanins extraction from red-purple pitaya. An optimum system composed of 100 % (w/w) ethanol, 200 g/L of dipotassium hydrogen phosphate (K_2HPO_4) with 15 min floatation time (flow rate of 20 – 30 cc/min) and applied up to 3 V of voltage using graphitic electrodes showed the highest separation efficiency of betacyanins concentration (98.383 ± 0.215 % for peel and 96.576 ± 0.0083 % for flesh, respectively) (Leong et al.,

2019a). **Table 2.6** summarized the advance technologies integrated with LBS for the extraction of biomolecules.

Table 2.6: Extraction of biomolecules using various types of advance technologies integrated in liquid biphasic system.

Assisted technology	Composition of LBS	Type of assisted employed	Type of feedstock	Biomolecule	Time	Extraction Efficiency, E (%)	Partition coefficient, K	Recovery yield (%)	Reference
Bubble-assisted LBS or Liquid biphasic flotation (LBF)	50 % (w/w) of 1-propanol and 250 g/L ammonium sulfate salt, $(NH_4)_2SO_4$	Flotation system (compressed air 0.5 bar)	*Burkholderia cepacian*	Lipase	30 min	88.0	-	93.27	(Mathiazakan et al., 2016)
	100 % ethanol, 20 mL of 200 g/L dipotassium phosphate K_2HPO_4	Flotation system (compressed air 0.5 bar)	*Hylocereous polyrhizus*	Lipase	15 min	E for peel and flesh were 88.361 ± 1.708%, 94.886 ± 0.060%.	K value of peel and flesh were 24.168 ± 2.949, 21.195 ± 1.030.	Recovery for peel and flesh were 95.488 ± 0.213, 94.886 ± 0.060.	(Leong et al., 2018)

Table 2.6 (Continue)

Assisted technology	Composition of LBS	Type of assisted employed	Type of feedstock	Biomolecule	Time	Extraction Efficiency, E (%)	Partition coefficient, K	Recovery yield (%)	Reference
Bubble-assisted LBS or Liquid biphasic flotation (LBF)	0.5 g/mL PEG 1000, 35 mL of 0.40 g/mL trisodium citrate $Na_3C_6H_5O_7$	Flotation system (30 mL/min flow velocity)	Whey	a-lactalbumin	42 min	87.54	-	-	(Jiang et al., 2020)
Ultrasound-assisted LBS	100 % (w/w) acetonitrile and 200 g/L glucose solution.	Ultrasound irradiated for 5 min of 5 s ON/10 s OFF pulse mode and flotation system	*Chlorella vulgaris FSP-E*	Protein	5 min	86.38	-	93.33 of protein recovered	(Sankaran et al., 2018a)

Table 2.6 (Continue)

Assisted technology	Composition of LBS	Type of assisted employed	Type of feedstock	Biomolecule	Time	Extraction Efficiency, E (%)	Partition coefficient, K	Recovery yield (%)	Reference
Ultrasound-assisted LBS	20 % (w/w) ethanol and 23.5 % ammonium sulphate	Ultrasound irradiated (300 W, 37 mins)	*Cistanche deserticola* Y. C. Ma stems	Phenylethanoid glycosides	37 min	Echinacoside and acteoside were 5.35 and 6.22 mg/g dry weight	-	Echinacoside and acteoside were 27.56 and 30.23 mg/g dry weight	(Dong et al., 2015)
	24.3 % (w/w) ethanol and 23.8 % (w/w) ammonium sulphate	Ultrasound irradiated (30 Hz, 500 W, 10 mins),	Wheat chaff	Xylooligosaccharides (sugar) and phenolic compound	10 min	72.79 ± 3.98	3.91	Recovery of sugar and phenols were 16 mg/g and 2.67 mg/g	(Đorđević and Antov, 2017)

Table 2.6 (Continue)

Assisted technology	Composition of LBS	Type of assisted employed	Type of feedstock	Biomolecule	Time	Extraction Efficiency, E (%)	Partition coefficient, K	Recovery yield (%)	Reference
Electricity-assisted LBS	Without LBS	PEF treatment (5 – 7.5 kV/cm, 1 – 10 pulses and a pulse length of 0.05 – 0.2 ms)	Cell wall *C. reinhardtii* strain (cc-124) and cell wall deficient mutant strain (cc-400)	Protein	10 min/pulse	-	-	Cell wall strain (cc-124) and cell deficient (cc-400) with average protein yield of 31 ± 6 protein and 11 ± 3 protein.	('t Lam et al., 2017)
	100 % (w/w) ethanol, 200 g/L of dipotassium hydrogen phosphate (K_2HPO_4)	PEF treatment (3 V of voltage using graphitic electrodes) and 15 mins flotation system	Peel and flesh of *Hylocereus polyrhizus*	Betacyanins	15 min	E for peel and flesh were 98.383 ± 0.215 and 96.576 ± 0.083	K for peel and flesh were 100.814 ± 7.324 and 24.883 ± 1.052	Betacyanins concentration (98.383 ± 0.215 for peel and 96.576 ± 0.0083 for flesh	(Leong et al., 2019a)

2.2.3 Key Parameters Affecting LBS

2.2.3.1 Type and molecular weight of polymer

In polymer-salt based LBS, the polymer phase component is crucial as it exhibits different degrees of hydrophobicity on target biomolecules partitioning. As the molecular weight of polymer increases, the hydrophobicity also increases due to the long hydrocarbon chain of monomers. This effect causes a reduction in free volume of the polymer-rich top phase, forcing the target biomolecules to be partitioned to the bottom phase. On the other hand, low molecular weight polymer will decrease the purification factor for target biomolecules as it will be partitioned together with contaminant proteins at the polymer-rich top phase (Lin et al., 2015, Ng et al., 2012). Therefore, it is important in selecting an optimum condition for the hydrophobicity of polymers to obtain the maximum recovery of target compounds.

The effect of molecular weight has been discussed with the used of polymers such as PEG and potassium phosphate salt for the recovery of cyclodextringlycosyltransferase (CGTase) from *Bacillus cereus* (Lin et al., 2015). In this work, the different molecular weights of PEG (e.g., PEG 4000, 6000, 8000, 10000 and 20000) were used in the LBF system for the CGTase extraction at a constant crude extract to volume ratio of 1.0:3.0. It was found that the maximum purification factor of 7.26 and 97.1 % recovery of CGTase were achieved composed of 18.0 % (w/w) PEG 8000 and 7.0 % (w/ w) potassium phosphates LBS. Well, as for the lowest molecular weight, PEG 4000 and highest molecular weight, PEG 20000 showed a purification factor of 2.25 and 3.23, respectively. This indicated that the low molecular weight polymer (PEG 4000) withdraw contaminant

biomolecules to the polymer-rich top phase and the high molecular weight (PEG 20000) would engender a more viscous phase, resulting in the decrease of free volume of polymer-rich top phase caused by volume exclusion effect. In most cases, it is recommended to start with a low molecular weight, depending on the product compatibility while optimizing the partitioning condition.

Also, one of the limitations of using PEG and salt as the phase-forming component in LBS is that most of them cannot be recycled for the next process. The non-recyclable phase-forming component makes the overall LBS in downstream processes to be unfavourable as it causes environmental pollution and increases cost operation (Phong et al., 2018). To improve the recyclability of phase-forming component in LBS process, another similar research replaced using thermo-separating polymer (EOPO) as the phase component for the purification and recovery of CGTase (Ng et al., 2012). The recovery of EOPO after recyclability was more than 80% verifying the viability of recyclable characteristics. This simple, rapid and recyclable feature show that the LBS process is a promising and attractive approach for the recovery and purification of target biomolecules.

2.2.3.2 Type and concentration of alcohol

The use of different alcohols (e.g., methanol, ethanol, 1-propanol, and 2-propanol) with different concentrations in the LBS will affect the overall recovery yield of target biomolecules. The exposure of active site from the implementation of organic solvent helps to maintain the enzyme's open conformation and bind the target compounds to the alcohol-rich top phase. A larger amount of alcohol is

favorable as it will enhance the target biomolecules buoyancy and stability towards the interface layer.

Santos et al. (2016) conducted an experiment on extraction of caffeine from coffee bean and guaraná seed and reported the possibilities to manipulate the partitioning of caffeine to either the alcohol-rich top phase and salt-rich bottom phase (Santos et al., 2016). For caffeine to be partitioned at alcohol-rich top phase, an increase in the concentration of 2-propanol caused the increment in the "caffeine-water" interaction. This effect will promote the biomolecules to be partitioned at the alcohol-rich top phase. Meanwhile, methanol was selected for caffeine to be partitioned at the salt-rich bottom phase. The purpose of selecting methanol was due to its low partition coefficient; therefore, increasing the tendency of caffeine to be partitioned at the salt-rich bottom phase.

A recent study on recovery of glycyrrhizic acid (GA) and liquiritin (LQ) from Chinese licorice root (*Glycyrrhiza uralensis Fisch*) reported that 87 % GA and 94 % LQ were successful obtained at alcohol-rich top phase under the optimum condition of 25 % (w/w) ethanol and 30 % (w/w) K_2HPO_4 in the LBS (Huang et al., 2018). The effect of alcohol concentrations from 14 to 34 % (w/w) and the extraction efficiency and partition coefficient were studied. By increasing the alcohol concentration to 26 % in the system, the extraction efficiency and partition coefficient increased for both GA and LQ biomolecules. However, the extraction efficiency and partition coefficient decreased when the alcohol concentration was increased to 34 %. This was due to the large amount of water-soluble alcohol in the alcohol-rich top phase interacting with the water molecules and causing the

biomolecules to be partitioned to the salt-rich bottom phase (Wang et al., 2011). This term was also referred as “volume exclusion” effect. In general, the selection of alcohol is mainly dependent on the target biomolecules from the complex crude extract. Each target biomolecule has their respective physico-chemical properties and therefore, it is difficult to govern a specific optimum condition for extraction and separation in LBS.

2.2.3.3 Type and concentration of salt

In the LBS, it is critical in selecting the type of salts as the phase-forming component since it can significantly affect the solubility and interaction of the target biomolecules. When the salt is added into a solution, the surface tension of water will increase which then leads to the increase of hydrophobic interaction between protein and water (Wingfield, 2001). Few studies had shown that a high saturation level of salt concentration will cause a reduction in solubility of target biomolecules due to the higher salting-out ability of salt (Smiglak et al., 2007, Zhao et al., 2014). Lu et al. (2016) reported that the ability of salt solution and hydrophilic alcohol solution to form a biphasic system was mainly dependent on the Gibbs free energy of salt hydration (Lu et al., 2016). The alteration in environmental phase system and behavior of biomolecules partitioning is utilized by the different salt component (Yang et al., 2010). Different salts used for the LBS were based on their capability to support hydrophobic interaction between biomolecules (Goja et al., 2013). According to the Hofmeister series, the salting-out ability of anions are arranged in the following order: SO_4^{2-} >HPO_4^{2-} > $C_6H_5O_7^{3-}$ >F^- >Cl^- >Br^- >I^- >NO_3^- >ClO_4^- (Raja and Murty, 2012). However, an optimum condition is required in order to

obtain the maximum recovery of target biomolecules. It is also important to select a biodegradable and eco-friendly salt to ensure a more sustainable green approach in utilizing the LBS.

The effect of various salts used has been studied with the use of potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$) and ammonium sulphate ($(NH_4)_2SO_4$) for the extraction of protein from *Chlorella sorokiniana* microalgae (Phong et al., 2017b). In this study, the salt concentration of 250 g/L were selected for each salt (KH_2PO_4, $MgSO_4$ and $(NH_4)_2SO_4$) as an optimum condition in the LBF. It was found that the KH_2PO_4, $MgSO_4$ and $(NH_4)_2SO_4$ exhibited high separation efficiency of 97.85 %, 97.74 % and 97.74 %, respectively. However, an observation was found using KH_2PO_4 solution where a white solid was formed and deposited around the interface at flotation time of 1.5 mins, showing its incapability for the separation process. This formation happened when the properties of salt having a low solubility. Thus, an addition process is required to melt the solid salt solution. Another observation found using $MgSO_4$ solution was the absence of interface in the LBF after a flotation time of 4 mins. In contrast, it was observed that only $(NH_4)_2SO_4$ solution could clearly render the highest recovery yield and purification values of 56.06 % and 68.99 %, respectively. The possible explanation was $(NH_4)_2SO_4$ has a lower molecular weight as compared with KH_2PO_4 and $MgSO_4$. As a conclusion, the extraction of protein is more favorable in the alcohol-rich top phase with increasing partitioning coefficient (K) when a low molecular weight salts is used (Asenjo and Andrews, 2012, Lin et al., 2013).

However, the selection of various salts is still dependent on the compatibility of LBS and interaction among biomolecules.

The study of salt concentration was continued by using ammonium sulphate at the concentration range of 100 to 300 g/L. The effect of increasing salt concentration tends to increase the protein recovery yield. As supported by Phong et al. (2017), stated that the salting-out effect would occur at a higher salt concentration, the presence of ions tended to decrease the solubility of protein in the salt-rich bottom phase (Phong et al., 2017b). A further increase in salt concentration would decrease the protein recovery percentage. It was recommended to start with a minimum salt concentration of 20 % (w/w) until the optimum condition was obtained rather introducing a high salt concentration abruptly.

2.2.3.4 pH system

The partitioning of target biomolecules can be affected by the pH system in LBS, due to a change in charges and solute properties of solute. The net charge of the target biomolecule becomes negative when the pH value is greater than the isoelectric point (pI) and positive when pH value is lower than the pI. If the net charge is equal to zero, both pH and pI values are equal (Karr et al., 1986). Generally, it is found that in higher pH system would induce a positive dipole moment causing the partition coefficient to increase; therefore, favor the partitioning of negatively charge target biomolecules towards the polymer-rich top phase (Andrews et al., 2010, Olivera-Nappa et al., 2004).

The partitioning of polyhydroxyalkanoate (PHA) from *Cupriavidus necator* H-16 in the thermoseparating-based LBS showed a good setup in altering the pH

system as compared with conventional PEG-based LBS (Chakraborty and Sen, 2016). PHA showed a purification factor and recovery yield of 3.67 % and 63.5 %, respectively, at the pH 6 which was better than the conventional PEG-based LBS that had zero recovery of PHA in the top phase when pH was less than 7. However, there was a sudden drop in PHA recovery yield of 46.4 % when the pH was adjusted to 8.0 to 8.8 in the system. In another study of extraction of BSA had shown that the different pH values could alter the net charge of targeted compound (Chow et al., 2015). It was reported that the pH value increased from 6.0 to 9.0 which was larger than the isoelectric point of BSA (pI = 4.8) resulted in a maximum recovery yield of 84.32%. However, the high pH value is not favourable in the LBS since it can induce the protein denaturation.

Another experiment of antioxidants (i.e., xylooligosaccharides and phenol) extraction from wheat chaff explored the effect of pH on LBS (Đorđević and Antov, 2017). The influence of pH value ranging from 2.5 to 7.0 was studied in the case of partitioning parameters of antioxidant such as recovery and partition coefficient. A maximum recovery of sugar ranging from 96 % to 99 % was obtained at pH 7.0 but the recovery of phenol decreased which could be explained by the phenol compound having a low pKa value of 4.5. In extend, at pH values near pKa such as pH 4.0 was reported that the partitioning of xylooligosaccharides was more towards the ethanol-rich top phase and phenol was more toward salt-rich bottom phase at the highest recovery of 75 % and 77 %, respectively. Hence, it is important to examine the effect of pH at the optimum condition to enhance the purification factor and recovery yield

of the target biomolecules as it could be damaged or denatured by varying the sensitivity of pH conditions.

2.2.3.5 Temperature

The effect of temperature is dependent on the type of phase-forming components used in the LBS and stability of target biomolecules from denaturation. A change in temperature also affects the viscosity and density of the interface in the LBS. In most cases, the optimum temperature within the range of 20 to 40 °C was utilized for maximum recovery and partitioning of target biomolecules. The effect of temperature on the extraction efficiency of C-phycocyanin (CPC) from *Spirulina platensis* microalgae was studied and the maximum extraction efficiency up to 99.0 % was achieved near the temperature range of 35 °C (Chang et al., 2018). It was found that by lowering the temperature to 298 K caused the rate of CPC recovery to decrease, resulting in a low extraction efficiency. The influence of temperature on extraction efficiency study of BSA and papain was evaluated (Pang et al., 2017). However, the studies showed that the extraction efficiency of both BSA and papain decreased when the temperature was increased. This phenomenon was due to the increasing temperature which could inhibit the interaction of amino acid and surface water of protein, resulting in less efficiency of protein extraction (Chakraborty and Sen, 2016). Hence, the effect of temperature should be taken into consideration as the extraction efficiency of the biomolecule is dependent on the range of temperature in the LBS.

2.4 How does ionic liquid plays a role in sustainability for biomass processing

Development of the next generation industrial chemical processes emphasizes aspects related to sustainability, green chemistry, and eco-efficiency. In recent years, researchers have expended a tremendous amount of effort in exploring ionic liquids (ILs), or the molten salts, as an environmentally friendly alternative to the conventional volatile organic solvents (VOCs). VOCs possess negative impact on the environmental and human health due to their undesirable inherent properties such as volatility, flammability and toxicity (Laus et al., 2005). Therefore, a shift to ILs technology in many industrial sectors is considered viable as ILs can mitigate these concerns and offer comparative performance as to the conventional solvents.

Physico-chemical properties of ILs, such as negligible vapor pressure, high melting point, and high thermal stability, are desirable for many fields of chemistry (Lee et al., 2015). Low vapor pressure of ILs mitigates the issue of air pollution due to the minimal evaporation of ILs during operation, as compared to VOCs. In addition, the high melting point and thermal stability allow ILs to withstand intensive operational conditions (i.e., high temperature and pressure). Since ILs comprises tuneable anions and cations, the interionic interaction forms hydrogen bonding that is important for solvation purpose; the unique combinations of ions in ILs earn them a title of designer solvent, as ILs can be specifically designed to fit a specific condition or application by altering their functional groups and selection of appropriate cation and anions (Wilkes, 2004). Till today, imidazolium-based ILs are

widely used for the dissolution of cellulose, extraction of bioproduct from microalgae, CO_2 capture, electrolysis and catalysis technologies.

Emerging technologies such as microwave- and ultrasound-assisted applications have been coupled with ILs for a greater advantage, such as the provision of a more economical route as compared to other heat-dependant processes (Khoo et al., 2020c). The heating effect adopted in microwave is mainly due to the dielectric polarization. This happens when a molecule irradiated with microwaves naturally aligns itself with the applied field at frequency near 2.45GHz. Consequently, the molecule continually attempts to align itself according to the rapidly change in electric field, and this produces heat energy which will be absorbed by the molecule (Kadam et al., 2013). By adopting ILs media, microwave-assisted processing can be rapid and more efficient. On the other hand, the utilization of ultrasound in chemical reaction produces ultrasonic propagation in the ILs media that can emulsify two immiscible liquids and speed up the reactions. The generation and collapse of cavitation bubbles release energy and promote mass transfer in heterogenous system of ILs, thereby enhancing the overall processes (Karimi et al., 2014). Despite the advantages brought by ILs in these processes, a good understanding of toxicology and sustainability of these ILs is important for a further effort in commercialization and marketing of these IL-driven technologies.

2.3.1 Dissolution of lignocellulosic and microalgae biomass

In the progression of 21st century, issues related to climate change and efficient energy management have driven the ideology for a sustainable and greener production. Many developed countries have applied the Sustainable Development

Goals (SDGs) as a trade-off and an important tool to eliminate and limit the use of hazardous organic solvent; one of the solutions is to replace them by the environmentally benign solvent. Over the years, ILs have made tremendous impact in various applications owing to their distinguish attributes (i.e., high thermal stability, high vapor pressure, recyclability, and tuneable ionic properties) but most importantly, as an ideally "green" solvent (Kudłak et al., 2015). Previous studies demonstrated the feasibility of these ILs for the pretreatment of lignocellulose biomass (Mäki-Arvela et al., 2010). Imidazolium is the common cation used in the conventional ILs for the dissolution of lignocellulosic biomass. There are several criteria of ILs' traits for an effective dissolution of lignocellulose. The selected ILs should possess a low melting point as this prevents the degradation of cellulose; other desirable characteristics include good stability and storability, easy recovery of ILs for recyclability purposes, non-toxicity, odourless, biodegradable. and low cost of synthesis. There are a number of patents filed for the applications related to the dissolution of cellulose and woods, biofuels production, and extraction of biopolymers (Argyropoulos, 2011, Hummel et al., 2010, Myllymaki and Aksela, 2008, Rahman et al., 2014, Scott and Piskorz, 1989). **Figure 2.6** shows an overview of the utilization of ILs from the cradle to grave.

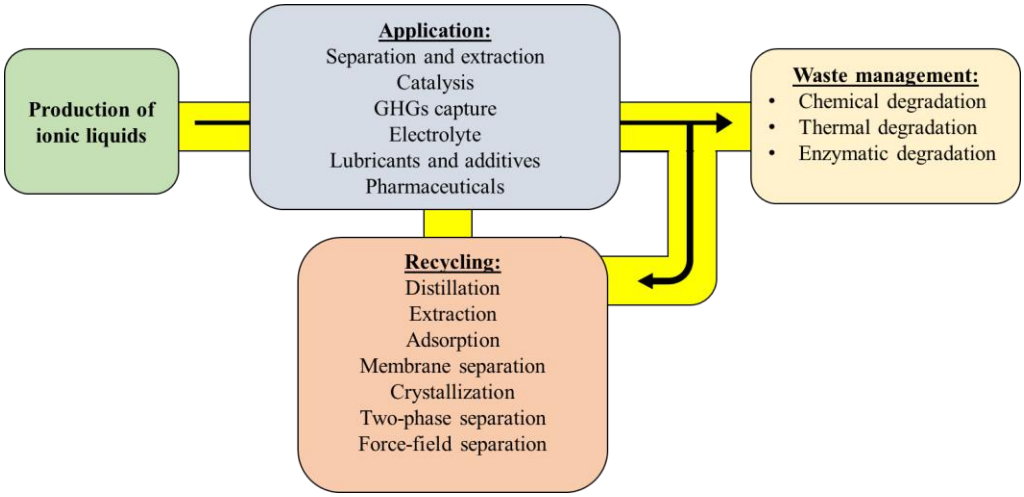


Figure 2.6: Overview of the utilization of ILs from cradle to grave.

2.3.1.1 Factors affecting the dissolution of lignocellulosic and microalgae biomass

It is important to evaluate the effects of size and functional group of the cation in ILs as they may improve the dissolution of lignocellulose biomass. Due to the compact extracellular biopolymer structure of lignocellulosic biomass, small cations are often efficient in solubilizing the cellulose. A recent study conducted by Alayoubi et al. (2020) concluded that 1-ethyl-3-methylimidazolium, $[Emim]^+$, which has a smaller cation than that in 1-butyl-3-methylimidazolium, $[Bmim]^+$, dissolved cellulose (up to 90%, w/w) more effectively (Alayoubi et al., 2020). The main reason behind this improvement is the steric hindrance effect preventing the large molecule in forming hydrogen bonding with cellulose. As the alkyl chain increases (i.e., C_2 to C_{10}), the dissolution power of solvent decreases significantly (Erdmenger et al., 2007). Aside from this, the dissolution of lignocellulose biomass

may be influenced by the functional groups' present in the cation. Cations containing hydroxyl group (-OH) in their structure may decrease the solubility of cellulose in the ILs. The tendency of -OH group to form hydrogen bonding with anions such as acetate and chloride is higher than that with cellulose; thus, this reduces the performances of ILs comprising -OH group (Zhao et al., 2008).

Anion in the ILs plays a significant role as a hydrogen-bond acceptor for an effective cellulose dissolution. An ideal anion should possess a good hydrogen bonding basicity. Chloride anions, Cl^-, are the smallest anion commonly used in the applications of cellulose dissolution. Small polarizing cation incorporated with small Cl^- is more interactive with cellulose because the oxygen atoms presence in cellulose are attacked by the -OH group (Zhang et al., 2005). A previous study by Vitz et al. (2009) confirmed the poor dissolution effect of IL made of $[Br]^-$ (Vitz et al., 2009). On the other hand, anions such as formate and acetate were reported as an excellent anion for cellulose dissolution (Zavrel et al., 2009). In addition, ILs with a higher hydrogen bond basicity exhibit a greater capability in cellulose dissolution, based on the Kamlet-Taft equation, where the quantification of solvation effects of solvents on an observable physical parameter X is shown in **Equation 1**, as described by (Mäki-Arvela et al., 2010).

$$\log X = c + x\,\pi^* + y\delta + a\alpha + b\beta \qquad \textbf{(1)}$$

where π*, δ, α and β define the polarizability, dipolarity, Bronsted acidity and basicity, respectively, whereas x, y, a, and b are parameters values of the solvents.

Besides, another approach in determining the solvation effects is the Hildebrand solubility parameters (Lee and Lee, 2005). This approach is much more favourable as the experiments can be performed to determine the viscosity of ILs, but this approach is limited to applications involving cellulose and lignocellulose.

Other factors that influence the dissolution of biomass are the process conditions, (i.e., temperature, time, stirring rate), particle size, and the presences of water, organic solvent, and salt. Since some of the ILs are miscible with water or organic solvent, the viscosity of ILs could be reduced by the addition of these solvents, which further decreases the solubility of cellulose in ILs (Liu et al., 2019a). The presence of water allows the re-formation of hydrogen bonding in cellulose that resists the interaction between the cation and anion of ILs (Lee et al., 2017c, Marks et al., 2019). On the contrary, the presence of water could improve dissolution of lignin and hemicellulose, depolymerization, and hydrolysis of lignin-carbohydrate complex of hardwood biomass (Roselli et al., 2017, Sun and Xue, 2018, Viell et al., 2016). Several studies have revealed that the addition of salts such as LiCl and Na_2SiO_3 in IL media helped in deconstructing and enhancing the selectivity of dissolution process to wood polymer (Pang et al., 2016, Sun et al., 2016, Xu et al., 2010). It has been proven that the implementation of ultrasound and microwave processing in the IL media could effectively enhance the dissolution of lignocellulosic biomass (Ab Rahim et al., 2020, Kohli et al., 2020, Rahim et al., 2019).

2.3.1.2 Molecular and mechanistic studies of ionic liquids

Mechanism studies is essential to evaluate the interaction occurred during the dissolution of lignocellulosic biomass by ILs. Up to date, numerous studies indicated that hydrogen bonding formation between anions and -OH group of cellulose is the main driving force for cellulose dissolution in ILs. A previous study by Zhang et al. (2010) evaluated the 1H Nuclear Magnetic Resonance, NMR spectrum of cellulose to identify the interaction of -OH group in cellulose with 1-ethyl-3-methylimidazolium acetate, [Emim][CH_3COO], as well as the longer chain of imidazolium-based ILs (Zhang et al., 2010). The 1H NMR spectrum of cellulose in DMSO-d6 revealed the proton signal of hydroxyl (OH^-) at chemical shift ranged from 4.5 ppm to 6.8 ppm. However, for a mixture of [Emim][CH_3COO], cellulose and DMSO-d_6, the peak broadened and gradually shifted toward the downfield, indicating the interaction of oxygen atom of OH^- with acidic proton of $Emim^+$. On the other hand, the downfield shifted of OH- was due to the formation of hydrogen bonding and electronegative atom between the hydrogen atom (hydroxyl of cellulose) and the anion CH_3COO^- (Zhang et al., 2010). These findings were supported by Endo et al. (2016), who investigated the interaction between cellulose and [Emim][CH_3COO] using a wide-angle X-ray scattering (WAXS) and ^{13}C NMR spectroscopy (Endo et al., 2016). The results showed that 40 mol% of [Emim][CH_3COO] completely deconstructed the crystalline structure of cellulose due to the anion bridging phenomenon induced by the hydrogen bonding (Endo et al., 2016). Aside from imidazolium-based ILs, di-isopropylethylammonium chloride, [DIPEA]Cl, and di-isopropylethylammoniumbenzoate, [DIPEA]Bn, are the ammonium-based ILs exhibiting an effective depolymerization of lignin for the

conversion of lignin into high-value aromatic compounds (Tolesa et al., 2020). Fourier-transform infrared (FT-IR) spectroscopy is an analytical method widely used in the analysis of cellulose dissolution; the induced structural change of polymer composed of oligomers, aliphatic and aromatic monomers could be interpreted from the spectra (Feng et al., 2016). A comprehensive review by Li et al. (2018) covers the mechanism and physicochemical aspect of cellulose dissolution in ILs (Li et al., 2018).

2.3.2 Ionic liquid technology for microalgae extraction

In recent years, microalgae have received great attention as one of the sustainable biomass feedstocks for the production of biofuels and biochemicals (Khoo et al., 2020a). Unlike other generation of biomass including terrestrial crops, microalgae can massively grow within two weeks, which are significant shorter in the growing stage. Microalgae also have great adaptability that allows them to consume carbon dioxide, CO_2, from the environment; they can be a good agent of bioremediation, which involves the up-taking of excess nutrients [i.e., ammonium ions (NH_4^+), nitrate ions (NO_3^-) and phosphate ions (PO_4^-)] from wastewater treatment plant (Leong et al., 2019b, Tang et al., 2020). Lipids from microalgae can be a promising source for biofuel production as the microalgal biofuels are more environmental-friendly than the fuels derived from petrochemicals feedstocks (Piemonte et al., 2016). Aside from their contribution to energy sector, microalgae are known to contain great proportions of high-value phytochemicals such as proteins, lipids and carotenoids for various biotechnological applications (Chew et al., 2017, Khoo et al., 2019b).

Some microalgae consist of rigid cell wall that demands the cellular disruption to release the intracellular component for further processing. Numerous strategies have been proposed for the extraction of bioactive compounds from microalgal biomass. Conventional extraction approaches mainly utilize the concentrated acid or alkali solutions, bead milling, homogenisation, supercritical CO_2, and grinding treatment for the disruption of microalgal cells. However, the applications of concentration acid or alkali are not favourable due to their inherent toxicity and hazardous characteristics being not applicable for direct human consumption. On the other hand, mechanical pretreatment methods like bead milling, grinding, and homogenisation are energy intensive processes (e.g., high temperature and pressure conditions) that renders a high treatment cost. Assisting technologies based on ultrasound, microwave, electric, liquid biphasic system and ILs have been implemented for the extraction of bioactive compounds from microalgae (Khoo et al., 2020d, Khoo et al., 2019a, Khoo et al., 2020c, Leong et al., 2019a). Among all the extraction approaches, ILs have been known to be an effective green solvent for the optimal extraction of various bioactive compounds from microalgae. **Table 2.7** shows the attributions of ILs to the 12 Principles of Green Chemistry.

Table 2.7: Attributions of ionic liquids to the 12 Principles of Green Chemistry

Principles	Definition	Justification
Prevention	It is better to prevent waste than to treat or clean up waste after it has been created.	ILs can be separated, recycled, biodegraded after its utilization.
Atom Economy	Synthetic methods should be designed to maximize incorporation of all materials used in the process into the final product.	The synthesis of ILs incorporates almost all of the starting raw materials into the final product.
Less Hazardous Chemical Syntheses	Synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.	Ils composed of distinguished characteristic (i.e., high melting point, negligible vapor pressure and high thermal stability) compared to conventional volatile organic solvents.
Designing Safer Chemicals	Chemical products should be designed to preserve efficacy of function while reducing toxicity.	The design of ILs is dependent on its application and thus its toxicity can be controlled and optimized.
Safer Solvents and Auxiliaries	The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used.	Task-specific ILs are designed to facilitate the limitation of organic solvents. Therefore, the use of ILs can either replace or act as a solvent or catalyst.
Design for Degradation	Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.	Researches have also proven the degradability of these ILs that ensures a safer disposal. Such degradation approaches involve chemical, thermal and enzymatic routes.

Table 2.7 (Continue)

Principles	Definition	Justification
Design for Energy Efficiency	Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.	Conventional approaches are somehow intensive and laborious due to high energy consumption. The implementation of ILs could overcome the limitation over conventional approaches by promoting and efficient and low energy consumption in the processes.
Use of Renewable Feedstocks	A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.	ILs can be reused and recycled for the subsequent processes. Unlike conventional organic solvents which are easily volatile and decomposable under harsh conditions.
Reduce Derivatives	Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.	The utilization of ILs as a mediated solvent is highly selective in the end product or process compared to the use of conventional organic solvents, which would result in the unnecessary by-product and derivatives.

Table 2.7 (Continue)

Principles	Definition	Justification
Catalysis	Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.	In fact, ILs are mediator, also known as co-catalyst, catalyst and solvent in various organic chemical synthesis.
Real-time analysis for Pollution Prevention	Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.	The degradation of ILs was ≥ 60% in approximately 28 days according to Organization for Economic Cooperation and Development (OECD) standard.
Inherently Safer Chemistry for Accident Prevention	Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.	Based on their distinguish properties (i.e., high melting point, negligible vapor pressure and high thermal stability) of ILs. They are relatively more stable than conventional organic solvents.

ILs have been used for direct extraction of intercellular bioactive compounds from the microalgal biomass via cell permeabilization approach. This approach is milder than the conventional mechanical pretreatment as the cellular structure of microalgae was not disrupted. In addition, ILs could avoid the degradation of biomolecules under high extraction temperature and pressure conditions. ILs allow the immersible bioactive compounds to permeate or diffuse across the cell membrane through ionic bonding between the cations and anions of ILs, as opposed to hydrophobic bonding between bioactive compound (Young et al., 2010). Besides, alternative assisting technologies such as microwave and ultrasound treatments have been implemented along with ILs to enhance the extraction process. However, the incorporation of these assisting technologies in IL-based extraction requires an intensive evaluation on the optimization stage as this is to ensure an ideal operating condition without damaging or degrading these highly sensitive bioactive compounds from microalgae. **Table 2.8** summarizes the assisting technologies used along with ILs for the extraction of biomolecules from microalgae. Up to date, there is no study utilizing electricity as an assisting tool in IL-based extraction of biomolecules from microalgae; this remains as a gap to be filled in future research.

Table 2.8: Assisting technologies used in ionic liquids-based extraction of biomolecules from microalgae

Assisted technologies	Type of ILs	Biomass	Conditions	Biomolecule	Recovery	References
Microwave	1 - butyl - 3-methylimidazolium hydrogen sulfate, [Bmim][HSO_4]	*Chlorella sorokiniana*	5 g of IL, 800 W, 120°C	Lipid	0.23 g/g dry algae	(Pan et al., 2016)
Microwave	[Bmim][HSO_4]	*Nannochloropsis salina*	5 g of IL, 800 W, 120°C	Lipid	0.10 g/g dry algae	(Pan et al., 2016)
Microwave	[Bmim][HSO_4]	*Galdieria sulphuraria*	5 g of IL, 800 W, 120°C	Lipid	0.19 g/dry algae	(Pan et al., 2016)
Microwave	1-ethyl-3-methylimidazolium chloride, [Emim]Cl	*Nannochloropsis gaditana*	3.3% wt IL-water, 800 W, 80°C	Lipid	13.9%	(Motlagh et al., 2020)
Microwave	2-hydroxyethylammonium acetate (2-HEAA), 2-hydroxyethylammonium formate (2-HEAF)	*Arthrospira platensis*	10 mL. g^{-1} of IL, 62 W, 2 min	Phycocyanin	8.40 mg/g	(Rodrigues et al., 2020)

Table 2.8 (Continue)

Assisted technologies	Type of ILs	Biomass	Conditions	Biomolecule	Recovery	References
Microwave	(2-HEAA) and (2-HEAF)	*Arthrospira platensis*	10 mL. g^{-1} of IL, 62 W, 2 min	Allophycocyanin	13.3 mg/g	(Rodrigues et al., 2020)
Microwave	1-octyl-3-methylimidazolium acetate, [Omim][OAc]	*Chlorella vulgaris*	700 W, 60°C, 5 min	Lipid	19.2%	(Krishnan et al., 2020)
Microwave	1-ethyl-3-methylimidazolium methylsulfate, [Emim][$MeSO_4$]	*Nannochloropsis* sp.	700 W, 65°C, 15 min	Biodiesel	36.79%	(Wahidin et al., 2018)
Ultrasound	1-butyl-3-methylimidazolium hydrogen sulfate, [Bmim][HSO_4]	*Galdieria sulphuraria*	5 g of IL, 120 W, 120°C, 1 h	Lipid	0.16 g/dry algae	(Pan et al., 2016)
Ultrasound	1-butyl-3-methylimidazolium methyl sulfate, [Bmim][$MeSO_4$]	*Chlorella vulgaris*	5 mL of IL, 60°C	Lipid	47 mg/g	(Kim et al., 2013)

Table 2.8 (Continue)

Assisted technologies	Type of ILs	Biomass	Conditions	Biomolecule	Recovery	References
Ultrasound	Cholinium 2-hydroxy-3-morpholinopropanesulfonate, [Ch][MOPSO]	*Chlorella vulgaris*	400 W, 5 s on/5 s off pulse, 2 min	Protein	9.9 ± 0.1%	(Lee et al., 2017b)
Ultrasound	[2-HEAA] and formate, [2-HEAF]	*Spirulina platensis*	25 kHz, 30 min	Allophycocyanin	6.34 mg/g	(Rodrigues et al., 2018)
Ultrasound	[2-HEAA] and [2-HEAF]	*Spirulina platensis*	25 kHz, 30 min	Phycocyanin	5.95 mg/g	(Rodrigues et al., 2018)
Ultrasound	[2-HEAA] and [2-HEAF]	*Spirulina platensis*	25 kHz, 30 min	Phycoerythrin	2.62 mg/g	(Rodrigues et al., 2018)
Ultrasound	1-butyl-3-methyl imidazolium chloride, [Bmim]Cl	*Spirulina platensis*	25 kHz, 30 min	Phycocyanin	0.36 mg/g	(Rodrigues et al., 2018)

2.3.2.1 Cell permeabilization and recyclability studies of ionic liquids for microalgae extraction

The mechanism of ILs in lignocellulose dissolution is relevant to the extraction of biomolecules from microalgae. ILs can solubilize polysaccharides such as cellulose and pectic from lignocellulosic biomass, indicating that ILs also play a role in permeabilizing the cell walls of microalgae. The ability of ILs to permeabilize cell is attributed to the formation of "pitting" or cavities on the cell surface, thereby facilitating the ionic bonding among the cations and anions of ILs and the intercellular target bioactive compounds (Desai et al., 2016). Hydrophilic ILs with low viscosity and high hydrogen bond capacity are especially more efficient in the solubilization process. Previous studies demonstrated that 1-ethyl-3-methylimidazolium-based IL with $[DBP]^-$, $[HSO_4]^-$, $[CH_3SO_3]^-$ and $[(CF_3SO_2)_2N]^-$ anions were able to recover up to 70 – 99% of astaxanthin from intact *Haematoccocus pluvialis* microalgae (Choi et al., 2019, Desai et al., 2016). The mechanism in dissolving cellulose is associated with the role of ILs as an electron donor-electron acceptor (EDA) between oxygen and hydrogen atoms of cellulose and the ions of ILs. Though, the dissolution capacity is based on the hydrogen bond basicity.

It is important to recycle these pretreated ILs as the cost of synthesizing these ILs is expensive. The performance of $[Emim][EtSO_4]$ after 5 cycles of extraction process achieved an average lipid yield of 98.0 ± 5.2% (w/w) from *Chlorella vulgaris* (Orr et al., 2016). Similarly, Liu et al. (2019) reported the capabilities of [Bmim]Cl and [Emim]Cl in sustaining their high extractability for astaxanthin, i.e.,

82.75% for [Bmim]Cl and 59.87% for [Emim]Cl, even after three subsequent pretreatment cycles (Liu et al., 2019b). Besides, a recent study by Lu et al. (2019) reported the feasibility of conventional [Bmim]Cl to be reused up to 10 times for the lipid extraction from *Chlorella pyrenoidosa* (Lu et al., 2019). However, the performance of lipid extraction dropped when the [Bmim]Cl was repeated used for five times. Similarly, performance of ILs was found to decrease significantly after several cycles of microalgae treatment, as evidenced in the subsequent extraction of astaxanthin from *H. pluvialis* microalgae by Desai et al. (2016) as well as the dissolution of *Gigantochloa scortechini* biomass by Rahim et al. (2019) (Desai et al., 2016, Rahim et al., 2019). The poor performance may indicate the incomplete recovery of ILs that were lost on the cell debris, causing a decrease in the concentration of recycled IL used in the next treatment. In fact, the performance of these recycled ILs may decrease after each cycle of cell permeabilization because of the residual of cell debris (i.e., cell wall, hydrolysed cellulose, chlorophyll, and impurities), water content contributed by distillation, and presence of antisolvents (e.g., ethyl acetate and methanol). Based on these issues, it is suggested that these recycled ILs should undergo a step of purification process to remove the excess impurities before subjecting these ILs for the subsequent treatment.

2.3.2.2 Sustainable of ionic liquids technology

Regardless of numerous efforts dealing with the potential of ILs for various applications, many published papers and magazine still debate on whether the toxicity issues of these ILs should be neglected (Bubalo et al., 2014, Pham et al., 2010). Statement made on the inherent toxicity of ILs will lead people to think that

all ILs are toxic. However, this is uncertain as the development of sustainable chemical products requires an in-depth evaluation on their toxicity potential along with proper procedure, knowledge and information about the impact on the environment, human health and animals (Ranke et al., 2007). For instance, nanoparticles have been implemented in several industrial sectors due to their ideal physico-chemical properties, but concerns over their biological impact (i.e., environmental and human health) are valid because of the ability of nanoparticles to permeate into cell membranes and affect living organism biological behaviour at a molecular level (AshaRani et al., 2009, Lewinski et al., 2008, Nel et al., 2006). Thus, the scientific communities should re-consider these generalizations on the toxicity of ILs by properly understanding the toxicology study. Ostadjoo et al. (2017) have comprehensively evaluated the toxicological data for imidazolium-based IL and this information is beneficial for researchers to further understand the 'green' levels of ILs (Ostadjoo et al., 2018). A recent published article has also revealed the potential of these ILs as the future solvent (Tullo, 2020). As mentioned previously, imidazolium-based ILs are a commonly used solvent for dissolution of lignocellulosic biomass. 1-ethyl-3-methylimidazolium acetate, [Emim][OAc] has been run at the pilot scale for the dissolution of cellulose in lignocellulose biomass (Hermanutz et al., 2008). By implementing ILs as the future solvent for biomass treatment or other applications, a more efficient process could then mitigate the intensive process operational conditions (i.e., high temperature and pressure) and could lower the overall toxicity impact (Li et al., 2013). In addition, the use of IL technology allows a higher quality product compared to those obtained from conventional approaches. There are already a good number of companies and

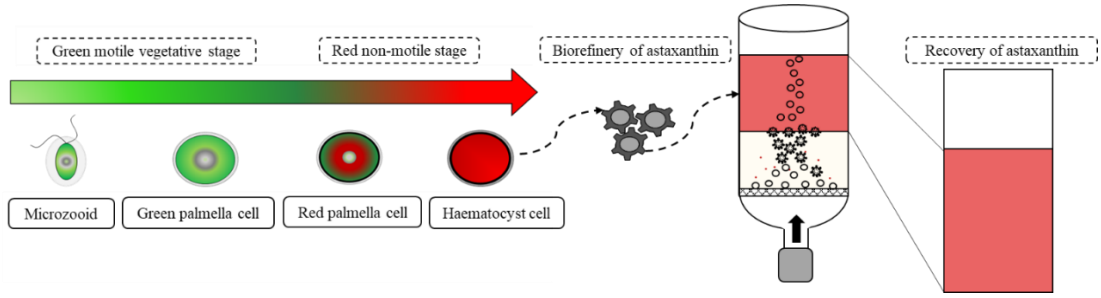
research institutions (e.g., BASF, BASIONICS and U.S. Department of Energy's Joint BioEnergy Institute) invested into ILs technology for cellulose dissolution and biofuel production (BASF, 2020, JBEI, 2015). In addition, chemical manufacturers such as Sigma Aldrich (Darmstadt, Germany) and Iolitec (Heilbronn, Germany) have been supplying various types of ILs for customers at scales ranging from grams to ton (Iolitec, 2019, Sigma-Aldrich, 2020).

2.3.2.3 Environmental sustainability of ionic liquids technology

It should be noted that ILs can reduce the burden of air pollution due to their low tendency to volatile and be released in vapour form. Nonetheless, some ILs have good solubility in water the aqueous mixture of ILs may be released into the environment via effluent discharge. Hence, the environmental fate of ILs faced a complex decision to-be or not-to-be implemented as an alternative industrial solvent due to their toxicity and biodegradability of ILs, which remains cautiously unquestionable. Environmental substitution of these ILs as catalyst or reaction media is vital as the utilization of conventional organic solvents in industrial has to be eliminated or drastically reduced because most of them are ecologically harmful and toxic in nature. In some cases, water is an impractical solvent due to its low solubility and it may even exist as a contaminant in many organic reactions (Breslow, 1991, Li, 2005). In addition, the environmental fate of these ILs has to be assessed to ensure a safer disposal after usage. The risk assessment of ILs could be done via an environmental analysis indicator or principles such as multidimensional risk analysis (MRA), life cycle assessment (LCA), 12 Principles of Green Chemistry, and Sustainable Development Goals (SGDs). So far, major questions on

environmental contamination, environmental fate, environmental substitution and safety aspect of these ILs have yet to be fully answered or addressed.

CHAPTER 3 EXTRACTION OF NATURAL ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS* USING LIQUID BIPHASIC FLOTATION SYSTEM



This chapter covers the application of liquid biphasic flotation system for the extraction of natural astaxanthin from *Haematococcus pluvialis* microalgae biomass. The performance of the liquid biphasic flotation system was optimized in achieving the maximal recovery of astaxanthin. The optimized liquid biphasic flotation system was scaled-up to evaluate the feasibility of extracting astaxanthin is larger scale. This chapter consists of thesis-version of work published in the Bioresource Technology (Khoo, K. S. et al., 2019, *Bioresource Technology*, 290: 121794 DOI: https://doi.org/10.1016/j.biortech.2019.121794).

Extraction of natural astaxanthin from *Haematococcus pluvialis* using liquid biphasic flotation system

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K.K.S. performed all the experiments; K.W.C., C.W.O., H.C.O. and T.C.L. provided guidance in experimental design and materials; K.K.S., K.W.C. and P.L.S. analyzed and interpreted the data; K.K.S. wrote the manuscript and P.L.S. edited the manuscript.

3.1 Abstract

Liquid biphasic flotation (LBF) is a separation technique integrating liquid biphasic system and bubble-assisted extraction method. Here we report the successful application of LBF for the efficient and rapid recovery of astaxanthin from *H. pluvialis* microalgae. The performance of LBF for the extraction of astaxanthin was studied comprehensively under different operating conditions, including types and concentrations of food-grade alcohol and salt, volume ratio, addition of neutral salt, flotation period, and mass of dried *H. pluvialis* biomass powder. The maximum recovery, extraction efficiency and partition coefficient of astaxanthin obtained from the optimum LBF system were 95.11 ± 1.35%, 99.84 ± 0.05% and 385.16 ± 3.87, respectively. A scaled-up LBF system was also performed, demonstrating the feasibility of extracting natural astaxanthin from microalgae at a larger scale. This exploration of LBF system opens a promising avenue to the extraction of astaxanthin at lower cost and shorter processing time.

Keywords: Astaxanthin; *Haematococcus pluvialis*; Liquid biphasic flotation; Recovery; Microalgae

3.2 Introduction

Astaxanthin, also known as 3,3'dihydroxyl-β,β′-carotene-4,4′-dione, is a bright red carotenoid. Astaxanthin belongs to xanthophylls groups, which is widely utilized in food, cosmetic, aquaculture, nutraceutical and pharmaceutical industries. The stereoisomers of astaxanthin include all-cis (3S, 3'S), cis-trans (3R, 3'S) and all-trans (3R, 3'R) (Yang et al., 2013). Astaxanthin is attractive for its highly valuable antioxidant capacity, which is responsible for its protective properties against cancer, diabetes, cardiovascular diseases, ulcer, immune response and inflammation (Yuan et al., 2011). Therefore, the commercial application of astaxanthin as a supplement in food and dietary has been growing in recent years.

Astaxanthin can be either derived from natural sources (e.g., microalgae, yeast, and fungi) or chemically synthesized (Ambati et al., 2014). Synthetic astaxanthin is less desirable for application in food industry because its production route is complex and involves petrochemical resources as raw material (Li et al., 2011a). Hence, natural astaxanthin originated from living organisms has been widely explored for the large-scale production of astaxanthin. Additionally, antioxidant activity of natural astaxanthin is significantly higher than that of synthetic astaxanthin (Pérez-López et al., 2014, Chew et al., 2017).

Haematococcus pluvialis (*H. pluvialis*) is one of the microalgae species containing an abundance of natural astaxanthin, which could be up to 3.8 to 5.0% of microalgae's dry weight depending on cultivation conditions (Wayama et al., 2013). Despite this advantage, the extraction of natural astaxanthin from *H. pluvialis* remains a challenge in the downstream processing. Conventionally, natural astaxanthin is extracted from the cells with solvents such as concentrated

hydrochloric acid, dimethyl sulfoxide, methanol, dichloromethane, sodium hydroxide and acetone (Boonnoun et al., 2014, Sarada et al., 2006, Zhang et al., 2014). These solvents are not favourable due to their inherent toxicity and hazardous characteristics for direct human consumption usage. Recently, natural astaxanthin has been successfully extracted from *H. pluvialis* using alternative approaches such as supramolecular solvent extraction, supercritical CO_2 extraction, magnetic-assisted extraction, ionic-liquid-mediated extraction (Cheng et al., 2018, Choi et al., 2019, Salatti-Dorado et al., 2019). However, these alternative methods often suffer from the high costs of equipment and operating conditions. For instances, supercritical CO_2 extraction requires high pressure condition (1000 – 5000 psia), while the ionic liquid is relatively expensive and toxic.

Liquid biphasic flotation (LBF) is a bubble-assisted separation system combining the working principles of solvent sublation (SS) and liquid biphasic system (LBS). LBF consists of two aqueous phases wherein the target compounds will be extracted from one phase (e.g. salt-rich bottom phase) to the other (e.g., organic-solvent phase) by the selective adsorption of target compounds on air bubble (e.g., nitrogen or oxygen) (Leong et al., 2018). The adsorption of target compound on the air bubble is based on the surface-active properties of compound and the interfacial properties of air bubble. The ascending gas stream in the bottom phase carries the adsorbed compounds to the top phase and is eventually released from the system, while the target compounds are accumulated in the top phase over the gas sparing period. LBF has been widely utilized in the recovery of biomolecules such as protein, lipase, and betacyanins (Chia et al., 2019, Leong et al., 2018). The properties of the phase-forming component used in this liquid biphasic extraction

affect their physico-chemical interaction with target biomolecules, rendering them to be partitioned to either the top phase or the bottom phase (Yau et al., 2015).

The application of LBF system in extraction of astaxanthin from *H. pluvialis* has not been explored and reported before. In this study, LBF system was applied for the recovery of astaxanthin from *H. pluvialis*. Alcohol and salt were selected as the types of phase-forming components based on the following reasons: (i) lower viscosity and less expensive than the conventional polymer-based LBS; (ii) more environmentally friendly due to their recyclability; (iii) ethanol and 2-propanol are food grade, which are safe for human consumption (Khoo et al., 2019b). The extraction condition for the recovery of astaxanthin from *H. pluvialis* was optimised by varying the operation parameters of alcohol/salt-based LBF.

3.3 Material and methods

3.3.1 Chemicals

Ethanol (≥ 99.9%), 2-propanol (≥ 99.9%) ammonium sulphate [$(NH_4)_2SO_4$], di-potassium hydrogen phosphate (K_2HPO_4), sodium bicarbonate (Na_2CO_3), magnesium sulphate ($MgSO_4$), sodium citrate ($Na_3C_6H_5O_7$) and sodium chloride (NaCl) were obtained from R&M Chemicals (Malaysia). All the above-mentioned chemicals used were of analytical grade. *H. pluvialis* powder (≤ 3% astaxanthin content) was purchased from Qingdao China Wanwuyuan Bio-Technology Co., Ltd. (China). Astaxanthin standard (≥ 97% HPLC grade) was purchased from Sigma-Aldrich (Germany).

3.3.2 Apparatus

The setup of LBF apparatus was adapted from previous work by Leong et al. (2018). A glass filter funnel apparatus was utilized for LBF system. A 50-mL glass chromatography column (diameter: 2 cm, and height: 20 cm). A sintered glass disk (porosity of G4 grade) was installed at the funnel site of the column for bubble generation. The bottom joint of column was connected with a rubber tube, and the air flow rate was monitored by an air flowmeter. The air bubbles with size range of 5-15 μm were created through the sintered glass disk.

3.3.3 Liquid biphasic flotation technique for extraction of astaxanthin

Two types of food-grade alcohol (i.e., ethanol and 2-propanol) and various types of salts were used in the LBF system for extraction of astaxanthin from the dried *H. pluvialis* biomass powder. The dried *H. pluvialis* powder was first mechanically disrupted by grinding with mortar and pestle. The disrupted microalgae cells were then dissolved in the salt solution and transferred to the LBF column. Subsequently, the alcohol was added gently to the column. Operating parameters such as types and concentrations of alcohol and salts, addition of NaCl, volume ratio, flotation period and mass of dried *H. pluvialis* biomass powder was studied systematically for their effects on the extraction of astaxanthin. These parameters of LBF system were optimized using one-factor-at-a-time (OFAT) approach. The initial setting and the variables of operating conditions for alcohol/salt-based LBF system for recovery of astaxanthin from dried *H. pluvialis* biomass powder are shown in **Table 3.1**.

Table 3.1: Initial operating conditions of LBF system

No.	Operating conditions	Unit	Initial setting	Other settings
1.	Type of alcohol	-	Ethanol	2-propanol
2.	Type of salt solution	-	$(NH_4)_2SO_4$	K_2HPO_4, Na_2CO_3, $MgSO_4$ and $Na_3C_6H_5O_7$
3.	Concentration of alcohol	% (w/w)	100	60, 70, 80 and 90
4.	Concentration of salt solution	g/L (w/w)	250	150, 200, 300 and 350
5.	Volume of alcohol	mL	15	10 and 20
6.	Volume of salt	mL	15	10 and 20
7.	Concentration of neutral salt	molarity	-	0.2, 0.4, 0.6, 0.8 and 1.0
8.	Flotation time	min	15	5, 10, 20 and 25
9.	Mass of dried biomass powder	g	0.01	0.0075 and 0.0125
10.	pH	-	7	4, 5, 6 and 8

3.3.3 Analytical procedures

3.3.4.1 Spectrophotometric determination of astaxanthin concentration

The stock solution of astaxanthin standard was first prepared at 10 µg/mL using ethanol or 2-propanol. The absorbance of astaxanthin was determined using a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) with alcohol as background reference. Based on the screening of absorbance spectrum ranging 300 – 600 nm, the maximum absorbance for astaxanthin was at optimal density, OD_{478}. The standard curve was then generated using the stock solution diluted in the range of 0 – 5 µg/mL. From the standard curves, Equations (1) and (2) were derived for the determination of astaxanthin concentration in ethanol and 2-propanol, respectively:

$$C_{A(Ethanol)}(mg/mL) = \frac{OD_{478} - 0.0035}{81.88} \quad (1)$$

$$C_{A(2-propanol)}(mg/mL) = \frac{OD_{478} - 0.0033}{72.78} \quad (2)$$

where $C_{A(Ethanol)}$ and $C_{A(2\text{-}propanol)}$ are the concentration of astaxanthin using ethanol and 2- propanol.

3.3.4 Calculations of partition coefficient, extraction efficiency, recovery yield

Partition coefficient (*K*) of astaxanthin in LBF system was calculated according to Equation (3) (Leong et al., 2018):

$$K = \frac{AS_T}{AS_B} \quad (3)$$

where AS_T and AS_B are the concentrations of astaxanthin (mg/mL) in alcohol-rich top phase and salt-rich bottom phase, respectively.

Volume ratio (V_R) of LBF system was calculated using Equation (4) (Chew et al., 2019a):

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

where V_T and V_B are the volumes of the top and bottom phases, respectively.

Extraction efficiency (E_{AS}) was used to evaluate the recovery of astaxanthin from salt-rich bottom phase to alcohol-rich top phase. The calculation of E_{AS} was based on Equation (5) (Chew et al., 2019a):

$$E_{AS} = \frac{K \times V_R}{1 + K V_R} \quad (5)$$

Total recovery of astaxanthin (AS_R, %) from *H. pluvialis* powder was calculated using Equation (6):

$$AS_R = \frac{AS_T V_T}{M_B C_{AS}} \times 100\% \quad (6)$$

where M_B is the total mass of *H. pluvialis* powder and C_{AS} is the percentage of astaxanthin in *H. pluvialis* powder (3%).

3.3.5 Statistical analysis

For the statistical analysis, the data presented was the average of triplicate reading. The values were expressed as mean ± standard deviation. The experiment was conducted three times to further verify the results. The data was subjected to one-way ANOVA using Microsoft Excel to evaluate the significant differences where $p \leq 0.05$.

3.4 Results and discussion

3.4.1 Effect of type of alcohol and salts

Ethanol and 2-propanol were chosen as the candidates of alcohol-based phase component in the LBF system for the extraction of astaxanthin from dried *H. pluvialis* biomass powder. As compared to the extraction methods utilizing hazardous concentrated hydrochloric acid, alkali, or solvents (i.e., dimethyl sulfoxide and dichloromethane), ethanol and 2-propanol are more suited to the astaxanthin processing due to their food-grade status. The performance of LBS systems made of different alcohol and salts is shown in **Table 3.2**. From the results, it can be seen that ethanol could form biphasic systems with most of the tested salts except for $MgSO_4$ and $Na_3C_6H_5O_7$. According to a past study, ethanol was not able to form biphasic systems with $(NH_4)_2SO_4$, K_2HPO_4 and Na_2CO_3 (Phong et al., 2017b). This could probably due to the stronger salting-out effect of ethanol at the higher concentrations (250 g/L) of alcohol and salt (Grundl et al., 2017). Another study conducted by Leong et al. (2018) showed that the higher concentrations of

ethanol and salt could induce the biphasic system due to the hydrophobic and hydrophilic interactions. Overall, the highest AS_R values obtained from LBF system made of ethanol and 2-propanol were 50.12 ± 3.97% and 74.55 ± 0.38%, respectively. The corresponding E_{AS} values were 93.75 ± 0.50 and 99.97 ± 0.11%, respectively. The good performance of LBS system made of 2-propanol could mainly due to its longer hydrocarbon chain and higher hydrophobicity, which may facilitate its interaction with astaxanthin in the alcohol-rich top phase. Hence, 2-propanol was selected to be further optimized in the LBF system.

Table 3.2: Effect of various types of alcohol and salts on the extraction of astaxanthin from *H. pluvialis*. The default operating conditions of LBF system were 10 mg of dried *H. pluvialis* biomass powder, 15 mL of alcohol, 15 mL of 250 g/L salt solution, 1:1 of V_R, and 15 min of flotation period.

Type of alcohol	**Type of salts**	**Biphasic formation in LBF**	AS_R **(%)**		E_{AS} **(%)**	K
			AS_T	AS_B		
Ethanol	K_2HPO_4	Yes	22.57 ± 4.13	0.17 ± 0.01	99.18 ± 0.02	32.04 ± 0.83
	$(NH_4)_2SO_4$	Yes	20.85 ± 0.16	1.50 ± 0.13	93.68 ± 0.52	3.49 ± 0.39
	Na_2CO_3	Yes	50.12 ± 3.97	3.49 ± 0.03	93.47 ± 0.04	5.68 ± 0.38
	$MgSO_4$	No	-	-	-	-
	$Na_3C_6H_5O_7$	No	-	-	-	-
2-Propanol	K_2HPO_4	Yes	73.18 ± 9.46	0.15 ± 0.03	97.75 ± 0.05	207.96 ± 48.72
	$(NH_4)_2SO_4$	Yes	75.55 ± 0.39	0.63 ± 0.27	99.10 ± 0.52	43.40 ± 19.36
	Na_2CO_3	Yes	104.28 ± 9.05	0.26 ± 0.11	99.74 ± 0.11	211.69 ± 42.73
	$MgSO_4$	Yes	42.86 ± 4.36	-	-	-
	$Na_3C_6H_5O_7$	Yes	62.13 ± 0.75	0.03 ± 0.02	99.51 ± 0.02	59.55 ± 11.55

In this study, various salts including $(NH_4)_2SO_4$, K_2HPO_4, Na_2CO_3, $MgSO_4$ and $Na_3C_6H_5O_7$ were tested in the alcohol/salt-based LBF system. For ethanol/salt-based LBF systems, the highest AS_R (50.12 ± 3.97%) was achieved with Na_2CO_3. However, the formation of salt crystal in Na_2CO_3-rich bottom phase indicates the non-equilibrium of both liquid phases in ethanol/ Na_2CO_3-based LBF system, making the system less desirable for the separation process. As for 2-propanol/salt-based LBF systems, the highest AS_R (74.55 ± 0.39%) was achieved with $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ has been a promising salt used in liquid-liquid separation of various biomolecules (Chew et al., 2019a). SO_4^{2-} ion from $(NH_4)_2SO_4$ interacts well with water, forming an excellent hydration capacity to exclude astaxanthin to the top phase. Moreover, the acidic solution of $(NH_4)_2SO_4$ (pH = 4.5 – 5.5) was conducive to astaxanthin, which is more stable in acidic condition. On the other hand, the alkaline solution of phosphate-based salts (pH = 6 – 8) might have caused the degradation of astaxanthin prior to the flotation process. Hence, $(NH_4)_2SO_4$ was selected for the following experiments.

3.4.2 Effect of the concentrations of alcohol and salt

The concentrations of alcohol and salt influence the equilibrium of biphasic system as well as the properties of the LBF (i.e., interfacial tension, density and viscosity); any change in the concentration of phase-forming components will cause the discordance that affects the solute partitioning (Chew et al., 2019a). In our studies, the partitioning of astaxanthin to the alcohol-rich top phase was conducted by altering the concentrations of alcohol [60 – 100% (w/w)] and salt (150 –350 g/L).

Figure 3.1(a) shows the effects of alcohol concentration on both AS_R and E_{AS} of astaxanthin. Based on our observation, 50% (w/w) of 2-propanol was not able to form a biphasic system with 250 g/L of $(NH_4)_2SO_4$ solution. This was due to the weak salting-out ability caused by the diluted 2-propanol which have more water molecules interacting with the salt ions and reduce the tendency to form a biphasic system (Leong et al., 2018). By increasing the concentration of alcohol to 60% (w/w), there was initially no formation of biphasic system but after some settling time, the two phases formed successfully. This is explained through the poorer partitioning between the alcohol-rich top phase and salt-rich bottom phase (K = 34.31 ± 1.67) collected in the tube after the LBF process. As the alcohol concentration increases, the K of the biphasic system also increases. It was also found that a higher concentration of alcohol would increase the volume of top phase (V_T = 20 mL) where the free volume available for the partitioning of astaxanthin to the alcohol-rich top phase is higher. When the concentration of 2-propanol increased to the range of 70-100% (w/w), the values of AS_R, E_{AS} and K also increased. Results showed that the optimal LBF system comprising 100% (w/w) of 2-propanol and 250 g/L of $(NH_4)_2SO_4$ gave the satisfactory results of AS_R (74.55 ± 0.38%), E_{AS} (99.75 ± 0.05%) and K (179.99 ± 7.19), respectively. The recovery of astaxanthin was significantly affected ($p < 0.05$) by the concentration of alcohol. Based on the results, 100% (w/w) of alcohol was selected for the subsequent study of 2-propanol/$(NH_4)_2SO_4$-based LBF system.

The concentration of salt was varied in the range of 150 to 350 g/L in 2-propanol/$(NH_4)_2SO_4$-based LBF systems. **Figure 3.1(b)** shows the effect of salt

concentration on both AS_R and E_{AS}. By lowering the concentration of $(NH_4)_2SO_4$ to 150 g/L, the AS_R value decreased significantly to 50.86 ± 0.14% but the E_{AS} value was maintained at 99%. It was also found that a lower concentration of $(NH_4)_2SO_4$ would cause an increase in volume of top phase. However, this is not favorable as an increase in the free volume would increase the probability of accommodating impurities from the dried *H. pluvialis* biomass in the alcohol-rich top phase (Lee et al., 2016). Meanwhile, by increasing the concentration of salt, the top phase volume (V_T = 17.5 mL) will be lowered at the same time. The statistical analysis showed that the recovery of astaxanthin was significantly affected ($p < 0.05$) by the different concentration of salt. Collectively, the LBF system comprising 100% (w/w) of 2-propanol and 350 g/L of $(NH_4)_2SO_4$ was optimal for the extraction of astaxanthin, as evidenced by AS_R (95.11 ± 1.35%), E_{AS} (99.84 ± 0.05%) and K (385.16 ± 3.87), respectively. According to our analysis, 350 g/L of $(NH_4)_2SO_4$ was chosen for the following experiments.

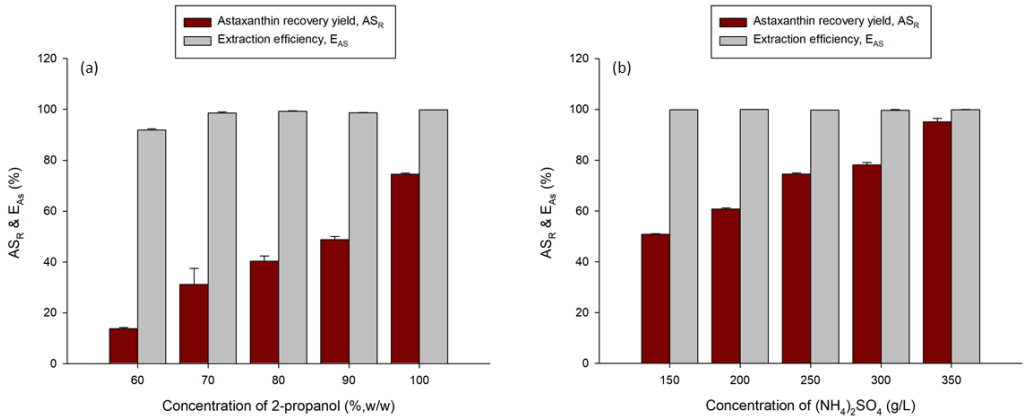


Figure 3.1: AS_R and E_{AS} of astaxanthin from *H. pluvialis* at different concentrations of **(a)** 2-propanol and **(b)** $(NH_4)_2SO_4$.

3.4.3 Effect of volume ratio

Based on previous experimental results, alcohol/salt-based LBF system comprising 100% (w/w) of 2-propanol and 350 g/L of $(NH_4)_2SO_4$ was further used in investigation of effect of V_R on the performance of astaxanthin extraction. V_R of LBF could affect the equilibrium of biphasic systems, which could therefore impact the AS_R, E_{AS} and K of astaxanthin. In this study, three different sets of V_R were used, namely 1:2, 1:1 and 2:1. **Figure 3.2(a)** shows the AS_R and E_{AS} of astaxanthin in the top phase of LBF system. As the volume of alcohol increased, AS_R to exceed beyond the initial concentration which is 3% astaxanthin in the dried *H. pluvialis* biomass powder. Based on our statement above, the results showed that an increase in free volume at the top phase would mainly partition not only astaxanthin. We assumed that the impurities were also partitioned towards the alcohol-rich top phase which causes the recovery yield to rise beyond the initial concentration of astaxanthin. The highest AS_R value (170.35 ± 3.96%) was obtained at V_R = 2:1, which include the impurities from the dried *H. pluvialis* biomass powder. However, increasing the V_R of salt phase caused a significant decrease in the volume of top phase. This eventually decreased the free volume of the top phase (V_T = 11.25 mL), resulting in a lower AS_R (48.01 ± 2.58%). A lower volume in the top phase would also a shorter path for the ascending air bubbles to escape the top phase and release the astaxanthin. On the other hand, 2-propanol/$(NH_4)_2SO_4$-based LBF system with V_R at 1:1 gave the highest of AS_R value and is optimal for the recovery of astaxanthin.

3.4.4 Effect of the addition of neutral salt

The optimized 2-propanol/$(NH_4)_2SO_4$-based LBF system was utilised for the study of the effect of the addition of neutral salt for the recovery of astaxanthin from dried *H. pluvialis* biomass powder. In our experimental studies, the addition of 3 mL of diluted NaCl solution (0.2 – 1.0 M) in 2-propanol/$(NH_4)_2SO_4$-based LBF system was carried out to determine their effects on the recovery yield of astaxanthin. Based on previous literature studies, the addition of NaCl increased the recovery yield and *K* of lipase using the alcohol/salt-based aqueous two-phase system (Ooi et al., 2009). It was postulated that the presence of Na^+ and Cl^- ions could change the electrostatic- and hydrophobic-interaction of astaxanthin and phase-forming components in the biphasic system. However, based on **Figure 3.2(b)**, there was no significant improvement in AS_R, yet a decline in AS_R and *E* values was obtained. Therefore, the addition of neutral salt does not necessarily improve the recovery yield of astaxanthin utilizing the LBF system. Our results showed that the LBF system comprising 100% (w/w) of 2-propanol and 350 g/L of $(NH_4)_2SO_4$ without addition of NaCl successfully obtained the maximum values of AS_R (95.11 ± 1.35%), E_{AS} (99.84 ± 0.04%) and *K* (85.16 ± 3.87), respectively.

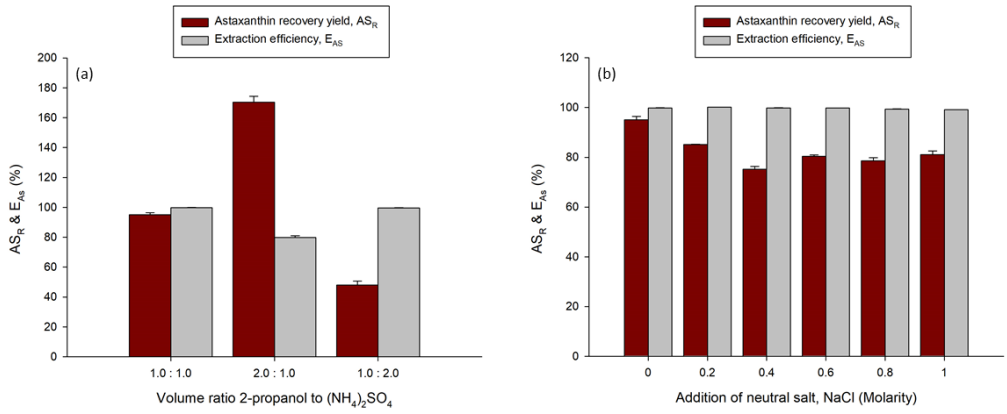


Figure 3.2: AS_R and E_{AS} of astaxanthin from *H. pluvialis* at different **(a)** volume ratio of 2-propanol to $(NH_4)_2SO_4$, and **(b)** concentration of NaCl added.

3.4.5 Effect of flotation period

According to Sankaran et al. (2018c), the effect of flotation period in the LBF system is an important aspect to be taken into consideration as the air-water interface per unit volume of aqueous solution was affected by the flotation period. Based on our experimental design, the flotation period was varied from 5 to 25 min in the LBF system composed of 10 mg of dried *H. pluvialis* powder, 15 mL of 100% (w/w) 2-propanol and 15 mL of 350 g/L $(NH_4)_2SO_4$ for the recovery of astaxanthin. Increasing the flotation period increases the transportation of the astaxanthin from the salt-rich bottom phase to alcohol-rich top phase. When the flotation period increased from 5 to 15 min, the AS_R increases. However, when the flotation period subsequently increased up to 25 min, the AS_R decreased to approximately about 5%. This finding was supported by previous research work on the effect on flotation period which indicate that by increasing the flotation time to extended period does

not enhance the recovery yield and extraction efficiency yet worsen the situation (Phong et al., 2017b). **Figure 3.3(a)** clearly shows the AS_R and E_{AS} of astaxanthin were affected by flotation period. The statistical analysis also showed that the flotation period was significantly affected ($p < 0.05$) for the recovery of astaxanthin. Hence, the flotation period of 15 min was chosen as the most appropriate duration of aeration for the extraction of astaxanthin.

3.4.6 Effect of the mass loading of dried *H. pluvialis* powder

Previous studies have shown that the concentration of loaded feedstock would affect the partitioning behavior of biomolecules in LBF system (Chew et al., 2019a, Selvakumar et al., 2010). An increase in mass of *H. pluvialis* biomass powder loaded in the LBF system would cause the biphasic system to be more viscous, making it difficult for the recovery and partitioning process. A high viscosity in medium of LBF system is not favorable for the continuous aeration of air bubble because an undesirable emulsification may occur in the bottom phase. Additionally, a smaller loading of biomass also reduced the effectiveness of recovery of bio-compounds (Chia et al., 2019). In our studies, the mass of dried *H. pluvialis* biomass powder was loaded in the range of 5 – 12.5 mg. Based on **Figure 3.3(b)**, neither an increase nor a decrease in the mass of loaded biomass powder affect the AS_R and E_{AS} significantly. However, increasing the mass of dried *H. pluvialis* biomass powder is not recommended as a higher mass of dried powder demanded a higher aeration rate for the air bubbles to transfer the astaxanthin to the top phase. Additionally, a higher biomass load would also cause the impurities to be extracted from the dried *H.*

pluvialis biomass powder (Show et al., 2011, Chew et al., 2019a). The mass of loaded *H. pluvialis* biomass significantly affected ($p < 0.05$) the recovery of astaxanthin in LBF system. Based on our quantification analysis, the mass of dried *H. pluvialis* biomass powder at 10 mg gave the most optimal values of AS_R and E_{AS} of astaxanthin. As a result, the optimal composition of biphasic system used in LBF system include 15 mL of 100% (w/w) 2-propanol and 15 mL of 350 g/L of $(NH_4)_2SO_4$ solution.

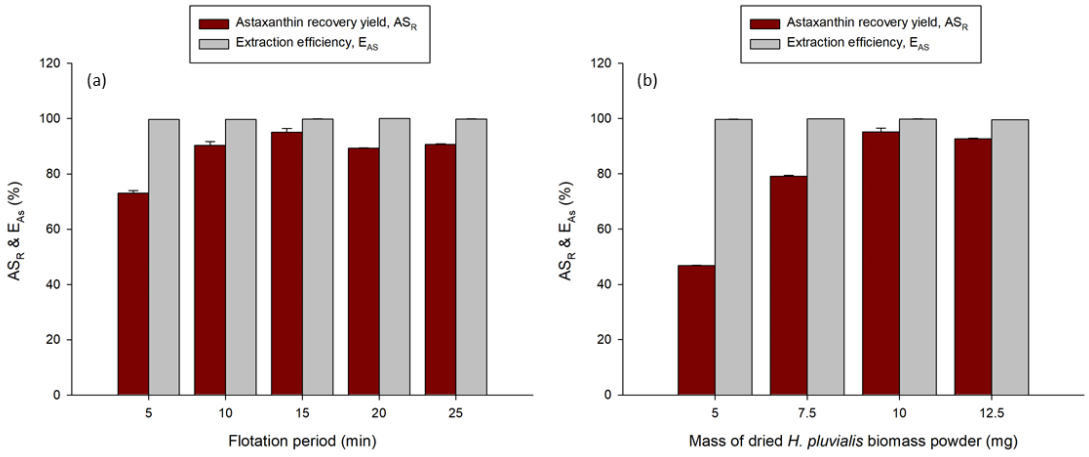


Figure 3.3: AS_R and E_{AS} of astaxanthin from *H. pluvialis* at different **(a)** flotation period and **(b)** mass of dried biomass.

3.4.7 Scaling-up approach on the LBF system

The LBF system was subsequently scaled up to examine the reliability and effectiveness of this extraction system for potential application at pilot scale. For this study, the optimized parameters based on the small-scale LBF system were kept constant. In the scale-up study, the volume for LBF system was increased up to ten folds. A total working volume of 300 mL composed of 100 mg of dried *H. pluvialis*

biomass, 150 mL of 100% (w/w) of 2-propanol and 150 mL of 350 g/L of $(NH_4)_2SO_4$ were used. **Table 3.3** shows the comparison study between the scaled-up LBS system and the initial scale of LBF system. The relatively higher values of AS_R (78.38 ± 0.93%) and E_{AS} (99.86 ± 0.05%) were obtained as compared to that of the initial scale of LBF. The yield obtained from the scale-up approach was 15% lower compared to the initial scale of LBF. This was because scaling up operation determined the actual reality of this initial system in the pilot scale compared to laboratory scale (Sankaran et al., 2018c). This also indicates that the large-scale operation of the LBF system using similar optimized parameter can yield better results and the LBF system can be scaled-up without compromising the yield of astaxanthin. This scaling up approach showed the potential of LBF as an alternative approach for the recovery of astaxanthin from *H. pluvialis*, after verifying its reliability in a larger scale setting as well as evaluating its feasibility to be applied in food industry.

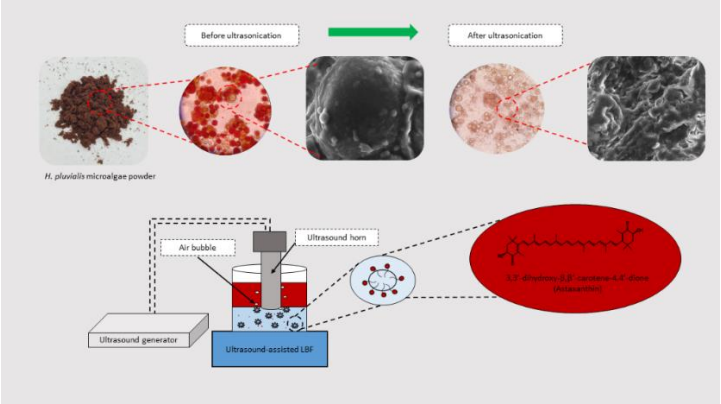
Table 3.3: Performance of astaxanthin extraction in 300 mL and 30 mL 2-propanol and $(NH_4)_2SO_4$ LBF systems

Volume of LBF systems (mL)	$\boldsymbol{AS_R}$ **(%)**	$\boldsymbol{E_{AS}}$ **(%)**
30	95.11 ± 1.35	99.84 ± 0.04
300	78.38 ± 0.93	99.86 ± 0.05

3.5 Conclusion

An efficient technology utilizing food-grade alcohol/salt-based LBF offers an alternative route for the recovery of natural astaxanthin from *H. pluvialis.* The optimized condition for the LBF system were 15 mL of 100% (w/w) 2-propanol, 350 g/L of $(NH_4)_2SO_4$, 1:1 of V_R, 15 min of flotation period and 10 mg of dried *H. pluvialis* biomass powder. The optimal LBF system successfully obtained the maximum values of AS_R (95.11 ± 1.35%), E_{AS} (99.84 ± 0.04%) and K (385.16 ± 3.87), respectively. The scaling-up approach verified the feasibility of this LBF system for the extraction of natural astaxanthin.

CHAPTER 4 INTEGRATED ULTRASONIC ASSISTED LIQUID BIPHASIC FLOTATION FOR EFFICIENT EXTRACTION OF ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS*



This chapter covers the integration of ultrasound-assisted technology with liquid biphasic flotation system for the extraction of astaxanthin from *Haematococcus pluvialis* microalgae biomass. The optimized condition from the previous chapter was further subjected for ultrasonication treatment to study the cell disruption effect of untreated and ultrasonication treated performance for the extraction of astaxanthin. Various operating conditions such as the effect of ultrasonication on cellular morphology, the position of ultrasound horn, pulse and continuous mode of ultrasonication, amplitude, air flowrate, air flotation time, and addition of *H. pluvialis* biomass powder were evaluated. This chapter consists of thesis-version of work published in the Ultrasonics Sonochemistry (Khoo, K. S. et al., 2020, Ultrasonics Sonochemistry, 67: 105052. DOI: https://doi.org/10.1016/j.ultsonch.2020.105052).

Integrated ultrasonic assisted liquid biphasic flotation for efficient extraction of astaxanthin from *Haematococcus pluvialis*

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Authors' Contributions:

K.K.S. performed all the experiments; K.W.C, G.Y.Y. and S.M. provided guidance in experimental design and materials; K.K.S., K.W.C. and C.W.O. analyzed and interpreted the data; K.K.S. wrote the manuscript; S.M and P.L.S. edited the manuscript.

4.1 Abstract

The purpose of this investigation is to evaluate the implementation of ultrasound-assisted liquid biphasic flotation (UALBF) system for the recovery of natural astaxanthin from *Haematococcus pluvialis* microalgae. Various operating conditions such as the effect of ultrasonication on cellular morphology, the position of ultrasound horn, pulse and continuous mode of ultrasonication, amplitude, air flowrate, air flotation time, and addition of *H. pluvialis* biomass powder were evaluated using the UALBF. Under the optimized operating conditions of ultrasound-assisted LBF, the maximum recovery yield, extraction efficiency, and partition coefficient of astaxanthin were 95.08 ± 3.02%, 99.74 ± 0.05%, and 185.09 ± 4.78, respectively. In addition, the applied scale-up operation has verified the practicability of this integrated approach for the effective production of natural astaxanthin.

Keywords: Ultrasound; Cavitation; *Haematococcus pluvialis*; Astaxanthin; Liquid biphasic flotation; Extraction.

4.2 Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a secondary carotenoid and is widely used in many industries (e.g., food, cosmetics, aquaculture, and pharmaceuticals) owing to its high antioxidant activity compared to synthetic astaxanthin (Ambati et al., 2014). The production of natural astaxanthin over synthetic astaxanthin is fundamentally vital as synthetic astaxanthin has been pinpointed by safety concerns due to its toxicity arising from using petrochemical resources as raw materials during its synthesis (Shah et al., 2016). The production of natural astaxanthin provides a higher antioxidant activity compared to other carotenoids (e.g., β-carotene, canthaxanthin, zeaxanthin, and lutein). *Haematococcus pluvialis* (*H. pluvialis*) compared to other microorganisms such as algae and yeast has the most accumulation of astaxanthin up to 3.8 to 5.0% of dry weight depending on the cultivation process conditions (Wayama et al., 2013). Regardless of the high accumulation of astaxanthin, the extraction and recovery of natural astaxanthin remain a hindrance in the biotechnological downstream processing.

Cell disruption is a necessary process to obtain a higher extraction efficiency of astaxanthin. Generally, two types of cell disruption i.e., either a mechanical approach or a non-mechanical approach are followed. The non-mechanical approach includes chemical, osmotic shock, and enzymatic treatment. On the other hand, the mechanical approach involves utilizing high-pressure homogenizer (HPH), bead milling, grinding, and ultrasonication pretreatment (Lee et al., 2017b). The advantages of using the ultrasonication approach are lower energy

consumption, which is beneficial for reducing the operation cost, greener processing, large-scale processing, and effective cell disruption to more widespread algal species (Wang et al., 2014). The principle of utilizing ultrasound is due to high shear forces arising from the cavitation bubbles of ultrasonic waves along with mechanical shearing, which promote the cell disruption of microalgae for high value-added extraction of biomolecules (e.g., proteins, lipids, and carotenoids) (Chemat et al., 2017b). The drawbacks associated with conventional extraction techniques via concentrated acid and alkali such as hydrochloric acid (HCl), dimethyl sulfoxide ($(CH_3)_2SO$), methanol (CH_3OH), dichloromethane (CH_2Cl_2), sodium hydroxide (NaOH), and acetone ($(CH_3)_2CO$). The characteristics of these concentrated solvents are noxious and hazardous, which are not suitable as direct supplement for humans. Alternative extraction techniques, such as supercritical CO_2 extraction and ionic-liquids-mediated extraction, are often subjected to high processing costs. Based on literature studies, ultrasound-assisted extraction has been proven to be a promising and effective approach for the separation and extraction of biomolecules over conventional extraction techniques (Goula et al., 2017, Jiang et al., 2017). Besides, the concept of "Green Food Processing" is also critical to protect both the environment and consumers in the food technology industry. This is to ensure an efficient production by contributing to environmental preservation in reducing the use of water and solvent, elimination of wastewater, fossil fuels energy, and generation of hazardous substances (Chemat et al., 2017a). Therefore, in this study, the implementation of ultrasound-assisted technologies has been proposed for efficient green cell disruption to obtain the maximum recovery of astaxanthin from the dried powder of *H. pluvialis* microalgae.

Based on earlier demonstrated experiments on the extraction of astaxanthin from *H. pluvialis* microalgae utilizing liquid biphasic flotation (LBF) system and grinding as cell disruption technique (Khoo et al., 2019a), the drawback encountered was the requirement of two processing steps. A separate pretreatment and extraction were required to obtain astaxanthin, which is time-consuming and not efficient, and hence, the consideration of incorporating a cell disruptive extraction method was applied. The proposed ultrasonication assisted LBF system utilizes the benefits of ultrasonication-assisted cell disruption, along with an effective LBF which perform together to extract and recover high amounts and good quality of astaxanthin. Ultrasonic-assisted LBF has also been applied for the extraction of various other biomolecules, such as proteins, lignans, and phenolic acid and polysaccharide (Khoo et al., 2020d, Sankaran et al., 2018a, Tham et al., 2019). This demonstrates the potential of utilizing this one-step ultrasonication integrated LBF system for the recovery of natural astaxanthin from the *H. pluvialis* microalgae.

In this study, an alternative approach of integrating ultrasound-assisted technology and LBF in a single-step was conducted for the retrieval of astaxanthin from the microalgae biomass of *H. pluvialis* was investigated. A slight modification of optimal operating conditions using alcohol/salt-based LBF from the previous experiment was utilized (Khoo et al., 2019a). This study focused on the single-step method for the recovery of astaxanthin, also known as a cell disruptive integrated process, compared to the earlier reported work. The parameters investigated include the effect of untreated and ultrasonication treated, the position of ultrasound horn, the pulse mode of ultrasonication, the resting mode of ultrasonication, amplitude of

ultrasound, flowrate of air bubbles, additional flotation time after ultrasonication, the additional mass of dried powder of *H. pluvialis* biomass and scalability. Moreover, a comparative study on the recovery of astaxanthin between LBF and ultrasound-assisted LBF system was assessed.

4.3 Materials and methods

4.3.1 Materials

Dried powder of *H. pluvialis* was obtained from Qingdao China Wanwuyuan Bio-Technology Co., Ltd. HPLC grade standard astaxanthin (purity ≥97%) SML0982 was obtained from Sigma-Aldrich (Malaysia). Food grade 2-propanol (purity ≥99.9%) and ammonium sulphate were obtained from R&M Chemicals (Selangor, Malaysia). Ultrapure water produced from Milli-Q integral water was used throughout the experiment. All the chemicals used were of analytical grade.

4.3.2 Equipment setup

A modified LBF column with a diameter and height of 7 × 15 cm, and a total volume of 500 mL was used for the separation. The bottom of the glass column was modified with a grade G4 porosity sintered disk and an air inlet, which is connected to a pump for generating air bubbles into the glass column. The airflow rate was controlled using an air flowmeter (RMA-26-SSV, Dwyer, USA). Ultrasound was introduced using an ultrasound regulator device (Bandelin Sonoplus UV2200, Germany) through a titanium horn sonotrode (TT-13/FZ) operating at a frequency

of 20 kHz with maximum input power of 200 W. The calorimetric measurement of ultrasonication power (P) was referred based on a previously performed experiment (Both et al., 2014), where the actual input power from the device is converted to heat which is dissipated in the LBF system as shown in the Equation (1).

$$P = m \cdot C_p \cdot \frac{dT}{dt} \qquad (1)$$

where m is the mass of the solvent in the system (g), C_p is the heat capacity of the solvent at constant pressure (J/g/^{o}C), and dT/dt is the temperature rise per second.

4.3.3 Ultrasound-assisted LBF for the recovery of astaxanthin

Ultrasound-assisted LBF was performed using a titanium ultrasound horn located in the interface of the LBF system. The ultrasound horn was connected to an ultrasound regulator device and was set to the initial operating condition. **Figure 4.1** shows a schematic setup of the ultrasound-assisted LBF system. In this study, one-factor-at-a-time (OFAT) approach was employed to examine the effect of various operating conditions for the recovery of astaxanthin from the dried powder *of H. pluvialis*. According to the previous experiment conducted using only LBF, the optimized conditions in the alcohol/salt-based LBF achieved an optimal AS_R (78.38 ± 0.93%) from dried *H. pluvialis* that was integrated with an ultrasound-assisted system (Khoo et al., 2019a). The initial operating conditions were 1 g of dried *H. pluvialis* powder mixed with 150 mL of 350 g/L of $(NH_4)_2SO_4$ salt solution. Then, 150 mL of undiluted food-grade 2-propanol was added into the LBF system. The flotation time and air flowrate were maintained constant at 15 min and 75-80

cc/min, respectively. The ultrasonication was employed initially at 10% amplitude with maximum power (200 W) and in a continuous duration of 15 min at ambient temperature (25 ± 1 ^{o}C). The initial position of the ultrasound horn was kept at the top phase of the alcohol-rich solution. The parameters involved were the morphology of microalgae cell, the position of ultrasound horn, pulse mode of ultrasonication, the amplitude of ultrasonication, air flowrate, air flotation time, and additional mass of dried powder of *H. pluvialis*. The aim of utilizing ultrasound was to overcome the additional steps in the two-step process of the grinding method and to make it into a single-step process. The application of ultrasonication was to disrupt the thick and rigid cell wall of *H. pluvialis* biomass. **Table 4.1** shows the initial operating conditions of the ultrasound-assisted LBF system.

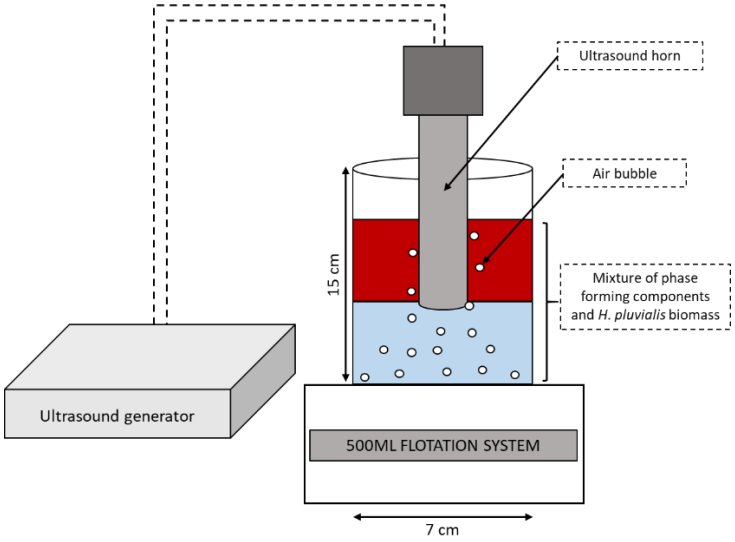


Figure 4.1: Schematic diagram of the ultrasound-assisted LBF system for the recovery of astaxanthin from *H. pluvialis* microalgae

Table 4.1: The initial operating conditions of the ultrasound-assisted LBF system

No.	Operating conditions	Initial setting	Variables	Unit	Justification
1.	The optimized LBF from the previous large-scale LBF experiment: a) 100 mg of dried *H. pluvialis* powder b) 150 mL of 100% food grade 2-propanol c) 150 mL of 350 g/L $(NH_4)_2SO_4$ d) 15 min of flotation time e) 75-80 cc/min of air flowrate			-	(Khoo et al., 2019a)
2.	Probe position	Top phase	Interface and bottom phase	-	Interface is between the top and bottom phases.
3.	Pulse mode	Non-stop	10/5, 15/5, 20/5, 25/5, and 30/5	sec	Pulse mode was applied to observe the mass transfer operation in the LBF system.

Table 4.1 (Continue)

No.	Operating conditions	Initial setting	Variables	Unit	Justification
4.	Amplitude	10	20, 30, and 40	%	The maximum ultrasound amplitude was 40%.
5.	Flowrate	75	100, 150, and 200	cc/min	An increase in the flowrate was to observe the effect in the recovery yield between air-bubble interface.
6.	Flotation time	15	20 and 25	min	Flotation time was considered to evaluate the additional recovery of astaxanthin after ultrasound radiation period.
7.	Mass of dried powder of microalgae	100	50, 75, and 125	mg	-

4.3.3.1 Comparative study between LBF and ultrasound-assisted LBF system for the extraction of astaxanthin

The extraction of astaxanthin from *H. pluvialis* microalgae was carried out using the optimized conditions of a 300 mL LBF based scale-up system from the previous work (Khoo et al., 2019a). Initially, 100 mg of dried *H. pluvialis* microalgae were assorted with 150 mL of 350 g/L of $(NH_4)_2SO_4$ solution and were poured into LBF. This was followed by gently adding 150 mL of 100% (w/w) 2-propanol into the system. The flotation time and flowrate were continuously adjusted at 15 min and 75 ± 1 cc/min, respectively. As for the ultrasound-assisted LBF system, the operation was carried out using the optimized conditions, as mentioned in Section 4.3.3, with the employed ultrasound specifications.

4.3.4 Analysis

4.3.4.1 Determination of the concentration of astaxanthin

The standard curve for astaxanthin was generated from the previous experiment by preparing multiple dilutions of astaxanthin at concentrations of 0-5 µg/mL using 2-propanol (Khoo et al., 2019a). The absorbance of astaxanthin was previously reported as OD_{478} and measured with a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan). The absorbance was recorded in triplicate by testing astaxanthin-containing alcohol. The concentration of recovered astaxanthin was calculated using the following Equation (2).

$$C_{AS(2-propanol)}(mg/mL) = \frac{OD_{478} - 0.0033}{72.78} \quad (2)$$

where $C_{AS(2\text{-propanol})}$ is the concentration of astaxanthin in 2-propanol.

4.3.5 Calculation of partition coefficient, separation efficiency, and recovery yield

The calculations of partition coefficient (*K*), separation efficiency (E_{AS}), and recovery yield (AS_R) were based on previous work (Khoo et al., 2019a). *K* is the recovered astaxanthin in the top phase over the bottom phase. The separation efficiency (E_{AS}) is the amount of astaxanthin recovered from the top phase over the astaxanthin present in the whole system. The recovery yield (AS_R) of astaxanthin is the amount of astaxanthin recovered from the total accumulation of astaxanthin in the *H. pluvialis* biomass powder.

4.3.6 Microscopic analysis of the cell morphology of *H. pluvialis* microalgae

For this study, the untreated and ultrasonication treated microalgae cells were collected and centrifuged. The pellets were then collected and resuspended and then subjected to microscopy observations. A compound microscope (Olympus CX21) with eyepiece power of 10× along with a magnification of 40× and 100× oil immersion objective was used to witness the cell morphologies. Approximately one drop of immersion oil was placed on the glass slide along with the untreated and pretreated cells for the observations. The images of cell morphologies before and after ultrasonication were captured for microscopic analysis.

4.3.7 FESEM characterization of the surface morphology of *H. pluvialis* microalgae

The surface morphology of *H. pluvialis* microalgae before and after cell disruption was analysed using Field Emission Scanning Electron Microscope (FESEM, Quanta 400, FEI, USA) with an Environmental Scanning Election Microscope (ESEM) detector. The SEM-imaging was carried out to observe the disruption of the rigid cell wall of *H. pluvialis* microalgae after employing ultrasonication as a cell disruption technique.

4.4 Results and discussion

4.4.1 Comparative study on the recovery of astaxanthin using LBF and ultrasound-assisted LBF system

A comparative study between LBF and ultrasound-assisted LBF was assessed for the extraction of astaxanthin from *H. pluvialis* microalgae. Based on the results, as shown in **Figure 4.2,** ultrasound-assisted LBF system obtained a higher AS_R (95.08 ± 3.02%) and K (185.09 ± 4.78) compared to LBF system with AS_R (78.38 ± 0.93) and K (142.58 ± 3.87). According to Khoo et al. (2019), in the alcohol/salt-based LBF, the salt solution represented the first disruptive step by osmotic shock and purifying phase (Khoo et al., 2019a). Then, the addition of alcohol as the top phase was done to extract astaxanthin biomolecules due to its lipophilicity characteristics. The LBF system promotes extraction through ascending gas bubbles, which capture the surface-active biomolecules from the

aqueous salt solution into the alcohol-rich top phase. On the other hand, an ultrasound-assisted LBF system possesses the flotation effect and demonstrates high shear forces produced from the cavitation bubbles of ultrasonic waves, which promote the cell disruption of *H. pluvialis* microalgae. It is apparent that the UALBF system for astaxanthin recovery obtained better AS_R, E_{AS}, and K values. These results were significantly higher due to the combined flotation and ultrasound effects (Dong et al., 2014, Dong et al., 2015, Sankaran et al., 2018a). Based on these results, in this study, an ultrasound-assisted LBF system was employed for various operating conditions.

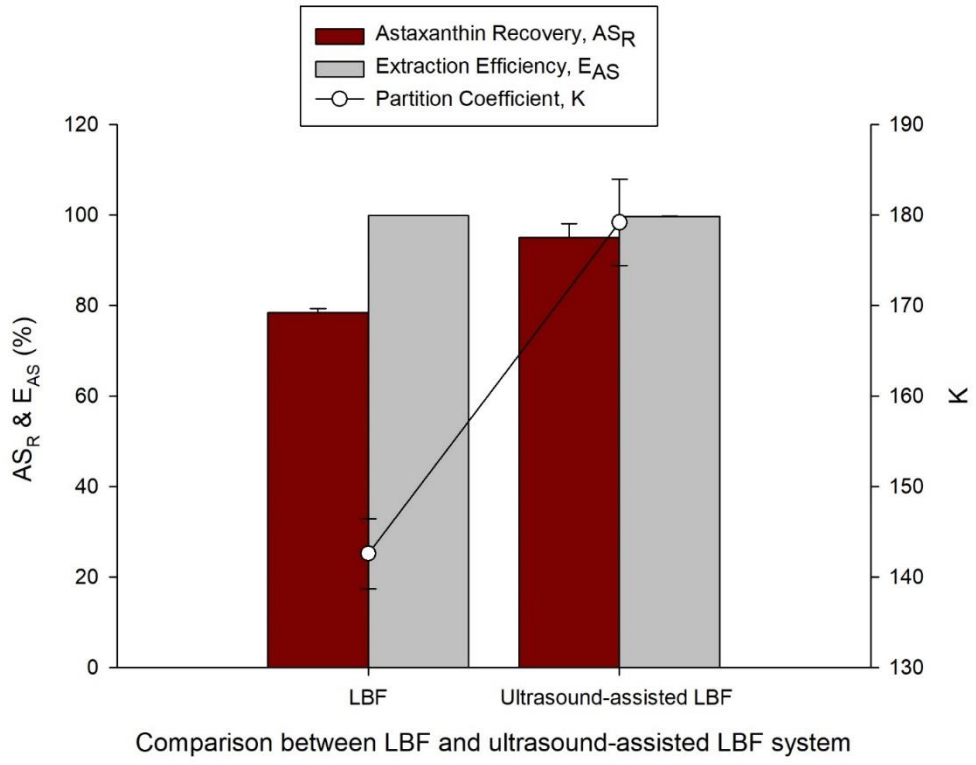


Figure 4.2: Comparative study between LBF and ultrasound-assisted LBF system for the extraction of astaxanthin from *H. pluvialis* microalgae

4.4.2 Effect of ultrasound-assisted LBF on the extraction of astaxanthin and cellular morphology

The effect of implementing ultrasound-assisted technology with the LBF system on the cell disruption intensity on *H. pluvialis* powder was examined. **Figure 4.3** shows the effect of before and after ultrasonication on the cell morphology of *H. pluvialis* microalgae at different magnification. Based on **Figure 4.3 (A1), (A2),** and (**A3)**, the microalgae cells before ultrasonication were spherical and intact with astaxanthin carotenoids in the cell wall. The rigidity of *H. pluvialis* microalgae was seen to consist of tri-layered cell wall structure, which includes trilaminar sheath, secondary wall, and tertiary walls (Praveenkumar et al., 2015). Compared to **Figure 4.3 (B1), (B2),** and (**B3),** the cellular morphology after subjected to ultrasonication showed that the cell walls of *H. pluvialis* were broken and damaged by the high shear forces produced from the cavitation bubbles of ultrasound waves. The ultrasonication effect distorts the cellular structure of the cell wall of *H. pluvialis*. Once the cell wall is broken, the intact astaxanthin in the cell wall is released, and the extraction could be facilitated by the ascending bubbles which transport astaxanthin biomolecules towards the top phase. This continuous ultrasonication process disrupts the cell wall into smaller cell debris, as shown in **Figure 4.3 (B3)**, which appears to be colourless with a ruptured cell wall. This observation was further confirmed by FESEM. Therefore, more astaxanthin will be recovered from *H. pluvialis* microalgae. This indicates that ultrasonication is an effective cell disruption technique for breaking the rigid *H. pluvialis* cell wall and has the potential when it is combined with the LBF system.

However, the concern remains at the degradation of metabolites present in the microalgae feedstock when subjected to ultrasonication treatment (Chemat et al., 2017a, Meullemiestre et al., 2016). Extraction using ultrasonication involves high temperature and pressure produced from the cavitation bubbles of ultrasonic waves. Similarly, the ultrasound horn may induce the formation of radicals, where the hydroxyl and C=C bonding of astaxanthin structure are easily degraded by the oxidation process caused by the formation of hydroperoxide due to the presence of O_2 (Goula et al., 2017). A study conducted by Sicaire et al. (2016) compared the peroxide value between ultrasound-assisted (presence of O_2) and modified-Argon ultrasound-assisted (exclude of O_2) processes for the extraction of oil from oleaginous seed (Sicaire et al., 2016). The peroxide value of the extract via ultrasound-assisted method was 11.93 ± 1.23 meq O_2/kg compared to modified-Argon ultrasound-assisted method, which has a lower peroxide value of 0.53 meq O_2/kg oil. Therefore, it is suggested that the presence of O_2 should be minimized to ensure an efficient way to prevent the degradation of metabolites during the ultrasonication extraction.

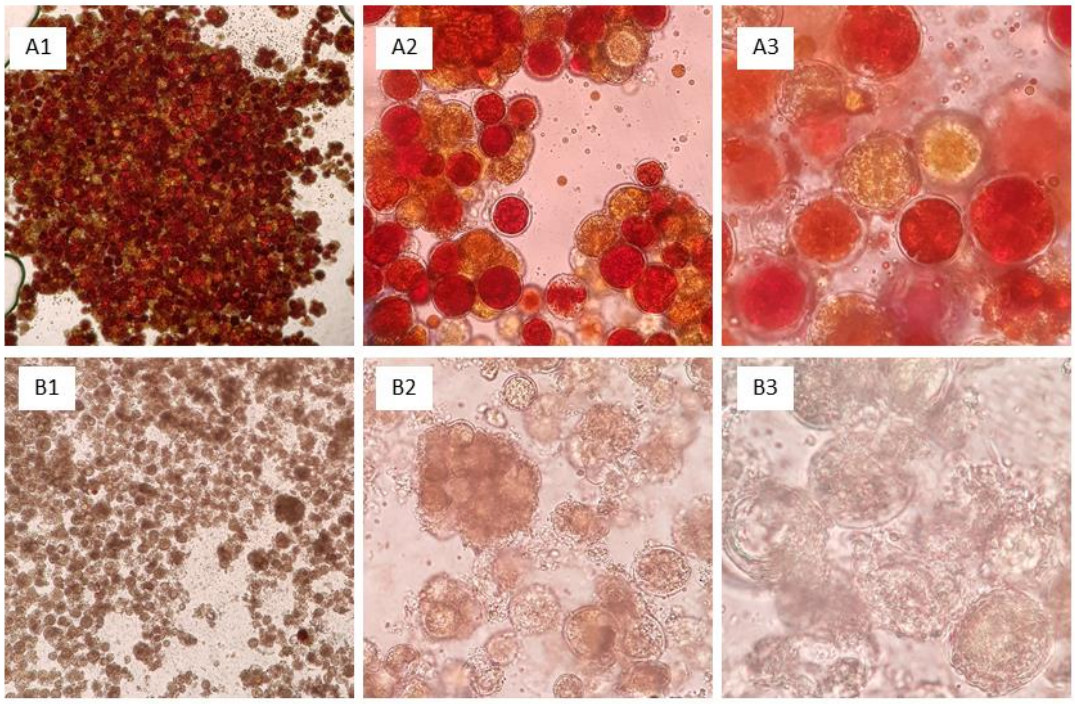


Figure 4.3: Microscopic images of cell morphology of *H. pluvialis* cell, **(A)** before ultrasonication and **(B)** after ultrasonication; where **(1)** 100x, **(2)** 400x, and **(3)** 1000x magnification.

4.4.3 Characterization and mechanistic studies on the surface morphology of *H. pluvialis* microalgae

The treated and untreated *H. pluvialis* microalgae were further subjected to FESEM analysis to examine their surface morphology after cell disruption. **Figure 4.4** shows the surface morphology before and after cell disruption utilizing an ultrasound-assisted LBF system at the magnifications of 3000×, 6000×, and 12000×. Based on **Figure 4.4 (A1)**, (**A2),** and (**A3),** the surface morphologies of *H. pluvialis* cell wall before subjected to ultrasonication were smooth, without wrinkles and cavities. This shows that astaxanthin is intact and conceals inside the cell wall of *H.*

pluvialis. Compared to **Figure B1**, **B2,** and **B3**, the surface morphologies of the cell wall of *H. pluvialis* after subjected to ultrasonication were deformed significantly, resulting in an irregular structure filled with cavities generated on the trilaminar sheath of the cell wall. This partially disrupted and porous *H. pluvialis* cell wall allows the escape of intact astaxanthin to be extracted by the organic solvent. Subsequently, the presence of ascending bubbles facilitates the transportation of surface-active astaxanthin biomolecules into the top phase. These results are in agreement with the observations shown in Section 3.2.

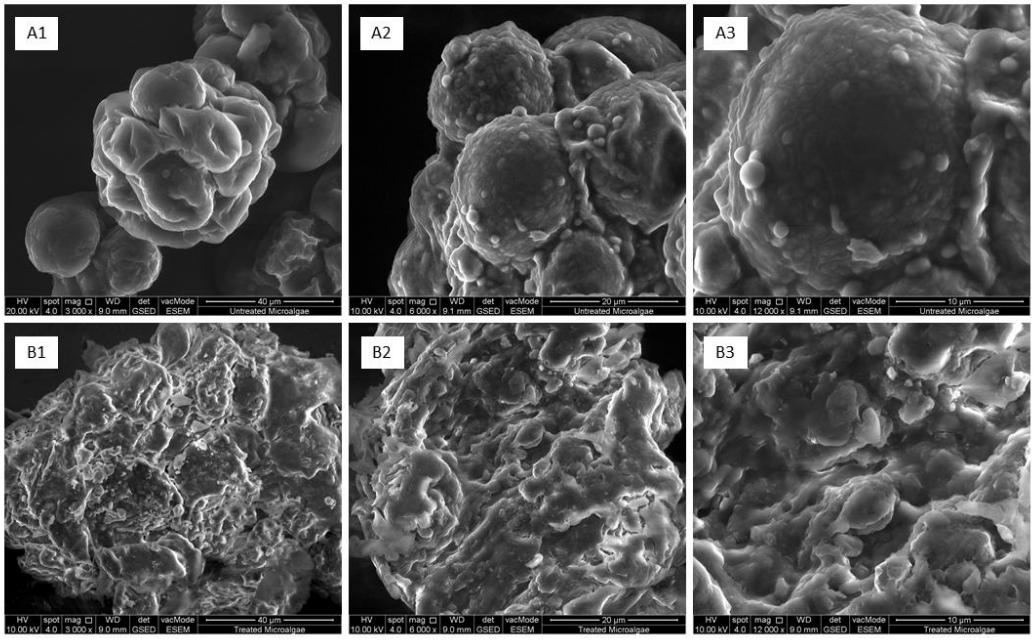


Figure 4.4: FESEM analysis on the surface morphology of *H. pluvialis* cell, **(A)** before ultrasonication and **(B)** after ultrasonication; where **(1)** 3000x, **(2)** 6000x, and **(3)** 12000x magnification.

The mechanism of ultrasonication treatment could be due to the following: combination, compression, and cavitation (**Figure 4.5**). The repetition of the above allows the cell to endure physical damage caused by the collapsing bubbles due to cavitation. Initially, the ultrasound horn generates small bubbles through the ultrasonic waves, which then attach to the cell wall of the microalgae. As more bubbles arise, the combination of bubbles increases in size and volume, which allows it to be compressed onto the surface of *H. pluvialis* cell wall. Once the bubble reaches its maximum volume, it collapses and releasing its energy in the form of high pressure and temperature, which led to the formation of small vapor-filled cavities on the surface of the microalgae cell wall (Khadhraoui et al., 2018). This eventually enhances the accessibility of astaxanthin in the cell wall to be facilitated by ascending bubbles which transport astaxanthin biomolecules towards the top phase. The severe degradation of cell walls was also observed. This showed that ultrasonication treatment has effectively damaged the cell wall for the recovery of astaxanthin from *H. pluvialis* microalgae.

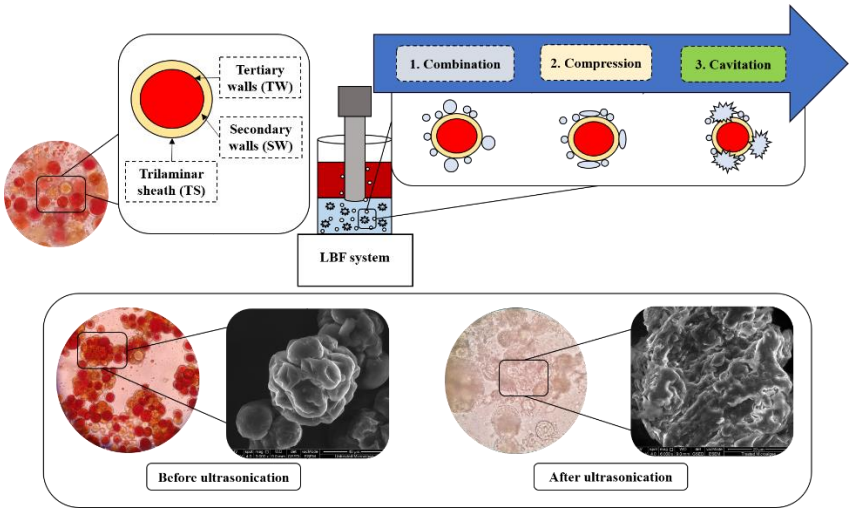


Figure 4.5: Mechanism studies on the surface morphologies of *H. pluvialis* cell wall during ultrasonication treatment.

4.4.4 Effect of the position of ultrasound horn in the LBF system

The location of ultrasound horn in the LBF system is crucial as it might affect the disruption of biomolecules, causing them to degrade and denature (Pchelintsev et al., 2016). The biphasic system consists of two phases. Hence, the position of ultrasound horn was evaluated in three different locations (i.e., alcohol-rich top phase, interface, and salt-rich bottom phase) to determine the suitable position for efficient cell disruption. The initial position of the horn was located at the top phase. According to **Figure 4.6(a)**, the maximum recovery yield of astaxanthin, AS_R was 85.30 ± 2.45% with the extraction efficiency, E_{AS} of 99.75 ± 0.0564%, and partition coefficient, K of 181.53 ± 6.88 were achieved when the ultrasound horn was in the interface of the LBF system. When the ultrasound horn was placed at the top phase, a lower AS_R (69.56 ± 1.98%) and K (155.40 ± 7.81) were observed. This was probably due to the absence of *H. pluvialis* biomass in the

alcohol-rich top phase resulting in a lower ultrasonication effect towards the biomass. Thus, when the ultrasound horn was located at the salt-rich bottom phase, the AS_R was higher as compared to the horn at the alcohol-rich top phase. However, AS_R was slightly lesser as compared to when the horn was placed in the interface. This could be due to the direct immersion of the ultrasound horn in the bottom phase, where the existing *H. pluvialis* cells were disrupted directly by the highly intense ultrasonication. On the other hand, when the ultrasound horn was placed in the interface, the cavitation energy produced by ultrasound evenly distributes between the alcohol-rich top and salt-rich bottom phases. This reduces the pressure and intensity directly towards the *H. pluvialis* cell (Sankaran et al., 2018a). Thus, the most appropriate position of the ultrasound horn was observed to be in the interface between the alcohol-rich top and salt-rich bottom phases of the LBF system.

4.4.5 Effect of continuous and pulse mode of ultrasonication and resting time

The effect of pulse and continuous mode of ultrasonication was evaluated to obtain an optimal condition for the recovery of astaxanthin from the *H. pluvialis* cell. The initial operating conditions of the ultrasonication were set at 15 min of continuous ultrasonication with an amplitude of 10%. The ultrasonication was conducted by varying the pulse modes at 10 s, 15 s, 20 s, 25 s, and 30 s with a fixed resting period of 5 s. Based on **Figure 4.6(b)**, the shorter pulse mode at 10 s/5 s gives a lower AS_R (49.41 ± 0.32%) and K (55.29 ± 10.83). This could be due to the rigidity of the *H. pluvialis* biomass cell wall, which consists of a tri-layered extracellular matrix (e.g., trilaminar sheath, secondary wall, and tertiary walls)

making the cells more resistant towards intense cell disruptive conditions (Khoo et al., 2019b, Shah et al., 2016). Based on the previous study, the yield of protein recovery from *Chlorella vulgaris* FSP-E microalgae decreases when subjected to a longer ultrasonication (Sankaran et al., 2018a). This could be due to the presence of a single cell wall in *Chlorella vulgaris* FSP-E, which degrades easily through longer ultrasonication. As more cavitation bubbles produced from the ultrasound waves start to break down, a large amount of energy is released into the medium to disrupt the cell wall of the microalgae. This study shows that a longer ultrasonication is required to produce more energy to rupture the tri-layered *H. pluvialis* cell wall into smaller cell debris to achieve a higher recovery yield of astaxanthin. According to the results, a continuous ultrasonication (15 min) achieved AS_R, E_{AS}, and K with 85.30 ± 2.45%, 99.75 ± 0.0564%, and 181.53 ± 6.88, respectively. Despite its high AS_R, the cost of the processes can be slightly higher during long-term usage compared to pulse mode condition at 30 s/5 s, which is equivalent to 12 min of ultrasonication and 3 min of resting period with AS_R, E_{AS}, and K of 83.73 ± 2.18%, 99.70 ± 0.05%, and 166.26 ± 8.35, respectively. Thereby, the pulse mode condition of 30 s/5 s (12 min of ultrasonication and 3 min of resting time) was considered for the subsequent studies.

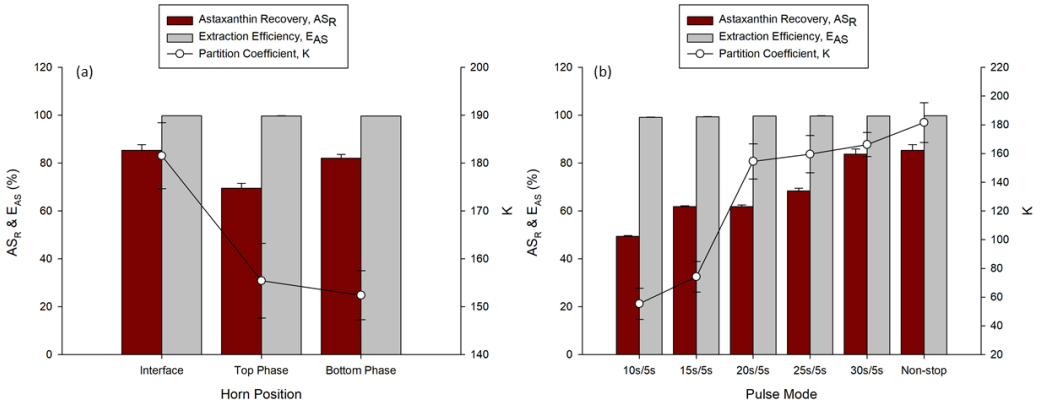


Figure 4.6: The effect of position of ultrasound horn in the LBF system and the effect of pulse mode and continuous ultrasonication on the recovery of astaxanthin from *H. pluvialis* **(a)** effect of position of ultrasound horn; **(b)** effect of pulse mode

4.4.6 Effect of the amplitude of ultrasound

The influence of amplitude of ultrasonication was evaluated to examine the energy provided to release astaxanthin from the tri-layered *H. pluvialis* cell wall. High amplitude leads to higher energy to rupture the tri-layered *H. pluvialis* cell wall. The initial operating condition was set at 10%, and the amplitude of ultrasonication was varied at 20%, 30%, and 40%. **Figure 4.7(a)** shows that the *K* decreased when the amplitude was set at 20%. This could probably due to the increment of ultrasound intensity narrowing the reaction zone on the biphasic system (Suslick, 1988). On the other hand, it is difficult to characterize and measure the forms of energy release during ultrasonication, which is one of the challenges associated while utilizing ultrasound. By changing the tip (e.g., silica and titanium) of the ultrasound horn, it also resulted in a different ultrasound intensity throughout the reaction zone. An inconsistency of increasing and decreasing of cavitation

activity could occur from the interference of multiple ultrasound waves in the biphasic system. Based on these findings, an increase in the amplitude enhanced the *K* of astaxanthin in the LBF system. At the amplitude of 40%, the AS_R, E_{AS}, and *K* successfully achieved 89.44 ± 1.06%, 99.74 ± 0.04%, and 182.37 ± 3.83, respectively. Therefore, an amplitude of 40% was selected for the remaining experiments.

4.4.7 Effect of air flowrate and flotation time in the LBF system

It is significant to evaluate the air flowrate as this parameter contributes to the area of air-water interface per unit volume of the biphasic solution. The initial operating condition was set at 75 cc/min for 15 min of flotation time. The air flowrate in the LBF was varied within 75 cc/min to 200 cc/min. **Figure 4.7(b)** shows that the flow rate at 200 cc/min achieved a low AS_R and *K* of 70.99 ± 8.92% and 139.25 ± 2.88, respectively. Based on Pakhale's prediction, the AS_R and *K* should increase proportionally with the air flowrate owing to the increment in the interfacial area and turbulence (Pakhale et al., 2013). The trend demonstrates that as the air flowrate increased from 75 cc/min to 100 cc/min, both AS_R and *K* increased. However, a decrement of AS_R and *K* was shown when the air flowrate exceeded 100 cc/min. This could be due to the high intensity of aeration that caused the biomass to move randomly in the biphasic system. Another factor that results in the low AS_R and *K* could be due to the cavitation phenomenon from the ultrasound wave, which might be reduced due to the cohesion of bubbles and sound waves. As the air flowrate increases, more bubbles will be aerated out from the system, which causes

a cohesion effect between the bubbles and the waves. This showed that a higher air flowrate in the LBF system might cause collision and disturb the cavitation process which eventually affects the cell disruption intensity, leading to a lower AS_R and K. The highest AS_R value (95.08 ± 3.02%) and K (185.09 ± 4.78) were achieved at an air flowrate of 100 cc/min. Therefore, an air flowrate of 100 cc/min with a flotation of 15 min was the optimal in the LBF.

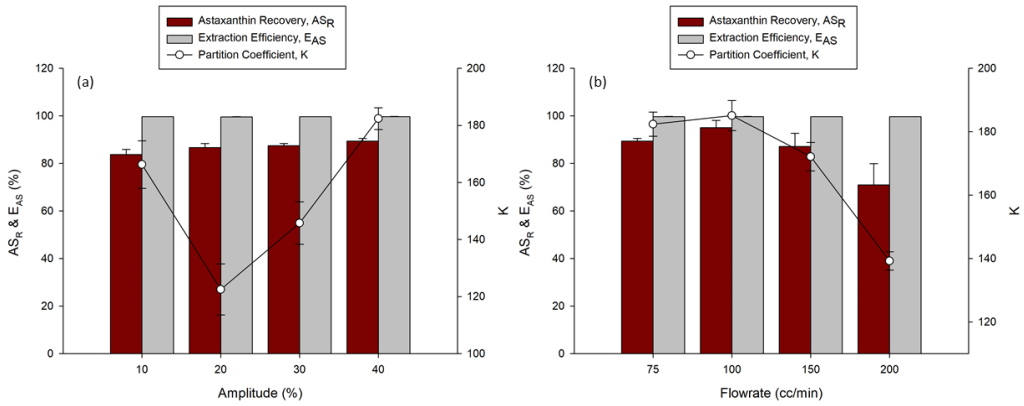


Figure 4.7: The effect of amplitude of ultrasonication and flowrate for the recovery of astaxanthin from *H. pluvialis* **(a)** effect of amplitude of ultrasonication; **(b)** effect of flow rate (cc/min)

Another critical aspect of air flotation time was evaluated as the air-water interface per unit volume was found to be influenced by the flotation time (Leong et al., 2018). In this study, the flotation time was evaluated within 15 to 25 min in the LBF system consisted of 150 mL of 2-propanol and 150 mL of 350 g/L $(NH_4)_2SO_4$ for the recovery of astaxanthin. The flotation time below 15 min was not

carried out as the ultrasonication period was set initially within 15 min. Based on **Figure 4.8(a)**, the AS_R and K decreased as the flotation time increased from 15 to 25 min. This was supported by Phong et al. (2017), who reported similar findings on the effect of flotation time, whereby extending the period does not necessarily improve the recovery yield (Phong et al., 2017a). The highest AS_R value (95.08 ± 3.02%) and K (185.09 ± 4.78) were obtained at a flotation time of 15 min. Based on the results obtained, 15 min of flotation time was selected as the suitable period of aeration for the recovery of astaxanthin.

4.4.8 Effect of the mass of dried powder of *H. pluvialis* microalgae

Based on earlier literature studies, it was identified that the mass of feedstock might affect the recovery yield and partition behaviour of organic molecules in the LBF system (Chew et al., 2019a, Chia et al., 2019, Leong et al., 2018). An increase in the mass of *H. pluvialis* biomass would create a more viscous biphasic system, where the high viscosity of the system will cause uneven partitioning effect resulting in a lower efficiency on the partition behaviour and separation efficiency of astaxanthin. Hence, this decreases AS_R and K in the ultrasound-assisted alcohol/salt-based LBF system. The initial mass of biomass was 100 mg of dried powder of *H. pluvialis* in a 300 mL LBF system. For this parameter, the mass of biomass was varied within 50 to 125 mg. Based on **Figure 4.8(b)**, the mass of 125 mg dried powder of *H. pluvialis* biomass attained a low AS_R (57.12 ± 0.03%) and K (56.01 ± 6.31). A higher mass caused the partitioning of astaxanthin to decrease due to the aggregation of unwanted products (i.e., contamination and impurities) within the

biphasic system. Moreover, a higher mass of dried powder of *H. pluvialis* biomass led to difficulties in controlling the air bubbles due to higher viscosity of the solution in the biphasic system. This limits the efforts from the bubbles to carry astaxanthin from the bottom to the top phase as there will be more astaxanthin due to the increase in the biomass. According to the results, the mass of 100 mg of *H. pluvialis* biomass achieved a maximum AS_R and K in the ultrasound-assisted LBF.

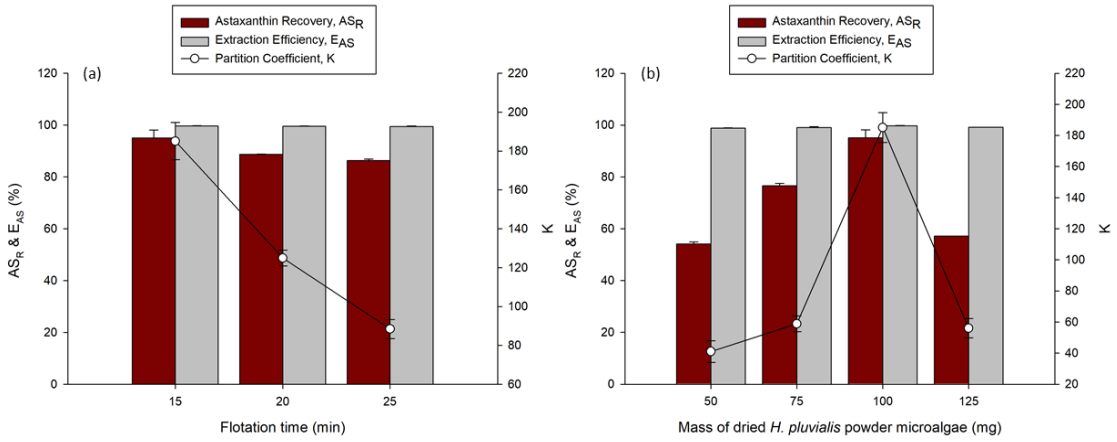


Figure 4.8: The effect of flotation time and the mass of dried powder of *H. pluvialis* biomass on the recovery of astaxanthin from *H. pluvialis*. **(a)** effect of flotation time (min); **(b)** effect of the mass of dried *H. pluvialis* biomass microalgae (mg)

4.4.9 Scale-up of ultrasound-assisted LBF system

The objective of this scale-up operation was to investigate the dependability and productiveness of this integrated system. The operating conditions for this scale-up study were based on the parameters optimized using a small-scale system. **Table 4.2** shows a comparison study between the initial small-scale system and scale-up

approach for the alcohol/salt-based ultrasound-assisted LBF system. In this scale-up study, a total working volume of 1.5 L system composed of 500 mg of dried powder of *H. pluvialis* biomass, 0.75 L of 100% 2-propanol, and 0.75 L of 350 g/L of $(NH_4)_2SO_4$ salt solution were utilised. Based on the outcome, it has been found that increasing the system into a larger volume causes AS_R and K values to decrease by about 12% and 27%, respectively. The scale-up ultrasound LBF system achieves a relatively lower AS_R (83.73 ± 0.70%) and K (157.83 ± 7.47) values compared to the small-scale ultrasound-assisted LBF. This demonstrates that the large-scale operation of ultrasound-assisted LBF under an optimized parameter can achieve better performance by proper optimization to enhance the scale-up of the system without compromising AS_R. Thus, this scale-up approach revealed the potential of ultrasound-assisted LBF for the recovery of astaxanthin from *H. pluvialis* microalgae to be reliable at a larger-scale, and it is found to be feasible to be applied in the industry.

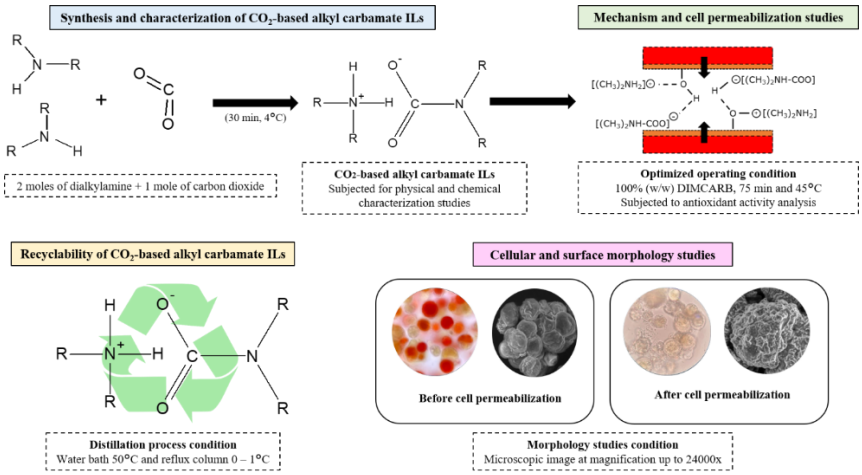
Table 4.2: Comparison study between the small- and large- scale ultrasound-assisted LBF system

Ultrasound-assisted LBF system	Total working volume (L)	AS_R (%)	K
Small-scale	0.3	95.08 ± 3.02	185.09 ± 4.78
Scale-up	1.5	83.73 ± 0.70	157.83 ± 7.47

4.5 Conclusion

An alternative single-step approach for the cell disruption of microalgae utilizing an ultrasound-assisted LBF system for the recovery of natural astaxanthin from *H. pluvialis* was performed. This integrated approach provides a one-step process for the effective recovery of astaxanthin compared to conventional extraction techniques. The optimal conditions of the ultrasound-assisted LBF system successfully achieved a maximum AS_R, E_{AS}, and K of 95.08 ± 3.02%, 99.74 ± 0.05%, and 185.09 ± 4.78, respectively. Moreover, the scale-up approach was conducted to investigate the reliability of this integrated approach for the extraction of biomolecules in the biotechnology industries.

CHAPTER 5 PERMEABILIZATION OF *HAEMATOCOCCUS PLUVIALIS* AND SOLID-LIQUID EXTRACTION OF ASTAXANTHIN BY CO2-BASED ALKYL CARBAMATE IONIC LIQUIDS



This chapter present the green and sustainable extraction approach using CO_2-based alkyl carbamate ionic liquids as a promising solvent to permeabilize the robust microalgae cell wall for an effective extraction of astaxanthin. Various parameters such as type of CO_2-based IL, concentration of IL, extraction time and temperature were optimized for the maximal extraction of astaxanthin. Recyclability studies of the selected CO_2-based alkyl carbamate ionic liquids were studied to verify its feasibility for multiple extraction cycles. This chapter consists of thesis-version of work published in the *Chemical Engineering Journal* (Khoo, K. S. et al., 2021, *Chemical Engineering Journal*, 411: 128510. DOI: https://doi.org/10.1016/j.cej.2021.128510).

Permeabilization of *Haematococcus pluvialis* and solid-liquid extraction of astaxanthin by CO_2-based alkyl carbamate ionic liquids

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Authors' Contributions:

K.K.S. performed all the experiments; C.W.O., K.W.C., S.C.F. and J.W.L provided guidance in experimental design and materials; K.K.S., C.W.O. and K.W.C. analyzed and interpreted the data; K.K.S. wrote the manuscript; C.W.O., Y.T., N.J., S-H.H. and P.L.S. edited the manuscript.

5.1 Abstract

Haematococcus pluvialis (*H. pluvialis*) microalgae are well known for their high content of astaxanthin, which is a super potent antioxidant with various remedial effects. Ionic liquids (ILs) have been proposed as a promising solvent to permeabilize the robust microalgal cell wall for an effective extraction of bioactive compounds. Nonetheless, the removal of ILs from the extracted compounds requires the additional treatment steps that complicate the overall extraction process and the recycling of ILs. To address these shortcomings, we demonstrated a sustainable extraction system that utilizes CO_2-based alkyl carbamate ILs to permeate *H. pluvialis* cells and extract astaxanthin. This class of ILs is readily distillable for an easy recovery of ILs from the extraction medium. Among the tested CO_2-based alkyl carbamate ILs [dimethylammonium dimethylcarbamate (DIMCARB), dipropylammonium dipropylcarbamate (DPCARB), dibutylammonium dibutylcarbamate (DBCARB) and diallyammonium diallycarbamate (DACARB)], DIMCARB gave the best performance of astaxanthin extraction. The optimized extraction conditions [100% (w/w) of DIMCARB, 75 min and 45°C] resulted in the high yield of astaxanthin (27.99 mg/g). The capability of DIMCARB to permeabilize the amorphous trilayered structure of cell wall was verified by the presence of pores on the cell surface of *H. pluvialis* as shown in the microscope images. The performance and recyclability of DIMCARB extraction system were also evaluated by conducting three successive rounds of astaxanthin extraction with the recycled DIMCARB solutions.

In addition, the antioxidant capacity of the extracted astaxanthin was well retained, showing that this type of distillable IL was suitable for the extraction of phytonutrients from algal source.

Keywords: Ionic liquids; Dialkyl carbamate; Astaxanthin; *Haematococcus pluvialis*; Extraction; CO_2

5.2 Introduction

Astaxanthin, or chemically known as 3,3′-dihydroxy-β,β′-carotene-4,4′-dione, is a secondary carotenoid widely recognized for its highly antioxidant capacity and its potential applications in food, cosmetic, aquaculture, nutraceutical and pharmaceutical industries (Khoo et al., 2019b). The robust antioxidant activity of astaxanthin deactivates the free radicals in human body, and astaxanthin was reported to be effective in the prevention of cancer, cardiovascular, inflammatory and neurodegenerative diseases (Naguib, 2000). Moreover, a recent review article highlights the putative pathogenesis of natural astaxanthin and the potential of astaxanthin in alleviating health adversaries triggered by Coronavirus disease 2019 (COVID-19) (Talukdar et al., 2020). However, the production of synthetic astaxanthin involves petrochemicals, which have always concerned consumers due to the inherent toxicity of these raw materials (Li et al., 2011a, Milledge, 2011). Synthetic astaxanthin has been utilized as feed additives in aquaculture sector but its application in food for human consumption has been impeded by the lack of human clinical trials showing its potential benefits.

Natural sources of astaxanthin including microalgae, yeast and crustacean's shells could be exploited without the safety concerns as mentioned above. *Haematococcus pluvialis* (*H. pluvialis*) is a microalgal strain that contains the highest accumulation of astaxanthin (≥ 3.8 - 5.0% of dried weight under the optimal accumulation condition) as compared to other microalgae such as *Chlorococcum*, *Chlorella zofingiensis* and *Neochloris wimmeri* in which the astaxanthin accumulation is in the range of 0.001 – 0.2% of dried weight (Ambati et al., 2014,

Wayama et al., 2013). Extraction of natural astaxanthin from *H. pluvialis* is a great challenge due to the robust microalgal cell structure; the amorphous multi-layered extracellular matrix (i.e., trilaminar sheath, secondary and tertiary wall) hinders the release of astaxanthin deposited in lipid droplets in the cytoplasm of cell (Wayama et al., 2013).

Majority of the studies related to the extraction of astaxanthin from *H. pluvialis* adopt the conventional volatile organic solvents (e.g., methanol and ethanol) in large quantity or the concentrated acid and alkaline to disrupt the microalgal cells (Sarada et al., 2006). Despite the high yield of product attained by the conventional solvent extraction, the limitations associated with the inherent toxicity of organic solvents, environmental impacts and sustainability have been frequently overlooked (Khoo et al., 2019b). The usage of green and non-toxic solvents in the processing of food and pharmaceutical ensures the safety of products for direct human consumption. In fact, the proper disposal or treatment of volatile solvents is highly desirable for minimizing the environmental footprint of extraction process (Ni et al., 2007). Extraction of astaxanthin has been attempted by using the emerging extraction technologies such as supercritical CO_2 extraction, ultrasound-, microwave- and magnetic-assisted extraction systems, and liquid biphasic systems made of ionic liquids (ILs) (Khoo et al., 2019a, Choi et al., 2019, Khoo et al., 2020c, Zhao et al., 2016a, Cheng et al., 2018). Up till now, the major bottleneck in the extraction of natural astaxanthin lies on the energy- and cost-intensive extraction conditions (i.e., expensive solvents; ultrasonication frequencies: ≥ 20 kHz; temperature: ≥ 70°C; pressure: 30 – 55MPa) that could damage these highly

sensitive carotenoids (Khoo et al., 2019a, Bustamante et al., 2011, Zhao et al., 2016a, Desai et al., 2016). In addition, the high cost of these energy-intensive processes is less economical at the industrial or large-scale operation.

ILs are well known for their distinctive properties like the tuneable chemical properties, high thermal stability, low vapour pressure and low melting point. Hence, they could function effectively as additives, alternative solvent, or electrolytes in the applications such as catalysis, biomass processing, and synthesis of pharmaceuticals and nanomaterials (Khoo et al., 2020f). The early adoption of ILs in the practical applications has mainly targeted on the traditional chemical industries. With the greater exploration of ILs over the years, ILs with the biodegradable and green properties have been gaining the attention of scientific community. These desirable features not only unlock the potential of green ILs in the formulation or processing of food and medical products, but also mitigate the environmental impact of ILs (Vekariya, 2017). ILs comprising the imidazolium- and pyridinium-based cations such as 1-ethyl-3-methylimidazolium-based ILs such as [Emim][DBP], [Emim][Cl], [Emim][BF_4], [Emim][$AlCl_4$], [Emim][CH_3SO_3], [Emim][SCN], [Emim][$EtOSO_3$], [Emim][DEP] and [Emim][OAc] have been used in the cell permeabilization and extraction of astaxanthin from *H. pluvialis* (Desai et al., 2016, Choi et al., 2019). However, the removal of ILs from the extracted astaxanthin remains a challenge because these ILs could not be distilled and evaporated like the organic solvents. The environmentally benign ILs made of cholinium cation and amino acid anion, or known as cholinium aminoates, can be a

potential substituent of these conventional ILs, but the recyclability of this IL is restricted.

CO_2-based alkyl carbamate ILs are a class of ILs utilizing CO_2 gas and secondary dialkylamine to form the dialkylammonium cation and dialkylcarbamate anion. They are readily distillable at a moderate temperature (55 – 200°C) depending on the length of the alkyl chain. In addition, CO_2-based alkyl carbamate ILs have the ability to dissolve a number of organic compounds and they can be easily evaporated from the extracted product (Vijayaraghavan and Macfarlane, 2014). This advantageous feature of ILs allows an easy separation of the target biomolecules from the ILs. They have also been successfully applied in electrochemical, biochemical, extraction and catalytic processes (Feroci et al., 2007, Peled et al., 2011, Zhou et al., 2007).

Here we addressed the challenges of IL separation from the extracted astaxanthin by adopting the CO_2-based alkyl carbamate ILs as an extractant. To authors' best knowledge, CO_2-based alkyl carbamate ILs have not been applied in the extraction of astaxanthin from *H. pluvialis*. The synthesized CO_2-based alkyl carbamate ILs were characterized using Fourier Transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC). The extraction of astaxanthin from *H. pluvialis* was optimized using one-factor-at-a-time (OFAT) approach on various operating parameters, namely type of CO_2-based IL, concentration of IL, extraction time and temperature. The IL-treated microalgae were subjected to the surface morphology analysis by a compound microscope and Field Emission Scanning Electron Microscope

(FESEM). Recyclability studies were also conducted to determine the efficiency of the recycled ILs for multiple rounds of extraction. In addition, the antioxidant properties of the extracted astaxanthin were evaluated using Trolox equivalent antioxidant capacity (TEAC) and total phenolics content (TPC).

5.3 Experimental Section

5.3.1 Materials

H. pluvialis intact cell powder was purchased from Qingdao China Wanwuyuan Bio-Technology Co., Ltd. (China); the content of astaxanthin in these freeze-dried cells was ≤ 3%. Standard astaxanthin (≥ 97% HPLC grade) was purchased from Sigma Aldrich (Germany). Dimethylammonium dimethylcarbamate (DIMCARB), dipropylamine, dibutylamine, diallyamine (≥ 99% purity), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), Folin-Ciocalteu (F-C) reagent, sodium carbonate (Na_2CO_3) and gallic acid were purchased from Sigma Aldrich (St. Louis, USA). Methanol (99.9% purity, HPLC grade) was obtained from Fisher Chemical (Selangor, Malaysia). Pallet dry ice was purchased from Synergy Dry Ice (Selangor, Malaysia). Ultrapure water generated from the water purification system (Milli-Q, Merck, Darmstadt, Germany) was used throughout the experiments.

5.3.2 Synthesis of CO_2-based alkyl carbamate ILs

Figure 5.1 shows the types of CO_2-based alkyl carbamate ILs used in this study. The synthesis of dipropylammonium dipropylcarbamate (DPCARB), dibutylammonium dibutylcarbamate (DBCARB) and diallyammonium diallycarbamate (DACARB) was based on the procedures reported elsewhere (Vijayaraghavan and Macfarlane, 2014, Song et al., 2018b). In brief, 2 moles of dialkylamine solution (e.g., dipropylamine, dibutylamine and diallyamine) were first transferred to a 250-mL round-bottom flask incubated in an ice bath (4°C). Next, the addition of 1 mole of pallet dry ice into the flask was done gradually to avoid the build-up of pressure in the flask. The synthesis reaction took place for ~30 min or until the added dry ice has completely sublimated into gas form. Theoretically, the secondary amines (R_2NH) are primarily in the ionic form due to their Lewis acid-base behaviour. As shown in Equation (1), the lone pair of nitrogen in secondary amines reacted with CO_2; this rendered the nonpolar R_2NH compound to the polar R_2N-COOH. Subsequently, the resulted carboxylic acid reacted with another secondary amine via H-proton exchange, resulting in the strong intermolecular force between dialkylammonium cation and dialkylcarbamate anion, as illustrated in Equation (2). The transfer of hydrogen ion from the carbamic acid was an exothermic reaction and the IL was obtained at liquid state at room temperature.

$$R_2NH + CO_2 \leftrightarrow R_2N\text{-}COOH \quad (1)$$

$$R_2N\text{-}COOH + R_2NH \leftrightarrow [R_2N\text{-}COO^-][R_2NH_2^+] \quad (2)$$

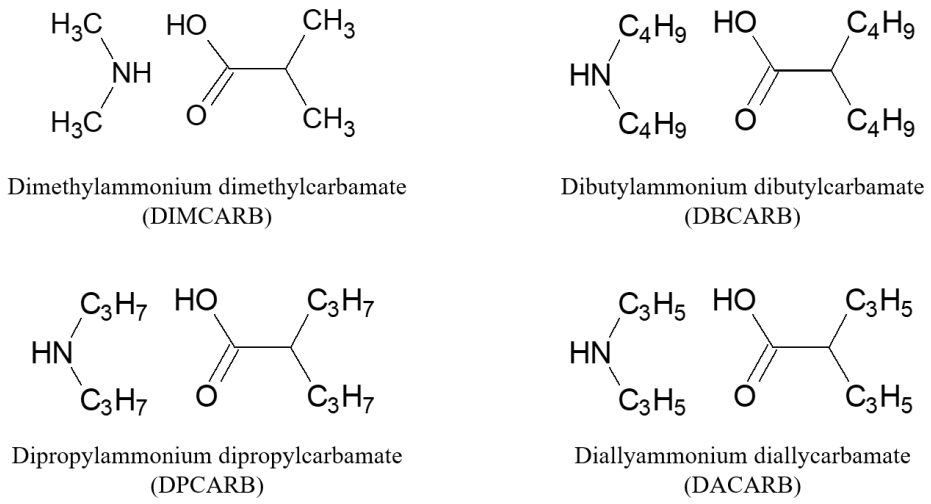


Figure 5.1: Chemical structures of CO_2-based alkyl carbamate ILs used in this study (Song et al., 2018b).

5.3.3 Characterization of CO_2-based alkyl carbamate ILs

The synthesized CO_2-based alkyl carbamate ILs were characterized in terms of structural properties, purity, and functional groups by a FTIR spectroscopy (Frontier, Perkin Elmer) using an average of 32 scans over 4000 – 400 cm^{-1}. The FTIR spectra are shown in **Figure Appendix 1** to **5**. The procedures and details of the characterization studies are given in the Supporting Information. The purity of the synthesized CO_2-based alkyl carbamate ILs was determined using a NMR spectrometer (Advance III 500 MHz, Bruker). 500 µL of MeOH-d_6 standard solution was mixed homogenously with 200 µL of ILs samples before being transferred into an NMR sample tube. The ^{1}H and ^{13}C NMR spectra were recorded at 25°C. The chemical shift (δ) was reported in parts per million (ppm) unit and the NMR spectra are shown in **Figure Appendix 6 to 9**.

The physicochemical properties of CO_2-based ILs such as density and viscosity properties were examined using a density meter (DMA 4500M, Anton Paar) and a viscometer (Brookfield DVE Viscometer). To analyse the thermal properties, the synthesized CO_2-based alkyl carbamate ILs were subjected to a differential scanning calorimeter (DSC Q2000, TA Instruments) operated over a temperature range of 30 – 200°C at 10 K/min of scanning rate and 20 mL/min of N_2 cooling rate. The result is shown in **Figure Appendix 10**. **Table 5.1** summarizes the properties of CO_2-based alkyl carbamate ILs.

Table 5.1: Properties of CO_2-based alkyl carbamate ILs.

CO_2-based alkyl carbamate ILs	Reference temperature [a], T_r (°C)	Thermal decomposition, T_d (°C)	Crystallization temperature [b], T_C (°C)	Distillation point [c], (°C)	Heat of distillation [c], (kJ/mol)	Density (g/cm^3)	Specific gravity	Viscosity (mPa s^{-1} / 25°C ± 1)
DIMCARB	73.67	84.67	-72.79	71	128	1.05	1.04	118.20 at 20 rpm [d]
DPCARB	94.33	98.00	-26.20	52	123	0.87	0.88	4.92 at 100 rpm
DBCARB	151.67	156.33	-37.30	59 - 176	127	0.82	0.83	9.63 at 100 rpm
DACARB	102.33	105.17	-32.01	59 - 113	128	0.90	0.89	10.62 at 100 rpm

[a] Reference temperature refers to the sample that began to decompose or to lose weight. [b] Heat was released (exothermic process) where heating rate was 10 $K.min^{-1}$. [c] Values adopted from Vijayaraghavan and Macfarlane (2014). [d] Stirring speed above 30 rpm was unable to be determined by the Brookfield DV-E viscometer due to the high viscosity of the DIMCARB.

5.3.4 Cell permeabilization and extraction of astaxanthin from *H. pluvialis*

The operating parameters such as various type of CO_2-based alkyl carbamate ILs, concentration of DIMCARB, incubation period and temperature were evaluated for their performances in permeabilization of microalgae biomass. These parameters were optimized using OFAT approach. **Table 5.2** shows the initial settings and variables of operating conditions for the cell permeabilization and the extraction of astaxanthin using CO_2-based alkyl carbamate ILs. Approximately 10 mg of dried *H. pluvialis* biomass was treated with 3 mL of ILs. The cells were separated from the ILs by centrifuging the mixture at 7500 rpm for 10 min. Methanol was selected as the co-extractive solvent due to its favourable solvent properties (e.g., dielectric constant and polarity index) towards the semi-polarity characteristics of astaxanthin (Foo et al., 2015). The permeabilized cells were immersed in 10 mL of methanol for 30 min at 40°C to extract the remaining astaxanthin from the cell. The mixture was re-centrifuged to remove the methanol, and the fresh methanol was added to the cells followed by vortexing for 5 min. The extracted methanol fractions were pooled along with the CO_2-based ILs to measure the total amount of astaxanthin extracted. A rotary evaporator was used to distillate the solvent and ILs from the astaxanthin. The astaxanthin was then resuspended and diluted with 3 mL of methanol to measure the amount of astaxanthin extracted. The sample was filtered using a 0.45µm PTFE disposable syringe-filter (Terumo, Malaysia) and was left overnight at 4°C before subjected to HPLC analysis.

Table 5.2: Initial settings and variables of operating condition for the extraction of astaxanthin from *H. pluvialis*.

Operating conditions	Units	Initial setting	Variables
Type of ILs	-	100% (w/w)	DIMCARB, DBCARB and DACARB
Concentration of DIMCARB	% (w/w)	100	90, 80, 70, 60 and 50
Time	Min	60	30, 45, 75 and 90
Temperature	°C	25	35, 45 and 55

5.3.5 Quantification of astaxanthin

The extracted astaxanthin was analysed using a high-performance liquid chromatography (HPLC) system (1200 Infinity, Agilent Technologies) coupled with variable wavelength detector. The samples were analysed using a ZORBAX Eclipse Plus C_{18} column (4.6 ×100 mm, 3.5 µm). The mobile phase is a solvent mixture made of methanol and water (97:3) filtered through a 0.45 µm membrane filter and degassed before use. The HPLC analysis was performed under the isocratic condition at room temperature for 5 min. The injection volume and flow rate were set at 20 µL and 1.0 mL/min, respectively. The chromatograms and absorbance values were recorded at 480 nm with the photodiode-array detection system. The peak area of the extracted astaxanthin was quantified using a calibration curve prepared from 1 mg/mL of standard astaxanthin (chromatograms are shown in **Figure Appendix 11**).

5.3.6 Microscopic analysis of surface morphology of *H. pluvialis*

The treated *H. pluvialis* cells were centrifuged, and the pellets were collected for the surface morphology analyses. The cell morphology was visualized using a compound microscope (CX21, Olympus) with an eyepiece power of 10× and two different objective lenses with 40× and 100× (oil immersion) magnification. The cells were also characterized under a higher magnification to scrutinise the surface morphology before and after the cell permeabilization using the ILs. A FESEM (Quanta 400, FEI) equipped with an Environmental Scanning Electron Microscope (ESEM) detector was used to perform the SEM imaging.

5.3.7 Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) of the astaxanthin extracted from *H. pluvialis* microalgae was evaluated in accordance to the ABTS radical ($ABTS^{\bullet}$) assay as adopted from other reported studies with minor modification (Régnier et al., 2015, Eren et al., 2019). The $ABTS^{\bullet}$ stock solution was freshly prepared by mixing 7 mmol/L of ABTS solution and 2.45 mmol/L of $K_2O_8S_2$ solution at 1:1 of volume ratio. The stock solution was incubated for 16 – 24 h at room temperature under dark condition. Then, the $ABTS^{\bullet}$ stock solution was diluted with methanol until an absorbance value of 0.70 ± 0.05 was achieved at OD_{734} and was later stored at room temperature. TEAC assay was conducted by mixing 100 µL of sample or Trolox solution (standard) or methanol (control) with 3.8 mL of the diluted $ABTS^{\bullet}$ solution. The sample was left incubated for 5 min at room temperature. The sample was analysed using a UV-Vis spectrophotometer (UV-1800, Shimadzu) at OD_{735}. The results were expressed as µmol of Trolox equivalents per g of *H. pluvialis* microalgae crude extract (TEs/g crude), where the percentage of scavenging on $ABTS^{\bullet}$ was calculated using Equation (3) (Régnier et al., 2015):

$$Scavenging\ percentage\ (\%) = \frac{Control - Sample\ or\ Standard}{Control} \qquad (3)$$

5.3.8 Total phenolics content analysis

Total phenolics content (TPC) of the astaxanthin extracted from *H. pluvialis* was analysed by F-C approach based on the past studies with minor modification

(Hossain et al., 2017, Hajimahmoodi et al., 2013). The diluted F-C reagent was freshly prepared by mixing the F-C reagent and the purified water at a volume ratio of 1:9. 100 µL of extracted astaxanthin or gallic acid (i.e., standard) was mixed with 500 µL of the diluted F-C reagent. The mixture was incubated for 5 min at room temperature under dark condition. An additional 2 mL of 75 g/L Na_2CO_3 solution was added into the mixture, followed by settling for 90 min at room temperature under dark condition. The sample was measured at OD_{760} using a UV-Vis spectrometer. The results of TPC were expressed as mg of gallic acid equivalents (GAs) per 100 g of *H. pluvialis* microalgae crude extract.

5.3.9 Recyclability studies on DIMCARB

The DIMCARB was distilled from the sample using a rotary evaporation following the conditions reported elsewhere (Song et al., 2018b, Khoo et al., 2020e). The sample (i.e., extracted astaxanthin and DIMCARB) was heated to 50°C, and the vapor was condensed at 0°C – 1°C. The recovered DIMCARB was characterized with ^{13}C NMR to evaluate the purity and structural properties of the IL. The recovered DIMCARB was subjected to the new batch of cell permeabilization studies. If necessary, an appropriate amount of fresh DIMCARB was added to the mixture for compensating the minor losses of DIMCARB during the distillation process. Recyclability of DIMCARB was tested by conducting three cycles of astaxanthin extraction using the recovered DIMCARB.

5.3.10 Statistical analysis

The yield of astaxanthin was obtained from the triplicate reading, and the values were expressed as mean ± standard deviation. The data was also subjected to one-way analysis of variance (ANOVA) followed by Tukey HSD *post-hoc* test using Microsoft Excel to evaluate the significant differences ($p \leq 0.05$).

5.4 Results and discussion

5.4.1 Effects of the type of CO_2-based alkyl carbamate ILs on astaxanthin extraction

Four different types of CO_2-based carbamate ILs were evaluated for their performances in dissolving the astaxanthin from the *H. pluvialis* that had not undergone the intensive mechanical cell disruption. The chemical structural and functional group of the synthesized DPCARB, DBCARB and DACARB was verified using NMR and FTIR before subjecting for the extraction process. The yield of astaxanthin was expressed as mg of astaxanthin extracted from 1 g of *H. pluvialis* biomass. According to **Figure 5.2**, DIMCARB gave the highest yield of astaxanthin (12.47 ± 0.30 mg/g) as compared to DPCARB, DBCARB and DACARB. This shows that the permeability of CO_2-based alkyl carbamate ILs into the cell wall of *H. pluvialis* was affected by the chemical properties of cation and anions. The screening results showed the promising potential of DIMCARB as a cell-permeabilizing solvent to extract astaxanthin. In a past study of astaxanthin extraction using a two-step approach, the mechanically disrupted cells were pretreated by acid and alkaline, followed by the solvent extraction (acetone, hexane,

methanol or ethanol); yet, the yield of astaxanthin was about 0.9 – 19.8 ± 0.05 mg/g (Mendes-Pinto et al., 2001, Dong et al., 2014). DPCARB and DACARB did not give a satisfactory extraction performance; this could be due to the steric hindrance effect caused by the non-polar parts of their alkyl side chains. In contrast, the higher yield of astaxanthin (7.06 ± 0.45 mg/g) obtained from the DBCARB extraction system was postulated to be associated with the hydrophobic nature of DBCARB that might be more compatible with the solubility of astaxanthin. Despite of that, the long alkyl side chain of DBCARB might have caused a steric effect wherein the permeation of the inner cell of *H. pluvialis* by cations and anions was difficult. Hence, the efficiency of cell permeabilization was reduced. On the contrary, the shorter alkyl side chain of DIMCARB renders a less hydrophobic nature of solvent and a greater miscibility that facilitate an effective permeabilization through the amorphous multilayered extracellular matrix into the inner part of *H. pluvialis* to extract the reddish astaxanthin pigment. In view of the highest yield of astaxanthin obtained, DIMCARB was selected to be further optimized under other operating conditions.

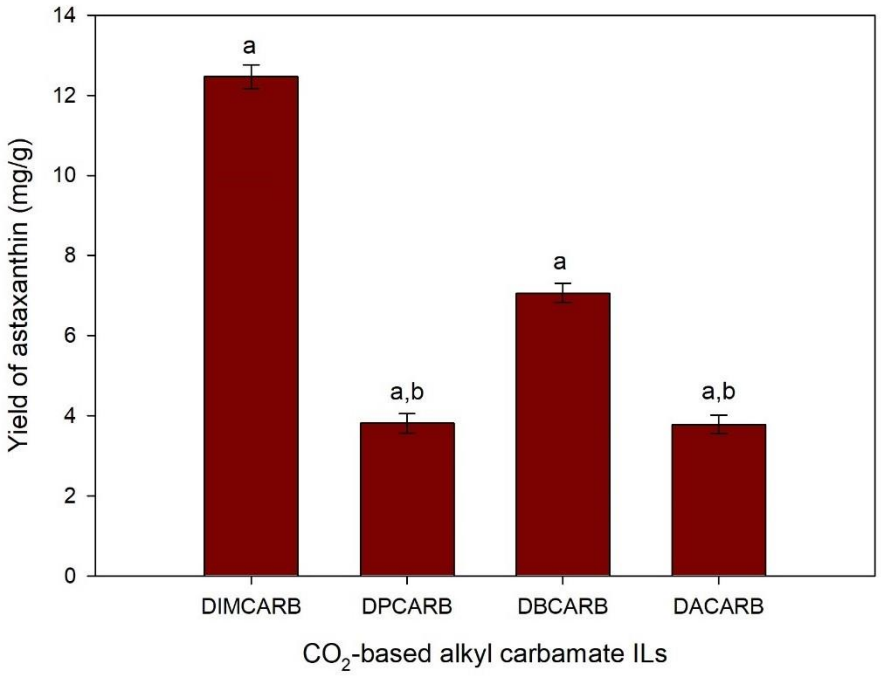


Figure 5.2: Yields of astaxanthin obtained from the extraction systems comprising different types of CO_2-based alkyl carbamate ILs. Experimental conditions: 100% (w/w) of IL; 60 min; 25 °C. The letter "a" indicates the significant difference ($p \leq 0.05$) among the groups, while the letter "b" marks the insignificant difference ($p > 0.05$) among the groups (one-way ANOVA followed by Tukey's test).

5.4.2 Effect of DIMCARB concentration on astaxanthin extraction

The concentration of DIMCARB was adjusted by methanol (diluent) to a concentration range of 50-90% (w/w). As shown in **Figure 5.3**, a reduction in concentration of DIMCARB from 100% (w/w) to 50% (w/w) resulted in the gradual decrease in the yield of astaxanthin from 11.26 ± 0.45 mg/g to 7.84 ± 0.25 mg/g. A low concentration of DIMCARB is not favorable for the cell permeabilization, which is a critical step prior to the releasing and dissolution of astaxanthin from the

cells to the solvent phase. A similar finding was reported by Vijayaraghavan et al. (2015) evaluating the polarity effects of a diluted IL mixture (50% of DIMCARB-ethanol) on the performance of curcuminoid extraction; the yield of curcuminoid did not increase significantly as well (Vijayaraghavan and Macfarlane, 2014). A probable explanation for the observed trend is the switching of this carbamate IL from the "ionic" state to a "molecular liquid" base (Phan et al., 2008); this may cause a drop in the polarity of IL, thereby reducing the extraction efficiency. Based on the results of statistical analysis, the yield of astaxanthin was found to be significantly affected ($p < 0.05$) by the concentration of DIMCARB. Based on the result, 100% (w/w) of DIMCARB was selected for the subsequent studies on the extraction of astaxanthin.

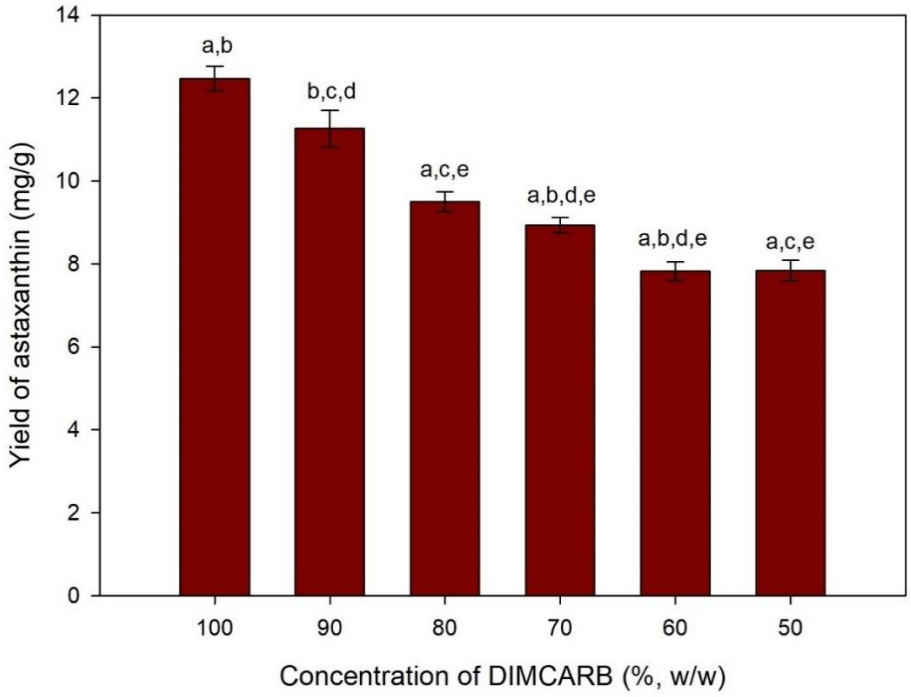


Figure 5.3: Yields of astaxanthin obtained from the extraction systems made of different concentrations of DIMCARB. Experimental conditions: 60 min; 25 °C. The letters "a and c" indicates the significant difference ($p \leq 0.05$) among the groups, while the letters "b, d and e" marks the insignificant difference ($p > 0.05$) among the groups (one-way ANOVA followed by Tukey's test).

Mechanism studies of cell permeabilization by these ILs is important in the evaluation of the interaction occurred during the biomass processing. Cellulose has the highly crystalline structure where the presence of branched H-bonding networks makes it insoluble in water and many organic solvents (Pinkert et al., 2009). Based on the literature, most of the studies reported that hydrogen bonding between the hydroxyl group (-OH) of cellulose from the cell wall of biomass form an electron donor-electron acceptor (EDA) complexes with the charged species of the ILs (Yu

et al., 2016, Desai et al., 2016, Choi et al., 2019). In this case, the ionic species of DIMCARB, $[(CH_3)_2NH_2]^+[(CH_3)_2NH\text{-}COO]^-$, interact with the oxygen and hydrogen atoms of the cellulose chain (see **Figure 5.4**). This interaction causes the removal of -OH group from the cellulose chain, resulting in the formation of cavities on the cellular surface of the microalgae. In fact, the results are in good agreement with previous study by Endo et al. (2016), who indicated that the deconstruction of cellulose's crystalline structure was attributed to the anion bridging phenomenon triggered by the hydrogen bonding, as evidenced from the ^1H NMR spectrum of -OH group of cellulose showing the gradual downshift from 4.5 ppm to 6.8 ppm (Endo et al., 2016).

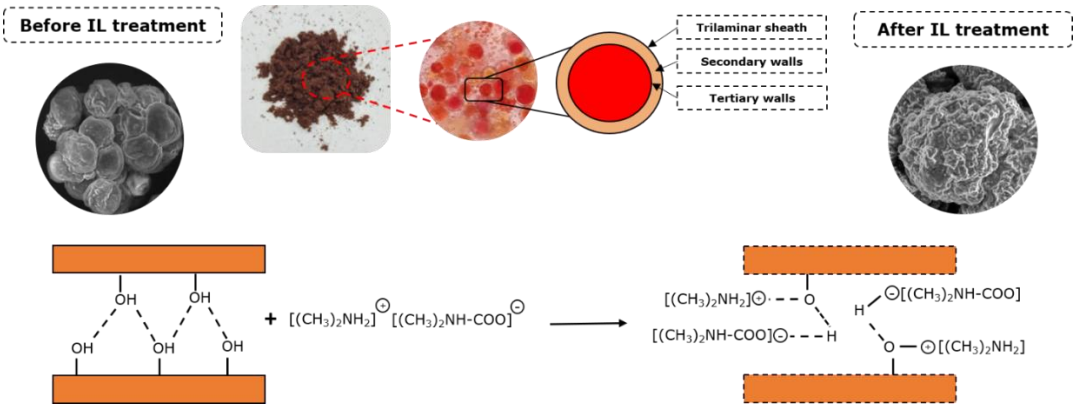


Figure 5.4: Proposed mechanism of the permeabilization of multi-layered cellular wall of *H. pluvialis* by DIMCARB.

5.4.3 Effect of extraction period on astaxanthin extraction

The incubation period is a critical consideration in the solvent extraction process because the permeation of *H. pluvialis* by DIMCARB is not instantaneous and the dissolution of astaxanthin in the bulk solvent is based on diffusion. **Figure 5.5** shows the extraction performance of DIMCARB when incubated for different periods of extraction. By increasing the extraction period from 30 min to 75 min, the yield of astaxanthin increased from 7.48 ± 0.18 mg/g to 14.18 ± 0.25 mg/g. An incubation period beyond 90 min did not increase the yield of astaxanthin, as the diffusion of astaxanthin from the microalgal cells to the solvent may be restricted by the maximal mass transfer of astaxanthin between inner and outer of the rigid cellular structure of *H. pluvialis* (Ruen-ngam et al., 2012). The cell wall of the encysted *H. pluvialis* is composed of cellulose and mannan compounds with numerous chains of β-linked D-glucose units formed between oxygen atoms and hydrogen network (Desai et al., 2016, Rahim et al., 2019). As a polar and hydrophilic IL, DIMCARB was postulated to facilitate the formation of hydrogen bonding that deprotonates the cellulose chain in the cell wall of *H. pluvialis*. This weakens the cellulose of the encysted *H. pluvialis* cell for the astaxanthin to be dissolved into the solvent. The results were subjected to the statistical analysis and the yield of astaxanthin was deemed to be significantly affected ($p < 0.05$) by the extraction period. Hence, 75 min of incubation period was selected as the optimum duration for the cell permeabilization of *H. pluvialis*.

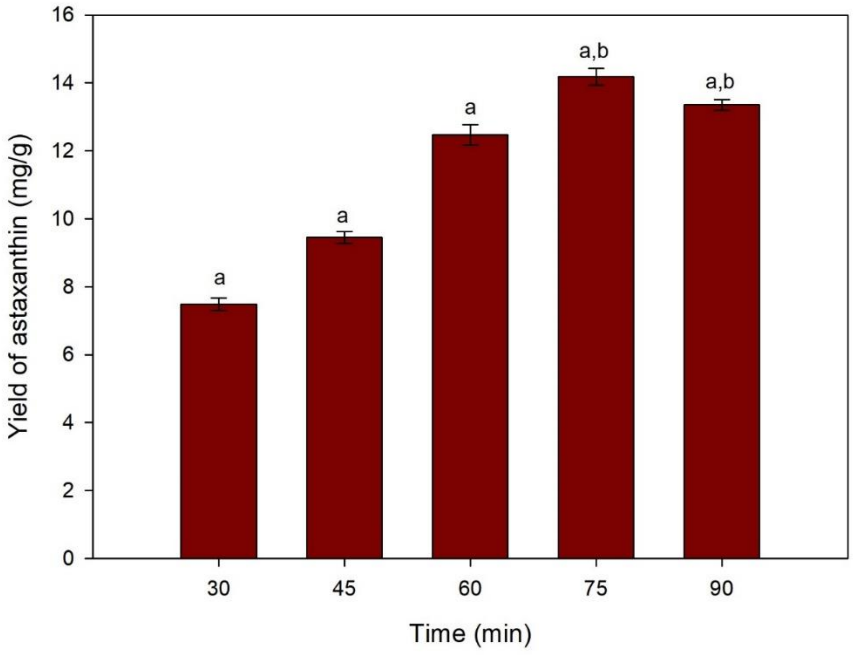


Figure 5.5: Yields of astaxanthin obtained from *H. pluvialis* treated by 100% (w/w) DIMCARB for different extraction periods at 25 °C. The letter "a" indicates the significant difference ($p \leq 0.05$) among the groups, while the letter "b" marks the insignificant difference ($p > 0.05$) among the groups (one-way ANOVA followed by Tukey's test).

5.4.4 Effect of temperature on astaxanthin extraction

The influence of temperature on the extraction of astaxanthin from *H. pluvialis* was investigated with 100% (w/w) DIMCARB and at different extraction temperatures. Numerous studies have reported that a mild extraction condition within 25°C to 55°C is recommended for the extraction process because astaxanthin is a highly heat-sensitive compound which can be degraded when subjected to a high temperature condition (Desai et al., 2016, Fan et al., 2019, Praveenkumar et al.,

2015). As shown in **Figure 5.6**, by increasing the temperature from 25°C to 45°C, the yield of astaxanthin increased from 13.36 ± 0.67 mg/g to 27.99 ± 1.01 mg/g. A similar trend of result was reported by Desai et al. (2016) on the extraction of astaxanthin using imidazolium-based ILs, stating that the increase in temperature affected the yields of astaxanthin obtained from [BMIM][DBP] extraction system (22 – 65%) and [EMIM][DBP] extraction system (36 – 70%) (Desai et al., 2016). On the other hand, the extraction of astaxanthin from *H. pluvialis* by DIMCARB at 55°C was unsuccessful due to the distillation of DIMCARB at this high temperature altered the optimized concentration of solvent thus, leaving the biomass alone in the extraction process during the extraction. This finding is rejected as the DIMCARB fail to perform at extraction temperature of 55°C. Based on the statistical analysis, the temperature is a significant factor of DIMCARB-based solvent extraction and it significantly affects ($p < 0.05$) the yield of astaxanthin. Thus, an extraction temperature at 45°C is optimal for the extraction of astaxanthin from *H. pluvialis*.

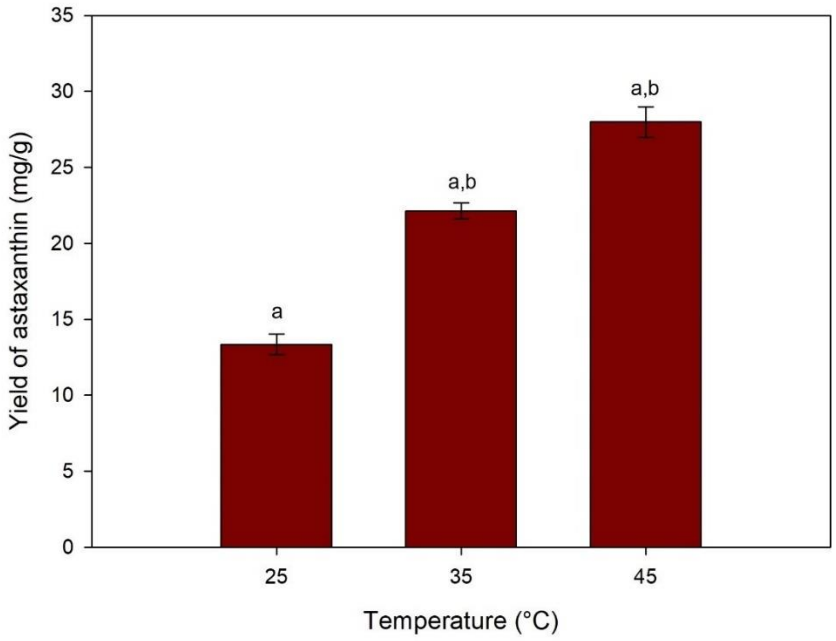


Figure 5.6: Yields of astaxanthin obtained from the extraction systems conducted at different temperatures. Experimental conditions: 100% (w/w) DIMCARB; 75 min. The letter "a" indicates the significant difference ($p \leq 0.05$) among the groups, while the letter "b" marks the insignificant difference ($p > 0.05$) among the groups (one-way ANOVA followed by Tukey's test).

5.4.5 Surface and cell morphology of *H. pluvialis* treated by DIMCARB

The permeabilization of *H. pluvialis* by DIMCARB for the extraction of astaxanthin was evaluated. **Figure 5.7** demonstrates the cell morphology of *H. pluvialis* before and after the cell permeabilization at different magnifications. Based on **Figure 5.7 (A1)**, **(A2)** and **(A3)**, the untreated *H. pluvialis* cells were spherical in shape and with the astaxanthin pigments remained intact in the cell. Some of the zooid cells were noted in **Figure 5.7 (A2)**, where the cells were

encapsulated by the mature cyst with a tri-layered cell wall composed of the trilaminar sheath, secondary wall, and a tertiary wall. The amorphous tri-layered cell walls of *H. pluvialis* was clearly shown in **Figure 5.7 (A3)**, where the reddish astaxanthin was encapsulated in the cell (Praveenkumar et al., 2015). After the permeabilization of the microalgae by DIMCARB, the cell morphology as shown in **Figure 5.7 (B1)**, **(B2)** and **(B3)** revealed the colourless cells in spherical shape, thereby confirming the leaching of astaxanthin to the surrounding environment. In an extraction study conducted by Desai et al. (2016), 1-ethyl-3-methylimidazolium dibutylphosphate (EMIM-DBP) was also successfully applied for the cell permeabilization of *H. pluvialis* (Desai et al., 2016). Due to the limitation of microscopic analysis, it was difficult to observe any formation of pore, "pitting" or cavities on the cellular surface morphology of the microalgae.

The untreated and treated *H. pluvialis* microalgae were further scrutinised under FESEM to investigate their surface morphology. **Figure 5.8** shows the surface morphology of cells before and after the treatment by DIMCARB. As shown in **Figure 5.8 (A1)** – **(A4)**, the untreated *H. pluvialis* cells have a smooth cell surface and were spherical in shape. After cell permeabilization, the cell appeared to have the wrinkled and rough surface, as shown in **Figure 5.8 (B1)** – **(B4**). As shown in the images (see **Figures 5.8 (B3)** and **(B4)**) captured at the higher magnifications, the presence of pore was believed to facilitate the leaching of astaxanthin from the intact *H. pluvialis* cell. The formation of cavities on the surface multi-layered wall of *H. pluvialis* was believed to be associated with the EDA complexes formed by

the hydrogen bonding interaction between cellulose and the charged species of DIMCARB.

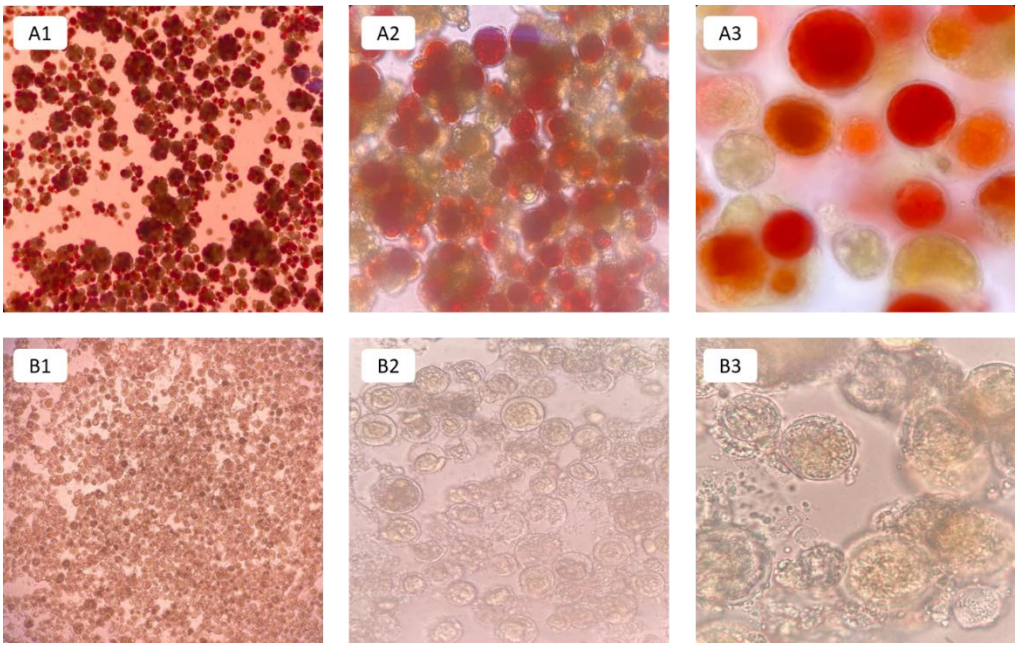


Figure 5.7: Microscope images of *H. pluvialis* cell **(A)** before, and **(B)** after the cell permeabilization by DIMCARB under the optimized conditions. Numbers 1-3 refer to the magnification levels at 100×, 400×, and 1000×, respectively.

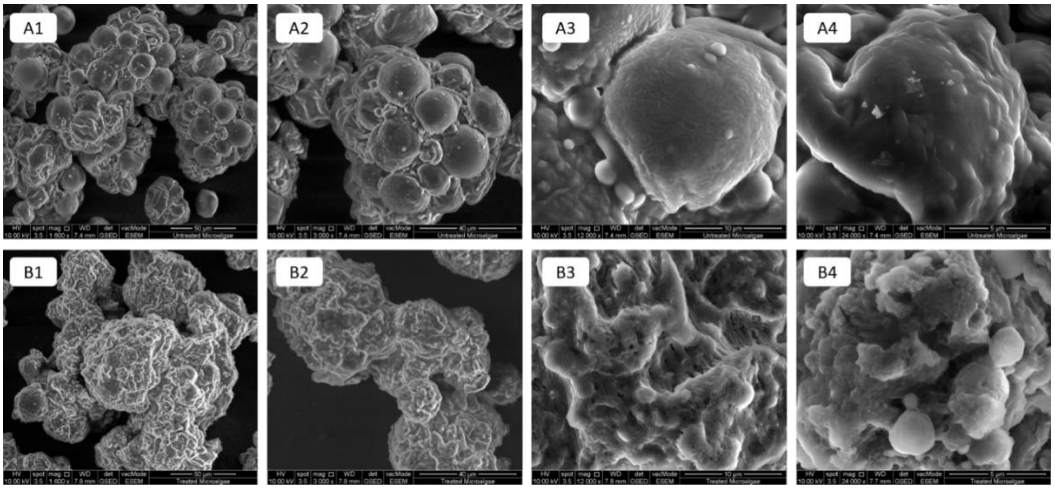


Figure 5.8: Surface morphology of *H. pluvialis* cell **(A)** before, and **(B)** after the cell permeabilization by DIMCARB. Numbers 1-4 refer to the magnification levels at 1600×, 3000×, 12000×, and 24000×, respectively.

5.4.6 Antioxidant activity of the extracted astaxanthin

The astaxanthin extracted from *H. pluvialis* was further evaluated in terms of its antioxidant activity. The TEAC assay was conducted to evaluate their antioxidant capacity in reducing of an oxidant probe through the indication of mixture's colour changing from light blue-green to light pinkish. It is essential to evaluate the antioxidant activity as it determines the effectiveness of antioxidant properties in the extracted astaxanthin. Based on the results, the TEAC value and the scavenging activities of astaxanthin are 209.61 ± 0.44 µM of TEs/g of crude extract and 72.13 ± 0.14%, respectively. Standard equation: percentage of scavenging (%) =0.0996[(Trolox)] + 2.5374 (R^2 = 0.9962). Aside from that, the presence of polyphenolic compound in the extracted astaxanthin was evaluated by TPC assay. In this study, the extracted astaxanthin showed a relatively high value

of TPC, namely 86.88 ± 0.54 mg GAEs/100g crude extract. Standard equation: OD_{760} =0.0032[(Gallic acid)] + 0.0519 (R^2 = 0.9997). The results obtained from both antioxidant assays showed that the antioxidant activity of the extracted astaxanthin has been well preserved.

5.4.7 Recyclability of DIMCARB for multiple cycles and comparison of extraction performance

The recovery and recyclability of DIMCARB are imperative as this could theoretically minimize the environmental and economic impact of IL for industrial application. Recyclability studies were conducted by using the optimized condition of DIMCARB-based extraction system for four cycles. The DIMCARB were distillate using rotary evaporator and collected for the subsequent treatment of *H. pluvialis*. Referring to **Table 5.3**, the yield of astaxanthin obtained from the three successive cycles of extraction systems was comparable to that from the initial round of extraction. However, at the third cycle of extraction, the yield of astaxanthin dropped to 16.12 ± 1.20 mg/g. This could be due to the dilution of distilled DIMCARB by the water droplets generated during the distillation process. Based on the ^{13}C NMR analysis (refer to **Figure A12**), DIMCARB used in the three rounds of extraction was found to be unaffected by the distillation.

Table 5.3: Recyclability of DIMCARB for the extraction of astaxanthin from *H. pluvialis*.

Recyclability of DIMCARB [a]	Yield of astaxanthin (mg/g)
Initial extraction	28.87 ± 0.11
1st cycle	29.22 ± 0.38
2nd cycle	26.71 ± 0.42
3rd cycle	16.12 ± 1.20

[a] Experimental conditions: 100% (w/w) DIMCARB; 75 min; 45°C.

The performances and operating conditions of other IL-based extraction systems for the recovery of astaxanthin from *H. pluvialis* were compared (see **Table 5.4**). For example, the recoveries of astaxanthin obtained from the conventional imidazolium-ILs were in the range of 65% – 85%. The extraction of astaxanthin from *H. pluvialis* by different types of imidazolium-based IL was conducted at high temperature and the duration of extraction was long (i.e., 55°C and 90 min). Unlike DIMCARB, the removal of imidazolium-based ILs from the extracted astaxanthin is challenging as the recycling approaches (i.e., extraction, adsorption, membrane-based separation and crystallization) involves complex and intensive processes which may easily denature the temperature-sensitive astaxanthin biomolecules (Desai et al., 2016, Liu et al., 2019b, Choi et al., 2019).

Table 5.4: Performances and operating conditions of different IL-based extraction systems for the recovery of astaxanthin from *H. pluvialis*

Type of ILs	Condition of *H. pluvialis*	Operating conditions	Yield of astaxanthin	References
DIMCARB	Intact cell (≤ 3%)	100% (w/w), 45°C, 75 min	27.99 ± 1.01 mg/g (92.67%)	This work
1-butyl-3-methylimidazolium dibutylphosphate, [BMIM][DBP]	Dried cell (≈3.2%)	40% (w/w), 45°C, 90 min	62.25%	(Desai et al., 2016)
1-ethyl-3-methylimidazolium dibutylphosphate, [EMIM][DBP]	Dried cell (≈3.2%)	40% (w/w), 45°C, 90 min	77.04%	(Desai et al., 2016)
1-butyl-3-methylimidazolium acetate, [BMIM][Acetate]	Dried cell (≈3.2%)	80% (w/w), 45°C, 90 min	120 μg ml^{-1}	(Desai et al., 2016)

Table 5.4 (Continue)

Type of ILs	Condition of *H. pluvialis*	Operating conditions	Yield of astaxanthin	References
1-butyl-3-methylimidazolium dicyanamide, [BMIM][DCA]	Dried cell (≈3.2%)	80% (w/w), 45°C, 90 min	66 μg mL^{-1}	(Desai et al., 2016)
1-butyl-3-methylimidazolium, chloride, [Bmim][Cl]	Intact cell cyst (≈3.5%)	40% (w/w) with methanol, 55°C, 90 min	85.42%	(Liu et al., 2019b)
1-ethyl-3-methylimidazolium, chloride, [Emim][Cl]	Intact cell cyst (≈3.5%)	40% (w/w) with methanol, 55°C, 90 min	65.29%	(Liu et al., 2019b)
[Emim] HSO_4	Intact cell NIES-144	6.7% (v/v), 30°C, 60 min	82.2 ± 1.4%	(Choi et al., 2019)
[Emim] CH_3SO_3	Intact cell NIES-144	6.7% (v/v), 30°C, 60 min	76.1 ± 0.3%	(Choi et al., 2019)

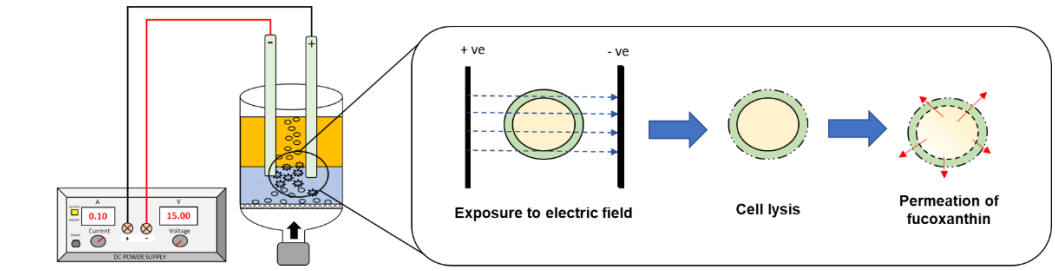
Table 5.4 (Continue)

Type of ILs	Condition of *H. pluvialis*	Operating conditions	Yield of astaxanthin	References
Ethanolammonium caproate, (EAC)	Powder (100 mesh)	Microwave-assisted: 210 W, 50 s, 5.2 mol L^{-1} of IL	34.4 $mg.g^{-1}$	(Fan et al., 2019)
[Emim] $(CF_3SO_2)_2N$	Intact cell NIES-144	6.7% (v/v), 30°C, 60 min	70.3 ± 3.0%	(Choi et al., 2019)
[Emim] $(CF_3SO_2)_2N$	Intact cell NIES-144	6.7% (v/v), 30°C, 60 min	70.3 ± 3.0%	(Choi et al., 2019)
Ethanolammonium caproate, (EAC)	Powder (100 mesh)	Microwave-assisted: 210 W, 50 s, 5.2 mol L^{-1} of IL	34.4 $mg.g^{-1}$	(Fan et al., 2019)

5.5 Conclusion

This work conceptualized a sustainable separation and extraction of astaxanthin using distillable CO_2-based alkyl carbamate ILs. The challenges associated with the mechanical disruption approach were overcome by using ILs for an effective solvent to permeabilize the robust tri-layered cell wall in *H. pluvialis* and to dissolve the astaxanthin. CO_2-based alkyl carbamate ILs are readily distilled from the extracted astaxanthin, in contrast to other conventional IL-based extraction method. Among the CO_2-based alkyl carbamate ILs tested, DIMCARB gave the highest yield of astaxanthin (27.99 ± 1.01 mg/g of astaxanthin) under the optimized extraction conditions, namely 100% (w/w) of DIMCARB, 75 min of incubation, and 45°C. The cell permeabilization by DIMCARB was indicated by the formation of cavities on the surface multi-layered wall of *H. pluvialis* as shown in FESEM images. The antioxidant activity of the extracted astaxanthin was confirmed by TEAC assay (209.61 ± 0.44 μM of TEs/g) and TPC assay (86.88 ± 0.54 mg GAEs/100g of crude extract). Moreover, the recyclability of DIMCARB distillate was proven by the three successive rounds of extraction using the recovered DIMCARB, and the performance of extraction was found to be unaffected significantly. In short, the CO_2-based alkyl carbamate ILs have shown a great potential as a sustainable solvent used in the extraction of astaxanthin. This opens up the possibility of using DIMCARB as a replacement to the traditional organic solvent in the downstream processing of bioactive compounds from other species of microalgae.

CHAPTER 6 ELECTROPERMEABILIZATION-ASSISTED LIQUID BIPHASIC FLOTATION FOR THE RECOVERY OF FUCOXANTHIN FROM *CHAETOCEROS CALCITRANS* MICROALGAE



This chapter unveil a greener and promising non-thermal technique by the incorporation of electropermeabilization-assisted technology with liquid biphasic flotation system for the disintegration of *Chaetoceros calcitrans* microalgae in the recovery of fucoxanthin. This study is to overcome the challenges adopted from previous study of intensive disruption adopted from ultrasonication treatment. Both optimization of the phase components from the liquid biphasic flotation system and electropermeabilization-assisted liquid biphasic flotation system were conducted. Comparison studies between the liquid biphasic flotation system and electropermeabilization-assisted liquid biphasic flotation system were evaluated. This chapter consists of thesis-version of work submitted in the *Separation and Purification Technology*.

Electropermeabilization-assisted liquid biphasic flotation for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae

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K.K.S. performed all the experiments; C.W.O., K.W.C. and S.C.F. provided guidance in experimental design and materials; K.K.S., C.W.O. and K.W.C. analyzed and interpreted the data; K.K.S. wrote the manuscript; C.W.O and P.L.S. edited the manuscript.

6.1 Abstract

Chaetoceros calcitrans (*C. calcitrans*) is a fucoxanthin-rich marine diatom, an emerging source of future sustainable antioxidants reservoir composed of carotenoids for various functional food industries. Many extraction technologies have been explored for the recovery of biomolecules from microalgae, however the issues such as energy intensive processes, poor environmental footprint, toxicity, large amount of solvent used, and sustainability should be thoroughly reconsidered. To address these challenges, this study proposed the utilization of electropermeabilization-assisted liquid biphasic flotation (LBF) system for the extraction of fucoxanthin from *C. calcitrans* microalgae. The optimization of LBF and electropermeabilization-assisted LBF system was comprehensively evaluated under various operating condition. Collectively, the optimized LBF and electropermeabilization-assisted LBF system achieved a significant maximal yield of extracted fucoxanthin (14.78 ± 0.79 mg/g and 16.09 ± 0.27 mg/g), extraction efficiency (98.85 ± 1.03% and 99.80 ± 0.05%) and partition coefficient (152.15 ± 1.13 and 236.72 ± 0.88). In addition, the extracted fucoxanthin from both systems exhibit satisfactory antioxidant activities. The exploration of this electropermeabilization-assisted LBF system for the extraction of fucoxanthin would provide a greener and highly efficient bioprocessing route which is worth for researchers to explore further.

Keywords *Chaetoceros calcitrans*; fucoxanthin; electropermeabilization-assisted; extraction; liquid biphasic flotation

6.2 Introduction

Bioactive compounds extracted from microalgae (i.e., fatty acid, polysaccharide, phycobilin, carotenoids and phenolic compounds) are a favourable food supplement that offers anti-inflammatory, anti-diabetic, anti-cancer, anti-malarial, anti-angiogenic and photo-protection activities (Mohamadnia et al., 2020, Sathasivam and Ki, 2018, Suchern et al., 2020). Fucoxanthin ($C_{42}H_{58}O_6$) is the major phytochemical constituting more than 10% of the total carotenoids accumulated in *Bacillariophyceae* and *Prymnesiophyceae* microalgae (Foo et al., 2017). The fucoxanthin content in in microalgae such as *Phaeodactylum tricornutum*, *Odontella aurita* and *Chaetoceros calcitrans* was reported to be higher than that in macroalgae like *Eisenia bicyclis*, *Laminaria digitate*, *Fucus vesiculosus*, *Sargassum muticum* and *Saccharina latissimi* (Kim et al., 2012a, Xia et al., 2013, Conde et al., 2015, Shannon and Abu-Ghannam, 2017). Fucoxanthin exhibits beneficial antioxidant properties that prevent chronic oxidative stress diseases that could lead to atherosclerosis, Alzheimer's disease and cancer (Halliwell, 2007).

Over the past decade, the search for green and sustainable production of phytonutrients derived from microalgae has been actively ongoing due to the greater demand of these natural additives or supplements in food, pharmaceutical, agricultural and nutraceuticals industries. Traditionally, the extraction of fucoxanthin from microalgae involves volatile organic solvents (e.g., methanol, acetone, hexane, tetrahydrofuron, ethyl acetate and dimethyl sulfoxide), which have always become a concern because of their environmental impact, toxicity, high

consumption volume and sustainability (Derwenskus et al., 2019, Suchern et al., 2020, Khoo et al., 2020e). Advanced extraction technologies in such as supercritical fluid extraction (SFE), ultrasound-assisted extraction, and microwave-assisted extraction have been explored for the extraction of fucoxanthin (Suchern et al., 2020, Guler et al., 2020). However, the high energy consumption by these extraction methods is deemed to be less economical in large-scale operation; furthermore, the harsh operating conditions like high temperature and pressure could denature or damage the highly sensitive carotenoids (Khoo et al., 2020b).

An alternative to the above-mentioned extraction methods is liquid biphasic flotation (LBF) system, which couples the liquid biphasic system (LBS) with the adsorptive bubble separation (Khoo et al., 2020d). LBF is composed of two immiscible aqueous phases (e.g., organic-solvent top phase and salt-rich bottom phase) wherein the target biomolecules in the bottom phase are selectively adsorbed on the ascending stream of air bubbles (e.g., nitrogen or oxygen) and are transferred to the top phase (Leong et al., 2018). The adsorption of the target compounds on the air bubbles is dependent on the surface-active properties of the compound and the interfacial properties of the air bubble (Show et al., 2013, Bi et al., 2010a). Consequently, the target compound can be accumulated in the top phase, while the impurities remain in the bottom phase. The applications of LBF in bioseparation field have been well demonstrated, as shown in many studies of LBF implemented in in the extraction, recovery and purification of biomolecules including proteins, lipase, lipid, astaxanthin, C-phycocyanin and betacyanins (Chew et al., 2019b, Chia et al., 2020, Leong et al., 2018, Show et al., 2013, Khoo et al., 2019b). Nonetheless,

the potential of LBF in simultaneous extraction and separation of biomolecules from the cells has not been widely explored.

Electroporation or electropermeabilization of cell membrane has been deemed to be a greener and promising non-thermal technique for the permeabilization of cell membrane (Flisar et al., 2014). The phenomenon of electropermeabilization occurs where the cells are exposed to the high-voltage electric field for a short period (Kotnik et al., 2015). Under the treatment of electric field, the integrity of cell membrane will be compromised, resulting in the formation of pores. This phenomenon facilitates the permeability of intracellular compound releasing its content into the extraction solvent across this membrane barrier (Polak et al., 2014). Electric field treatment is a greener and non-thermal process where an external electric field is applied to permeabilize the membrane due to its dipole nature of the membrane molecules (Eleršek et al., 2020, Eing et al., 2013). Owing to its non-thermal nature, electropermeabilization-assisted extraction of carotenoids from microalgae is highly attractive because it favourably prevents the degradation of these temperature-sensitive carotenoids. In terms of sustainability, electropermeabilization allows these microalgae to reseal and regenerate (i.e., cell recovery and enclosed of cell pore formation), thus this reduces the amount of waste generated in the environment after the extraction process (Buckow et al., 2013, Eleršek et al., 2020). In terms of energy consumption, electroporation approach was deemed to be more economical as compared to SC-CO_2, ultrasound-assisted, microwave-assisted and high-pressure homogenization (Golberg et al., 2016, Günerken et al., 2015).

This present work demonstrated the extraction of fucoxanthin from *Chaetoceros calcitrans* (*C. calcitrans*) microalgae by a LBF system assisted by electropermeabilization technology. To the best of the authors' knowledge, electropermeabilization-assisted extraction technology has not been applied in the bioprocessing of fucoxanthin. To attain the highest yield of fucoxanthin, the LBF system first optimized based on various parameters (e.g., types and concentration of food-grade alcohol and salt, flotation time and flotation rate). By adopting the optimized condition of the LBF system, the electropermeabilization technology was incorporated into the LBF system and the operating conditions such as electropermeabilization period, position of copper electrode, voltage applied, and biomass concentration was further investigated. The antioxidant properties, including scavenging activity, Trolox equivalent antioxidant capacity (TEAC) and total phenolic content (TPC) of the extracted fucoxanthin obtained from the optimized LBF system and electropermeabilization-assisted LBF system were also evaluated. Lastly, LBF and electropermeabilization-assisted LBF systems were assessed and compared in terms of fucoxanthin yield, extraction efficiency and partition coefficient values.

6.3 Experimental Section

6.3.1 Materials

Analytical ethanol (≥ 99.9%), 2-propanol (≥ 99.9%), ammonium sulphate [$(NH_4)_2SO_4$], potassium hydrogen phosphate (K_2HPO_4), sodium bicarbonate (Na_2CO_3), sodium citrate ($Na_3C_6H_5O_7$) and disodium phosphate (Na_2HPO_4) were

obtained from R&M Chemicals (Malaysia). Standard fucoxanthin was purchased from Sigma Aldrich (Germany). Iron (III) chloride ($FeCl_3 \cdot 6H_2O$), manganese chloride ($MnCl_2 \cdot 4H_2O$), boric acid (H_3BO_3), ethylene diamine tetraacetic acid disodium (Na-EDTA), sodium hydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), sodium nitrate ($NaNO_3$), zinc chloride ($ZnCl_2$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), ammonium molybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$], copper sulphate ($CuSO_4 \cdot 5H_2O$), silica ($Na_2SiO_3 \cdot 9H_2O$), cobalamin (vitamin B_{12}), ammonium formate (NH_4HCO_2), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, potassium persulfate ($K_2O_8S_2$), Folin-Ciocalteu (F-C) reagent, sodium carbonate (Na_2CO_3) and gallic acid ($C_7H_6O_5$) were purchased from Sigma Aldrich (St. Louis, USA). Methanol (≥ 99.9%, HPLC grade) were purchased from Fisher Chemical (Malaysia). All the chemicals used in this experiment were of analytical grade, unless mentioned otherwise. Copper electrodes (diameter: 6.35 mm and length: 25 cm) purchased from a local electric supplier (Semenyih, Selangor Darul Ehsan, Malaysia) prior to their application in LBF apparatus setup.

6.3.2 Methods

6.3.2.1 Cultivation and harvesting of *C. calcitrans* microalgae biomass

Marine diatom, *C. calcitrans* (UPMC-A0010) was bulk cultivated using a 120-L of annular photobioreactors decontaminated under UV-sterilized seawater supplemented with Conway cultivation medium composition [1.3 g/L of $FeCl_3 \cdot 6H_2O$, 0.36 g/L of $MnCl_2 \cdot 4H_2O$, 33.6 g/L of H_3BO_3, 45 g/L of Na-EDTA, 20

g/L of $NaH_2PO_4 \cdot 2H_2O$, 100 g/L of $NaNO_3$, 2.1 g/L of $ZnCl_2$, 2.0 g/L of $CoCl_2 \cdot 6H_2O$, 0.9 g/L of $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 2.0 g/L of $CuSO_4 \cdot 5H_2O$, 46.5 g/L of $Na_2SiO_3 \cdot 9H_2O$ and 1 mL of vitamin B_{12}]. The cultivation condition was exposed to continuous condition with light intensity of 150 µmol/m^2/s (light/dark 12:12 cycle), room temperature and within pH 8.0 – 8.5 for 14 days (Foo et al., 2015). The microalgae biomass was harvested and de-watered using tubular separator (model J-1250, Hanil Science, Industrial Co. Ltd., Korea) followed by a rinsing cycle with 1.0 mol/L of NH_4HCO_2 to eliminate the excess salt crystals. The microalgae biomass was freeze-dried and sieved through a 250-micron sized sieved. The biomass was then stored in the autoclaved sample bottles at -80°C prior to analysis.

6.3.2.2 Optimization of liquid biphasic flotation (LBF) system

The optimization of LBF system was conducted using two types of food-grade alcohol (i.e., ethanol and 2-propanol) and various types of salts for the extraction of fucoxanthin from the *C. calcitrans* microalgae biomass. The lyophilized biomass was first suspended in the salt solution. The mixture was transferred to the 500-mL LBF column and the food-grade alcohol was added subsequently into the column. The LBF system composed of 100 mL working volume and the operating parameters such as types and concentration of food-grade alcohol and salts, flotation time and flotation rate were evaluated thoroughly for the extraction of fucoxanthin from *C. calcitrans*. The optimization of LBF were optimized using one-factor-at-a-time (OFAT) approach in selecting the optimal variables of the operating condition for the subsequent studies. **Table 6.1** shows the

initial and various variables of operating conditions for the LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae.

Table 6.1: Initial settings and variables of operating condition for the LBF system.

No.	Operating conditions	Initial setting	Variables	Units
1.	Type of alcohols	Ethanol	2-propanol	N/A
2.	Type of salts	$(NH_4)_2SO_4$	$Na_3C_6H_5O_7$, K_2HPO_4, Na_2HPO_4 and Na_2CO_3	N/A
3.	Concentration of 2-propanol	100	60, 70, 80 and 90	% (w/w)
4.	Concentration of $(NH_4)_2SO_4$	250	150, 200, 300 and 350	g/L
6.	Flotation time	10	5, 7.5, 12.5 and 15	min
7.	Flotation rate	100	50, 75, 125 and 150	cc/min

6.2.2.3 Integrated of electropermeabilization-assisted LBF system

To enhance the performance of LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae. The electropermeabilization-assisted LBF system was designed by introducing two copper electrodes where one acted as anode (-ve) and another one as cathode (+ve) along with the optimized LBF system (see **Figure 6.1**). The copper electrodes were connected to a regulated dual DC power supply (GW Instek, GPS-3303 model) to supply the electricity continuously. The operating parameters of the electropermeabilization-assisted LBF system includes operating time of electropermeabilization, position of copper electrode, voltage applied, and

biomass concentration were accessed systematically for the extraction of fucoxanthin from *C. calcitrans* microalgae. Similarly, the parameters were subjected to OFAT approach in selecting the ideal condition for the following study. **Table 6.2** listed the initial condition and variables of operating condition of electropermeabilization-assisted LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae.

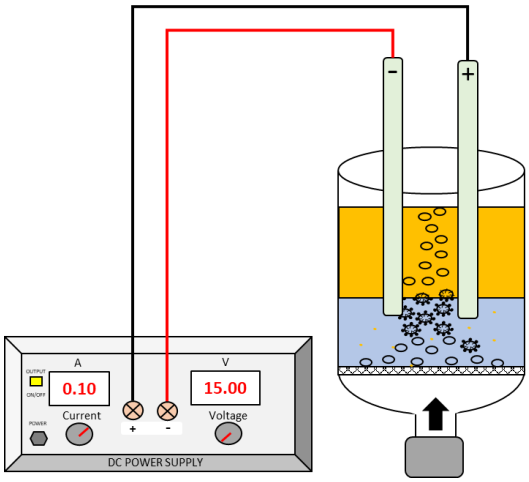


Figure 6.1: Schematic diagram of electropermeabilization-assisted liquid biphasic flotation system

Table 6.2: Initial settings and variables of operating condition for the electropermeabilization-assisted LBF

No.	Operating conditions	Initial setting	Variables	Units
1	Operating time electropermeabilization	10	2.5, 7.5 and 10	min
2	Position of copper electrode	Interphase	Top and bottom	N/A
3	Voltage applied	10	5, 15, 20 and 25	Voltage (V)
4	Biomass concentration	50	30, 40, 60 and 70	mg

6.3.3 Spectrophotometric determination

6.3.3.1 Determination of extracted fucoxanthin concentration

The stock solution of fucoxanthin standard was prepared at 50 µg/mL using ethanol and 2-propanol. The absorbance of fucoxanthin was determined using a UV-vis microplate spectrophotometer (Epoch, BioTek). The screening of absorbance ranging from 300 – 600 nm, the highest absorbance for fucoxanthin was found at OD_{445} and the standard curve was prepared by diluting the stock solution into six different concentration (0, 1.56, 3.12, 6.25, 12.5 and 25 µg/mL). Based on the standard curves, the fucoxanthin concentration was derived based on **Equation (1) and Equation (2) in** ethanol and 2-propanol, respectively. The extracted fucoxanthin samples were examined in triplicate, and the results were expressed as mg of fucoxanthin per g of microalgae biomass (mg/g).

$$C_F(Ethanol)\left(\frac{ug}{mL}\right) = \frac{OD_{445}-0.1425}{0.0703} \quad (1)$$

$$C_F(2-propanol)\left(\frac{ug}{mL}\right) = \frac{OD_{445}-0.0671}{0.0793} \quad (2)$$

where $C_{F(Ethanol)}$ and $C_{F(2\text{-}propanol)}$ are the concentration of fucoxanthin using ethanol and 2-propanol.

6.3.3.2 Calculations of partition coefficient and extraction efficiency and recovery yield

The partition coefficient (*K*) of extracted fucoxanthin in the LBF system was calculated based on Equation (3):

$$K = \frac{C_{F(T)}}{C_{F(B)}} \quad (3)$$

where $C_{F(T)}$ and $C_{F(B)}$ are the concentrations of extracted fucoxanthin (μg/mL) in the alcohol top and salt bottom phase, respectively.

The volume ratio (V_R) of the LBF system was determined via Equation (4):

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

where V_T and V_B are the volumes of the top and the bottom phases, respectively.

The extraction efficiency (*E*) of extracted fucoxanthin in the LBF system was calculated based on Equation (5):

$$E = \frac{K \times V_R}{1+KV_R} \quad (5)$$

6.3.3.3 Quantification of extracted fucoxanthin using high-performance liquid chromatography analysis

The extracted fucoxanthin from the optimized LBF and electropermeabilization-assisted LBF system was subjected to a High-Performance Liquid Chromatography, HPLC system (1200 Infinity, Agilent Technologies) coupled with a variable-wavelength detector (VWD) and an autosampler. A C_{18} column (ZORBAX Eclipse Plus, 4.6 × 100 mm, 3.5 µm) was used as the stationary phase in the HPLC system. The injection volume and flow rate were set at 20 µL and 0.7 mL/min, respectively. For the mobile phase, a mixture of methanol and water (97:3) was used. It is ensure that the mobile phase and sample were filtered through a 0.45-µm membrane prior to the HPLC operation (Khoo et al., 2020e). The fucoxanthin peak of the standard and the extracted sample was recorded at 3.2 min of retention time at wavelength of 445 nm (Foo et al., 2015).

6.3.4 Evaluation of antioxidant activities

6.3.4.1 Trolox equivalent antioxidant capacity (TEAC) assay

The extracted fucoxanthin from *C. calcitrans* of the optimized LBF and electropermeabilization-assisted LBF system were analyzed using TEAC assay as conducted in the previous work by Khoo et al. (2021). The preparation of $ABTS^{\bullet}$ radical, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical ($ABTS^{\bullet}$) stock solution was freshly prepared by mixing 7 mmol/L of $ABTS^{\bullet}$ solution and 2.45 mmol/L of $K_2O_8S_2$ solution at a volume ratio of 1:1. The freshly prepared $ABTS^{\bullet}$ stock solution was incubated for 18 – 24 h at room temperature

under dark condition. Before using the ABTS$^{\bullet}$ stock solution, it must be further diluted with methanol until an absorbance value of 0.70 ± 0.05 at 734 nm was obtained and the diluted ABTS$^{\bullet}$ stock solution was stored at room temperature prior to usage. TEAC assay was conducted by mixing 100 µL of sample (extracted fucoxanthin or Trolox standard solution) or methanol (as control) with 3.8 mL of diluted ABTS● stock solution. The sample was incubated for 5 min at room temperature and was analysed using a UV–Vis spectrophotometer (UV-1800, Shimadzu) at 735 nm. Trolox standard solutions were prepared in the concentration range of 31.25 – 2000 µM/L. The results were recorded in triplicate and were expressed as µM of Trolox equivalents per g of *C. calcitran* microalgae crude extract (µM TEs/g CE). Percentage of scavenging activity was calculated using Equation (6):

$$Percentage\ scavenging\ (\%): \frac{Control - Sample}{Control} \times 100\% \quad (6)$$

6.3.4.2 Total phenolic content (TPC) assay

TPC assay of extracted fucoxanthin was quantified using Folin-Ciocalteu (F-C) method adapted in literature study (Hajimahmoodi et al., 2013). In brief, the F-C reagent was diluted 10-fold with deionised water (1:9). TPC assay was conducted by mixing 100 µL of sample (extracted fucoxanthin or gallic acid standard solution) with 500 µL of diluted F-C reagent. The sample was incubated for 5 min at room temperature under dark condition. After 5 min, 2 mL of 75 g/L of Na_2CO_3 was added into the mixture and was left to settle for 90 min at room temperature under dark condition. The sample was then measured at an absorbance at 760 nm using a UV–

Vis spectrophotometer. The gallic acid was used as standard solution prepared at concentration range at 7.81–500 mg/L in this assay. The results were expressed as mg of gallic acid equivalents per 100 g of *C. calcitran* microalgae crude extract (mg GAEs/g CE).

6.3.5 Statistical analysis

The statistical analysis was conducted by evaluated the means of triplicate reading collected from the experiment. All the collected data was subjected to one-way ANOVA followed by Tukey HSD post-hoc test using Microsoft Excel to evaluate the significant differences ($p \leq 0.05$).

6.4 Results and discussion

6.4.1 Optimization of the LBF system for the extraction of fucoxanthin from *C. calcitran* microalgae

6.4.1.1 Effect of types of food grade alcohol and salts on the extraction of fucoxanthin from *C. calcitran* microalgae

In this study, LBFs made of various combinations of food-grade alcohol (i.e., ethanol and 2-propanol) and salts (K_2HPO_4, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, Na_2HPO_4 and Na_2CO_3) were selected to evaluate the LBF system for the extraction of fucoxanthin from *C. calcitran* microalgae. The selection of food-grade alcohol was to avoid the use of hazardous conventional organic solvents such as hydrochloric acid (HCl), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO) and dichloromethane (DCM), as food-grade status solvents are more preferable for the fucoxanthin bioprocessing

(Khoo et al., 2019a). The selection of alcohol was based on the solvent closest to polarity of the desired bioactive compound in which the final extract contains the highest recovery yield and subsequently higher antioxidant activities (Foo et al., 2015). Besides, fucoxanthin is a hydrophobic compound which tends to partition towards the hydrophobic environment (alcohol top phase) for its solubility. Based on the results, the highest yield of extracted fucoxanthin using ethanol and 2-propanol achieved 9.18 ± 0.28 mg/g and 11.91 ± 0.15 mg/g, respectively. The corresponding E values for ethanol and 2-propanol were 92.79 ± 0.19% and 97.76 ± 0.21% with K values of 4.07 ± 0.11 and 36.92 ± 0.20, respectively. It was found that the LBF system composed of alcohol with Na_2HPO_4 salt was unable to formulate a biphasic system which could probably due to the salting-out effect of alcohol at higher concentration (250 g/L) of aqueous salt solution. The excellent performance of LBF system composed of 2-propanol was due to its longer hydrocarbon chain and hydrophobicity interaction which is compatible for the extraction of fucoxanthin possess with semi-polar characteristics into the alcohol top phase.

The various type of salts consists of phosphate-, sulphate-, bicarbonate- and citrate-based were tested in the LBF system for the extraction of fucoxanthin from *C. calcitran* microalgae. It was found that sulphate-based salt has a higher *E* and *K* in the LBF system. As for bicarbonate-based salt, the formation of salt crystal precipitate was found at the bottom aqueous solution making the LBF system to be less desirable for the separation process. Similarly. the initial mixture of alcohol with Na_2HPO_4 salt revealed the formation of biphasic system, however after a period

of aeration, the bottom aqueous phase of the LBF system undergoes crystallization where fine crystal is formed. Despite of its good *E* values, the formation of salt crystal in the LBF system indicates the non-equilibrium of both liquid phases in alcohol/salt-based LBF system, in fact this may cause clogging or diminish the aeration on the sintered disk reducing the performance of the LBF system (Khoo et al., 2019a). $(NH_4)_2SO_4$ has been an auspicious salt used in liquid-liquid extraction of various biomolecules due to its SO_4^{2-} ions that interacts well with water forming an excellent hydration capacity to salt-out fucoxanthin to the top phase. Collectively, the LBF system comprising 100% (w/w) of 2-propanol and 250 g/L of $(NH_4)_2SO_4$ was selected for the subsequent study for the extraction of fucoxanthin from *C. calcitrans* microalgae. **Table 6.3** shows the performance in terms of extracted fucoxanthin yield, *E* and *K* of LBF systems comprised of various alcohol and salts.

Table 6.3: Effect of various type of alcohol and salts on the extraction of fucoxanthin from *C. calcitrans* microalgae. The initial operating condition of the LBF system were 50 mg of lyophilized *C. calcitrans* microalgae biomass, 50 mL of 100% (w/w) alcohol, 50 mL of 250 g/L of salts, 1:1 volume ratio, 10 min flotation time and 100 cc/min flowrate.

Type of alcohols	Type of salts	Biphasic formation	Extracted fucoxanthin (mg/g)	E (%)	K
Ethanol	K_2HPO_4	Yes	7.26 ± 0.10	91.52 ± 0.15	3.40 ± 0.20
	$(NH_4)_2SO_4$	Yes	9.18 ± 0.28	92.79 ± 0.19	4.07 ± 0.11
	$Na_3C_6H_5O_7$	Yes	6.07 ± 0.12	89.47 ± 0.25	2.68 ± 0.15
	Na_2HPO_4	No	-	-	-
	Na_2CO_3	Yes	6.53 ± 0.22	90.03 ± 0.18	2.90 ± 0.21
2-Propanol	K_2HPO_4	Yes	10.81 ± 0.55	80.28 ± 0.59	1.57 ± 0.16
	$(NH_4)_2SO_4$	Yes	11.91 ± 0.15	97.76 ± 0.21	36.92 ± 0.20
	$Na_3C_6H_5O_7$	Yes	11.40 ± 0.31	83.84 ± 0.23	2.67 ± 0.12
	Na_2HPO_4	No	-	-	-
	Na_2CO_3	Yes	8.55 ± 0.27	90.15 ± 0.28	4.30 ± 0.18

6.4.1.2 Effect of concentration of alcohol and salt of the LBF system

It is essential to evaluate the concentration of alcohol and salt as it influences the equilibrium of the LBF system. The change in concentration of the phase-forming components may shift the properties (e.g., interfacial tension, density and viscosity) of the biphasic system and this affect the solute partitioning (Khoo et al., 2019a, Chew et al., 2019a). The partitioning of the fucoxanthin was conducted by varying the concentration of 2-propanol within 60 to 100% (w/w) and $(NH_4)_2SO_4$ concentration within 150 to 350 g/L in the LBF system. Based on **Figure 6.2**, as the concentration of 2-propanol increased from 60% (w/w) to 90% (w/w), the yield attained from the extracted fucoxanthin increased from 10.09 ± 0.70 mg/g to 14.02 ± 0.21 mg/g, respectively. By observation, 60% (w/w) of 2-propanol concentration possess a longer time for the formation of biphasic system and this phenomenon can be explained due to its poor partitioning (K = 12.53 ± 0.88) between the alcohol and salt phase components. As the concentration of 2-propanol increases, the K of the biphasic system also increases from 12.53 ± 0.88 to 117.18 ± 1.53, respectively. In this case, it was found that the 90% (w/w) of 2-propanol has a higher volume on the top phase (V_{top}= 69 mL) compared to other concentration. This could due to the free volume available allowing the partitioning of fucoxanthin to the alcohol-rich top phase of the LBF system. The results showed that optimal LBF system composed of 90% (w/w) of 2-propanol and 250 g/L of $(NH_4)_2SO_4$ achieved the highest yield of extracted fucoxanthin (14.02 ± 0.21 mg/g), E (97.81 ± 1.15%) and K (117.18 ± 1.53), respectively. The effect of concentration of 2-propanol on the extraction of fucoxanthin was subjected to the statistical analysis; where the yield of extracted

fucoxanthin was significantly affected ($p < 0.05$) by the concentration of 2-propanol. Based on the results, 90% (w/w) of 2-propanol was selected for the subsequent extraction studies.

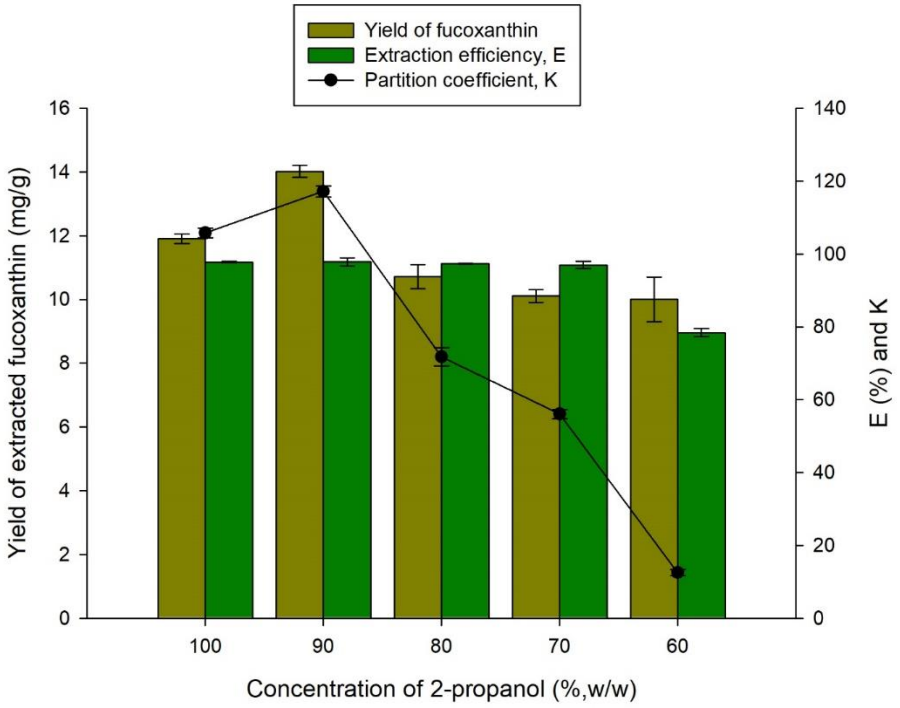


Figure 6.2: Effect of various concentration of 2-propanol for the extraction of fucoxanthin from *C. calcitrans* microalga using LBF system.

The concentration of $(NH_4)_2SO_4$ was varied in the range within 150 to 350 g/L along with 90% (w/w) of 2-propanol LBF system. **Figure 6.3** shows the effect of $(NH_4)_2SO_4$ on the yield of extracted fucoxanthin, *E* and *K* of *C. calcitran* microalgae. When the concentration of $(NH_4)_2SO_4$ varies within 150 g/L to 250 g/L, the *K* value increased from 19.91 ± 1.16 to 117.18 ± 1.53, respectively but the *E* value was maintained at 97%. However, by increasing the concentration of

$(NH_4)_2SO_4$ up to 350 g/L would influence the *K* value drastically (56.90 ± 1.07). Meanwhile. tth concentration of $(NH_4)_2SO_4$ of 350 g/L, reduces the yield of extracted fucoxanthin (10.04 ± 0.65 mg/g) in the LBF system. This is due to the high salt concentration that reduces the volume of 2-propanol top phase (V_{top} = 54 mL) for the extracted fucoxanthin to be accumulated. This finding was similar to Gómez-Loredo et al. (2014) stating that the reduction of free volume at the top phase decreases the yield of extracted hydrophobic compound to be partitioned at the top phase (Gómez-Loredo et al., 2014). As a result, 250 g/L of $(NH_4)_2SO_4$ salt concentration was chosen for the following experiment.

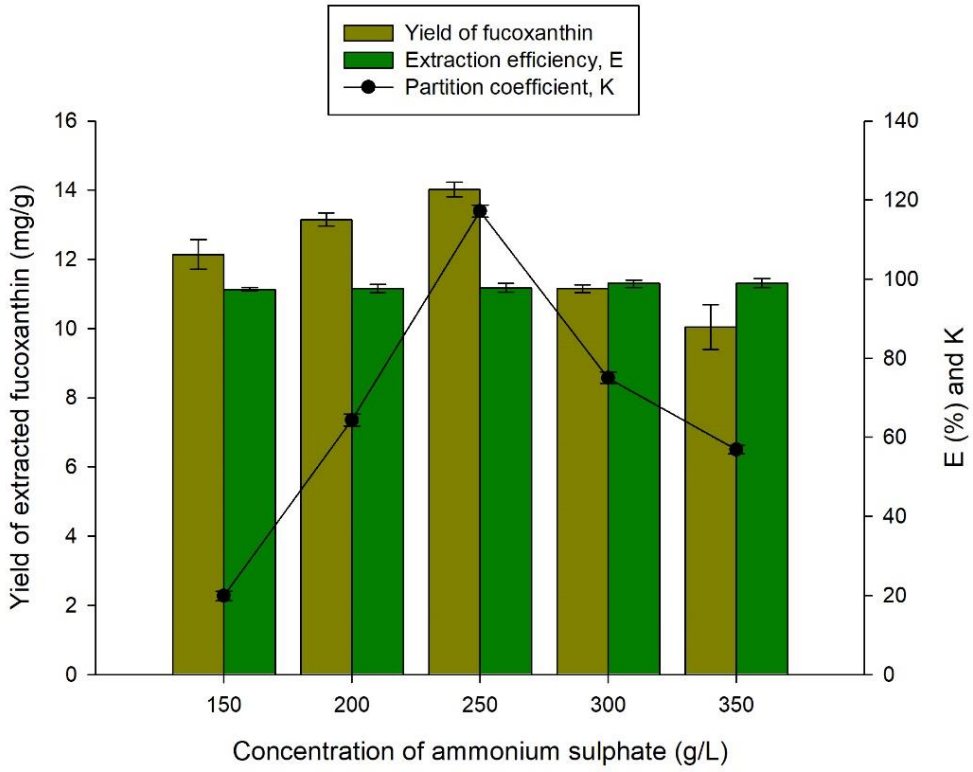


Figure 6.3: Effect of various concentration of ammonium sulphate for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

6.4.1.3 Effect of flotation time and flow rate

Based on Sankaran et al. (2018), the air flotation time and flow rate are the critical aspects to be evaluated in the LBF system as the air-water interface per unit volume of aqueous solution in time (Sankaran et al., 2018b). The mechanism interaction between the adsorptive bubble and the analyte in regard to the extraction process of the LBF system was previously evaluated using a mathematical model (Tham et al., 2019). **Figure 6.4** shows the effect of flotation time on the yield of extracted fucoxanthin, E and K of *C. calcitran* microalgae. As the flotation time increase from 5 min to 10 mins, the yield of extracted fucoxanthin increases within 10.78 ± 0.22 mg/g to 14.02 ± 0.21 mg/g, and K of 20.77 ± 0.39 to 117.18 ± 1.53, respectively. Though, by subsequently increasing the flotation up to 15 min, the yield of extracted fucoxanthin decreases approximately by 7%. This indicated that the prolonged flotation time does not enhance the recovery yield and extraction efficiency (Phong et al., 2017c). Likewise, the mathematical model reported by Tham et al. (2019) claimed that the prolonged flotation time may reduce the yield due to the reverse-extraction process cause by the free fall acceleration of the bubble, phase components and solute interaction deteriorating the extraction process (Tham et al., 2019). Therefore, the flotation time of 10 min was selected as the optimal duration for the extraction of fucoxanthin from *C. calcitrans* microalgae.

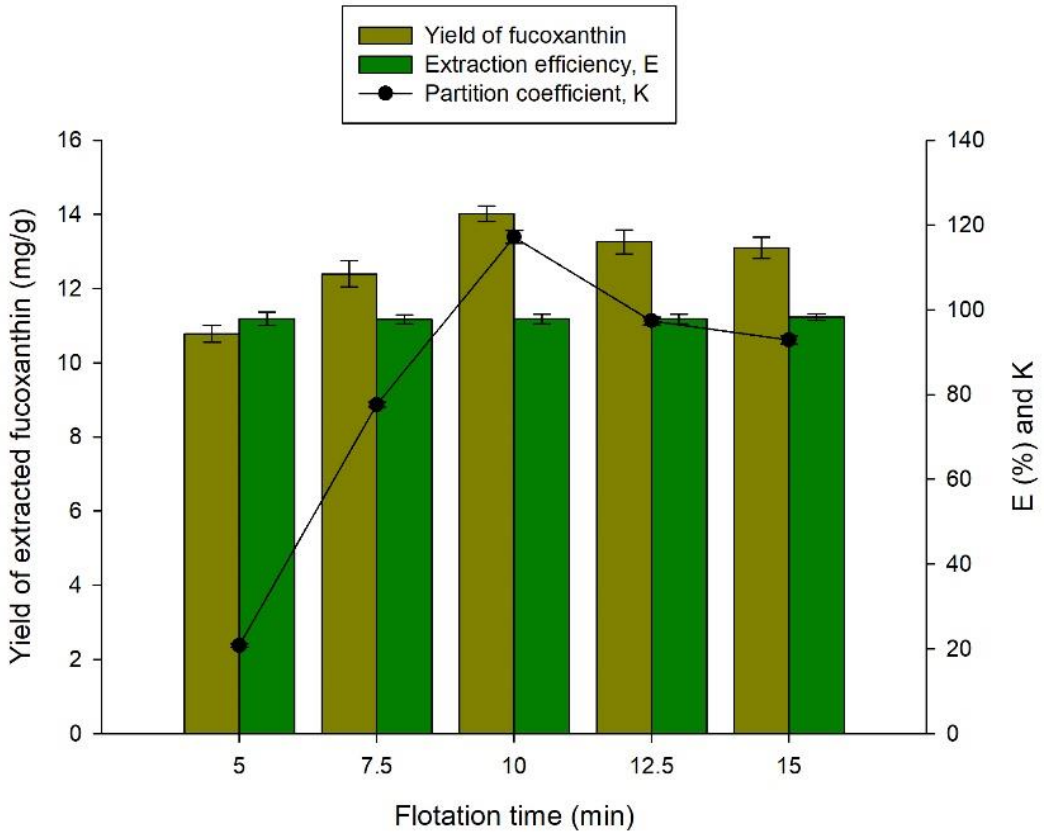


Figure 6.4: Effect of flotation time for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

The flow rate was varied within the range of 50 to 150 cc/min for the extraction of fucoxanthin from *C. calcitrans* microalgae. According to **Figure 6.5**, as the flow rate increases up to 125 cc/min, the yield of extracted fucoxanthin achieved 12.92 ± 0.57 mg/g to 14.78 ± 0.79 mg/g with *K* of 69.99 ± 1.85 to 152.15 ± 1.13, respectively. When the flow rate increases beyond 150 cc/min, the yield of extracted fucoxanthin and *K* reduces in the LBF system. This indicates that the interface of the LBF system is disturbed by the harsh turbulence that result in re-dissolution of extracted fucoxanthin into the bottom phase (Bi et al., 2010b). This

was supported by Li and Dong (2010), claiming that the high flow rate in the LBF system was unable to rupture at the similar rate, instead this causes the accumulation of bubbles on the top phase of the LBF system (Li and Dong, 2010). Hence, the flow rate of 125 cc/min was selected as the optimal condition for the extraction of fucoxanthin from *C. calcitrans* microalgae using the LBF system.

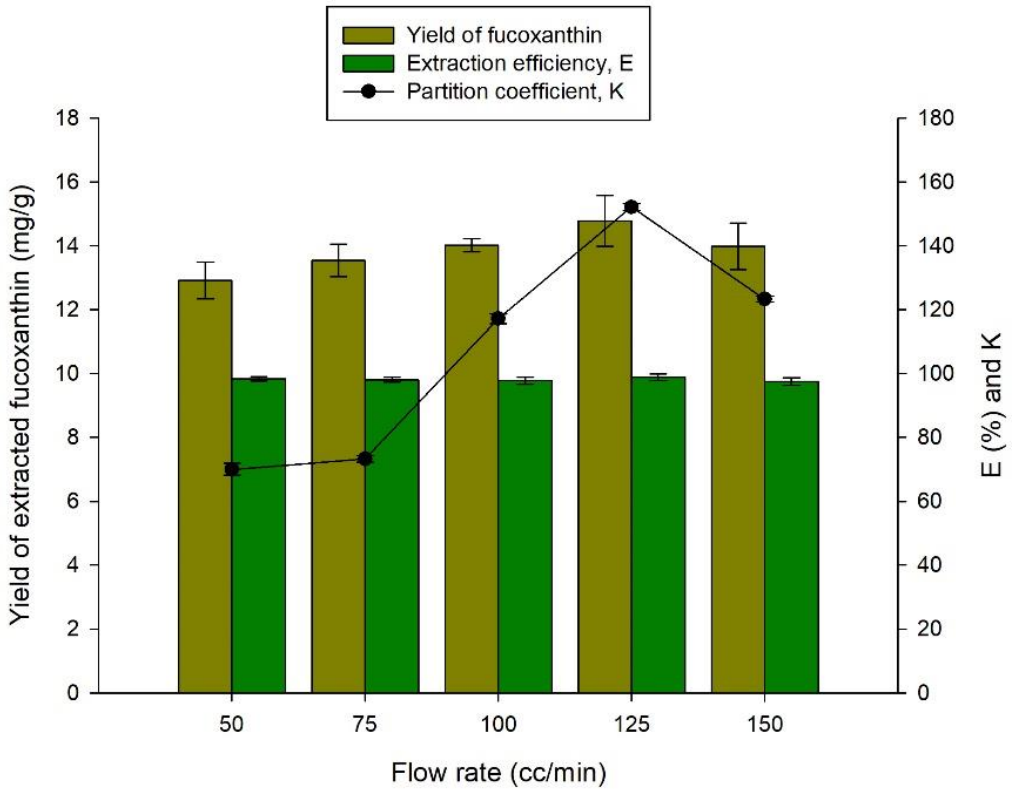


Figure 6.5: Effect of flow rate for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

6.4.2 Optimization of electropermeabilization-assisted LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae

6.4.2.1 Effect of the electropermeabilization-assisted period in the LBF system

The implementation of electropermeabilization-assisted in the LBF system was to enhance the extraction of fucoxanthin from *C. calcitrans* microalgae. In this study, the electropermeabilization-assisted period for extraction of fucoxanthin from *C. calcitrans* microalgae was varied within 2.5 to 10 min in the LBF system. **Figure 6.6** shows the effect of electropermeabilization-assisted period in the extraction process of the LBF system. The yield of extracted fucoxanthin has increases when the electropermeabilization-assisted period varies from 2.5 to 5 min, which then decreased drastically beyond 5 min. The prolonged electropermeabilization-assisted period might denature these highly sensitive carotenoids caused by the electrochemical reaction where the biomass is in contact with the electrodes. Hence, the electropermeabilization-assisted period of 5 min was selected for the subsequent study since it achieves the highest yield of extracted fucoxanthin of 15.44 ± 0.32 mg/g with E value of 99.80 ± 0.05% and K value of 229.21 ± 1.17, respectively.

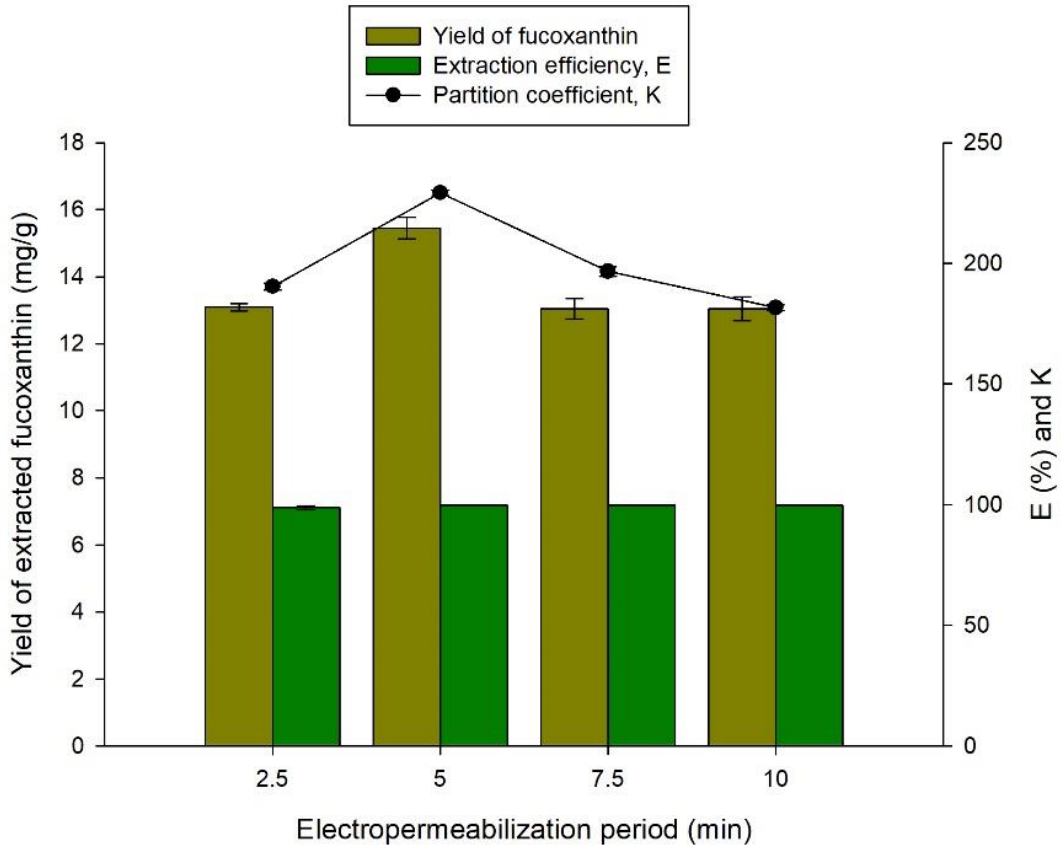


Figure 6.6: Effect of electropermeabilization period for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

6.4.2.2 Effect of the position of copper electrode in the electropermeabilization assisted LBF system

The position of copper electrode in the LBF system is crucial as this may influence the cell permeabilization of the biomass for release of intercellular compounds via the formation of pores in the cell membrane. Therefore, the position of the copper electrode was positioned at three different phase such as top phase, interphase, and bottom phase in the LBF system. Initially, the position of electrode was placed at the interphase based on previous literature studies for the

permeabilization of the biomass (Khoo et al., 2020b, Sankaran et al., 2018a). **Figure 6.7** shows the effect of the position of copper electrode in the LBF system for extraction of fucoxanthin from *C. calcitrans* microalgae. By placing the electrode at the top phase of the LBF system, the yield of extracted fucoxanthin (13.04 ± 0.18 mg/g) was reduced with *K* value of 193.73 ± 1.03. This was probably due to the absence of biomass located at the top phase for the electropermeabilization effect to take place. When the electrode was located to the bottom phase, the yield of extracted fucoxanthin increased by 16.09 ± 0.27 mg/g with *K* value of 236.72 ± 0.88, respectively. The possible explanation could be the large electric field available in the overall LBF system compared to the electrodes positioned at the top and middle phase that restricted the electric field availability throughout the LBF system (Sankaran et al., 2018b). Therefore, the position of copper at the bottom of the LBF system was selected for the subsequent studies.

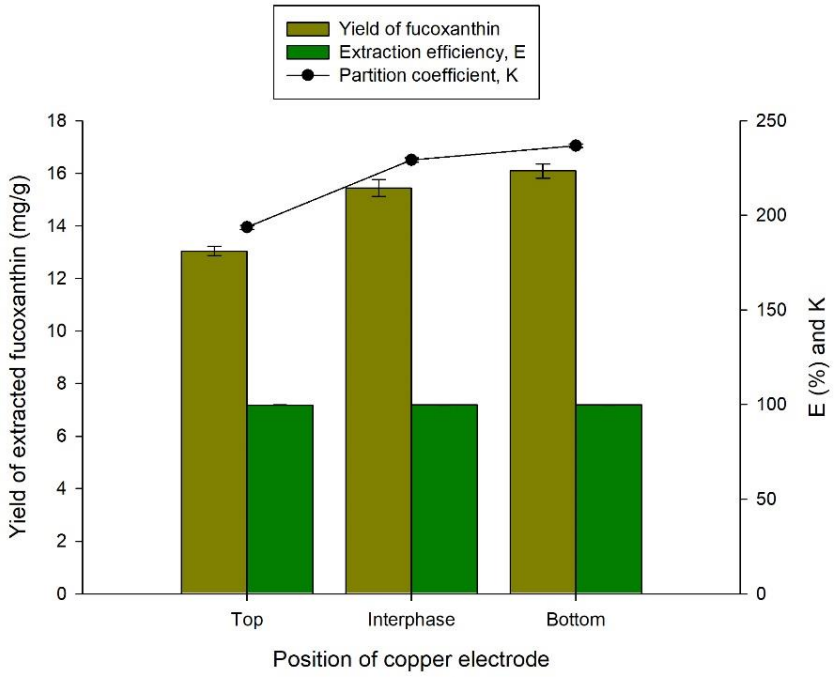


Figure 6.7: Effect of the position of copper electrode for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

6.4.2.3 Effect of voltage applied in the electropermeabilization-assisted LBF system

The effect of voltage was applied in the range of 5 to 15 V in the LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae. According to **Figure 6.8**, the increase of voltage applied in the LBF system from 5 to 15 V significantly increased the yield of extracted fucoxanthin by 11.99 ± 0.36 mg/g to 16.09 ± 0.27 mg/g along with increased *K* value of 174.67 ± 1.07 to 236.72 ± 0.88, respectively. When the voltage increased beyond 15 V, the yield of extracted fucoxanthin was reduced greatly. This possible explanation could be due to the intense electricity discharge from the shock wave and collapse of cavitation bubbles

which release its energy that would denature these highly sensitive carotenoids (Roselló-Soto et al., 2015). In fact, the incorporation of electropermeabilization process also enhance the *E* and *K* values in the LBF system, where these negatively charge *C. calcitrans* microalgae are driven towards the anode of the copper electrode (Lin et al., 2013). Thus, 15 V is the optimal voltage applied in the electropermeabilization-assisted LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae.

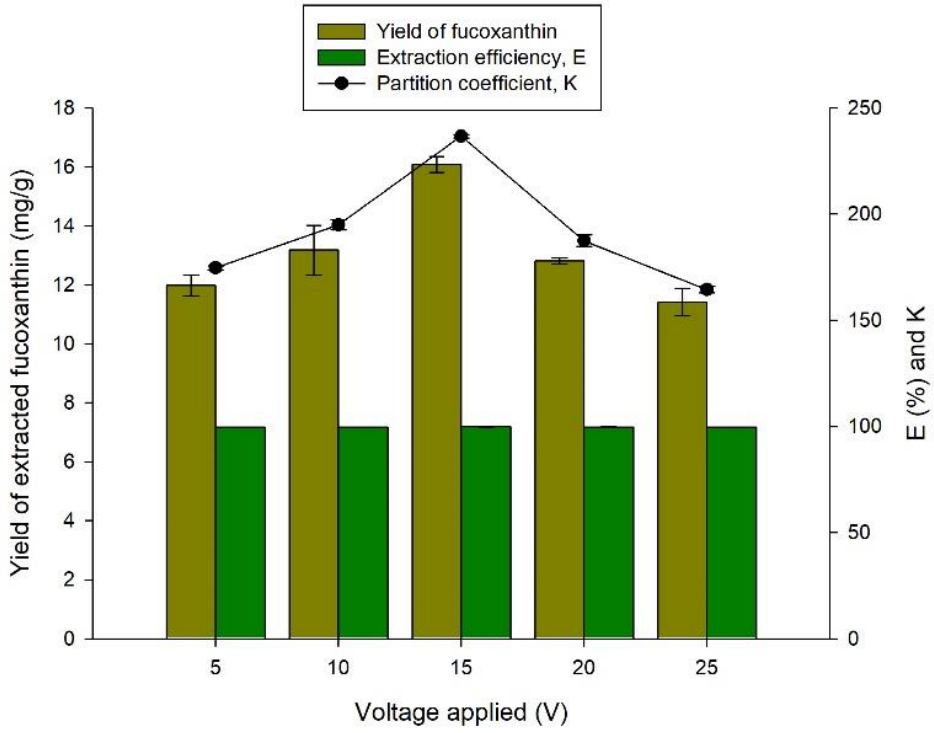


Figure 6.8: Effect of the voltage applied for the extraction of fucoxanthin from *C. calcitrans* microalgae using LBF system.

6.4.2.4 Effect of biomass concentration in the electropermeabilization-assisted LBF system

According to literature, the effect of biomass concentration may affect the yield of extracted fucoxanthin and *K* value of the bioactive compound in the LBF system (Chew et al., 2019a, Khoo et al., 2020b, Chia et al., 2020). By increasing the biomass concentration in the LBF system may create a more concentrated biphasic system, which would cause an uneven partitioning effect that results in a lower efficiency in the recovery of fucoxanthin from *C. calcitrans* microalgae. In addition, the effect of concentrated biphasic system may also mitigate the flotation of air bubbles, therefore this declines the performance of the LBF system. The initial mass of biomass was 50 mg in a 100-mL LBF system, and the mass of biomass was varied within 30 to 70 mg. Based on **Figure 6.9**, the highest yield of extracted fucoxanthin, *E* and *K* value was obtained at 50 mg of microalgae biomass loading with 16.09 ± 0.27 mg/g, 99.80 ± 0.05% and 236.72 ± 0.88, respectively. On the other hand, it can be seen that higher loading of biomass (70 mg) has a lower yield of fucoxanthin (11.31 ± 0.49 mg/g) and *K* value (187.89 ± 2.46). The decrease in *K* value was due to the accumulation of unwanted contaminates and products within the LBF system.

This trend of result was similar with Sankaran et al. (2018), who stated that the overall composition and properties of the LBF system was influence by the concentrated amount of biomass loading (Sankaran et al., 2018b). In fact, a higher biomass loading may influence the volume ratio of 2-propanol to $(NH_4)_2SO_4$ salt in the biphasic system which affects the fucoxanthin partitioning and degenerate the performance of the LBF system (Sankaran et al., 2018a). As a result, a mass of 50

mg of biomass concentration is the optimal condition to achieve the maximum yield of extracted fucoxanthin, *E* and *K* values.

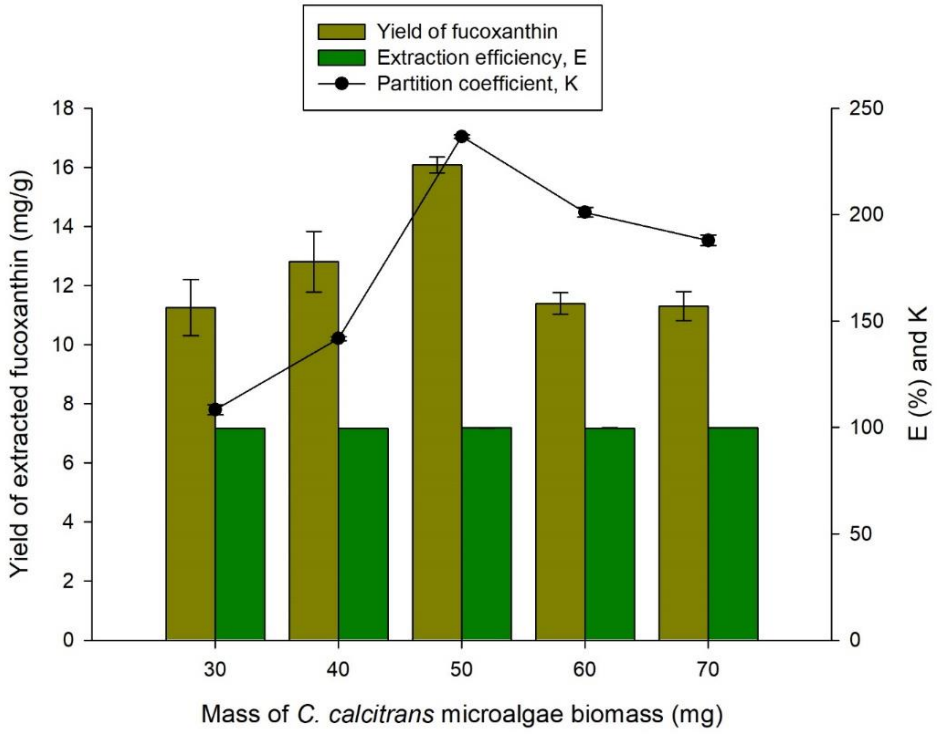


Figure 6.9: Effect of mass of *C. calcitrans* microalgae for the extraction of fucoxanthin using electropermeabilization-assisted LBF system.

6.4.3 Antioxidant activities of extracted fucoxanthin from *C. calcitrans* microalgae

The antioxidant activities of extracted fucoxanthin was evaluated based on both optimized LBF and electropermeabilization-assisted LBF system. TEAC assay was to determine the antioxidant capacity of the extracted fucoxanthin and TPC assay verified the presence of phenolic compounds in the sample. The extracted fucoxanthin from LBF and electropermeabilization-assisted LBF system exhibited

48.76 ± 0.19 μM TE/g CE and 49.28 ± 0.22 μM TE/g CE, respectively with standard curve of y = 0.0453x + 10.109 with R^2 of 0.9992. As for TPC, the extracted fucoxanthin from LBF and electropermeabilization-assisted LBF system achieve 55.53 ± 0.11 mg GAEs/g CE and 60.35 ± 0.12 GAEs/g CE, respectively with standard curve of y = 0.0031x + 0.043 with R^2 of 0.9986. The values of scavenging activity, TEAC and TPC of the extracted fucoxanthin were compared with previous finding using conventional organic solvent extraction and was found to be higher than that (Foo et al., 2015). **Table 6.4** shows the antioxidant properties of extracted fucoxanthin obtained from LBF and electropermeabilization-assisted LBF system.

Table 6.4: Antioxidant properties of extracted fucoxanthin by LBF and electropermeabilization-assisted LBF system.

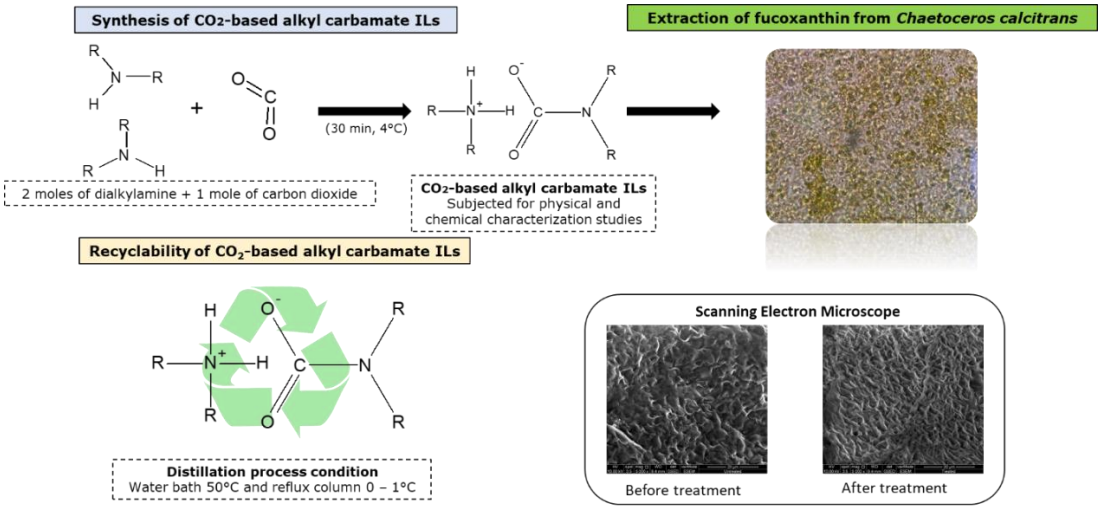
Extraction system	Percentage scavenging (%)	TEAC (μM TE/g CE)	TPC (mg GAEs/g CE)
LBF	75.31 ± 0.25	48.76 ± 0.19	55.53 ± 0.11
Electropermeabilization-assisted LBF	75.85 ± 0.82	49.28 ± 0.22	60.35 ± 0.12

6.5 Conclusion

This work shows the incorporation of electropermeabilization-assisted technology with LBF system for the extraction of fucoxanthin from *C. calcitrans* microalgae. The implementation of electropermeabilization has shown an effective disintegration of *C. calcitrans* microalgae for the extraction of fucoxanthin which is

deemed to be more economical than the current extraction technologies. Besides, the advantages of electropermeabilization-assisted allows a greener and non-thermal process for the disintegration and extraction of sensitive bioactive compounds from microalgae. The results obtained from the optimized LBF condition [90% (w/w) of 2-propanol, 250 g/L of $(NH_4)_2SO_4$, flotation time of 10 min and flotation rate of 125 cc/min] with electropermeabilization-assisted LBF condition [electropermeabilization period of 5 min, interphase position of copper electrode, 15 V of voltage applied and biomass concentration of 50 mg] has successfully achieved an excellent yield of extracted fucoxanthin of 14.78 ± 0.79 mg/g and 16.09 ± 0.27 mg/g, respectively. The extracted fucoxanthin from both systems has exhibited satisfactory antioxidant properties verified by TEAC and TPC assay.

CHAPTER 7 BIOPROCESSING OF *CHAETOCEROS CALCITRANS* FOR THE RECOVERY OF FUCOXANTHIN USING CO_2-BASED ALKYL CARBAMATE IONIC LIQUIDS



This chapter demonstrated a rapid cell permeabilization and extraction of fucoxanthin from *Chaetoceros calcitrans* microalgae by CO_2-based alkyl carbamate ILs. Various extraction conditions such as type of CO_2-based IL, concentration of IL, incubation period and temperature were investigated for the maximal extraction of astaxanthin. The cellular and surface morphology of the untreated and IL-treated biomass was analysed by Scanning Electron Microscopy and light microscope. Studies on recyclability studies were assessed to verify its feasibility for multiple extraction cycles. This chapter consists of thesis-version of work published in the *Bioresource Technology* (Khoo, K. S. et al., 2021, *Bioresource Technology*, 322: 124520. DOI: https://doi.org/10.1016/j.biortech.2020.124520).

Bioprocessing of *Chaetoceros calcitrans* for the recovery of fucoxanthin using CO_2-based alkyl carbamate ionic liquids

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Authors' Contributions:

K.K.S. performed all the experiments; C.W.O., K.W.C. and S.C.F. provided guidance in experimental design and materials; K.K.S., C.W.O. and K.W.C. analyzed and interpreted the data; K.K.S. wrote the manuscript; C.W.O and P.L.S. edited the manuscript.

7.1 Abstract

Ionic liquids (ILs) have emerged as an alternative solvent used in the bioprocessing of microalgae for recovery of valuable biomolecules. The aim of this work is to extract fucoxanthin from *Chaetoceros calcitrans* (*C. calcitrans*) by using the readily distillable CO_2-based alkyl carbamate ILs. The degree of cell permeabilization was analysed by quantification of extracted fucoxanthin and the analyses of cell surface morphology. Among the tested CO_2-based alkyl carbamate ILs, diallylammonium diallylcarbamate (DACARB) extraction system gave the maximal yield of fucoxanthin at 17.51 ± 0.10 mg/g under the optimal extraction conditions [90% (v/v), 3 min and 25°C]. Moreover, the extracted fucoxanthin fraction exhibited the satisfactory antioxidant activities. The recyclability of DACARB was demonstrated in the multiple batches of fucoxanthin extraction. Hence, CO_2-based alkyl carbamate ILs can prospectively substitute conventional organic solvents in the downstream processing of bioactive compounds from microalgae.

Keywords: *Chaetoceros calcitrans*; fucoxanthin; carotenoids; extraction; ionic liquids; CO_2

7.2 Introduction

A tremendous research effort has been devoted to the exploration of microalgae as a favourable source of bioactive compounds over the synthetic versions of these natural compounds produced via chemical routes. Bioactive compounds such as fatty acid, polysaccharides, phycobilin, carotenoids and phenolic compounds have been well characterized for their beneficial health effects, such as antioxidant, anti-inflammatory, anti-diabetic, anti-cancer, anti-malarial, anti-angiogenic and photo-protection activities as demonstrated in medical research (Mohamadnia et al., 2020, Sathasivam and Ki, 2018). Microalgae species including *Chlorella* and *Spirulina* have been granted "GRAS" status (generally recognized as safe), and they can be easily processed into powder form (Panahi et al., 2016, Soni et al., 2017, Chew et al., 2019a). Owing to the their high content of carotenoids (e.g., astaxanthin, fucoxanthin and lutein), microalgae have been a favourite candidate in formulation of food supplement and nutraceuticals (Khoo et al., 2019b).

Fucoxanthin, an epoxycarotenol with molecular formula of $C_{42}H_{58}O_6$, is one of the major carotenoids contributing ≥ 10% of the total production carotenoids in *Bacillariophyceae* and *Prymnesiophyceae* microalgae (Peng et al., 2011, Foo et al., 2017). Fucoxanthinol, amarouciaxanthin A, and halocynthiaxanthin are among the types of fucoxanthin found in most of the marine brown algae. The unique structural features of fucoxanthin include an allenic bond, a conjugated carbonyl, a 5,6-monoepoxide and an acetyl group; these attributes make fucoxanthin an antenna pigment carotenoid in the main light-harvesting complexes, which are responsible for the transfer of energy (> 80%) to the photosynthetic electron transport chains in

both micro- and macroalgae (Mikami and Hosokawa, 2013, Zarekarizi et al., 2019). In fact, various antioxidant analyses including 1'1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-based radical scavenging, ferric reducing antioxidant properties (FRAP), β-carotene bleaching assay and iron chelating assay revealed the roles of fucoxanthin as a powerful antioxidant scavenger and a reducing agent (Foo et al., 2015, Xia et al., 2013, Foo et al., 2017).

The extraction of fucoxanthin from microalgae typically involve the conventional organic solvents like methanol, ethanol, isopropanol, chloroform, acetone, hexane, tetrahydrofuron, ethyl acetate and dimethyl sulfoxide (Foo et al., 2015, Guler et al., 2020, Sivagnanam et al., 2015). The extraction yield is governed by the properties of solvent including dielectric constant and polarity index; the mid-polar solvent is suitable for the fucoxanthin extraction because of the semi-polar characteristics of fucoxanthin (Guler et al., 2020). Although conventional extraction approach provides a higher yield of products, other aspects such as environmental impact, toxicity, consumption volume, and sustainability of these organic solvents should be thoroughly considered. Especially, conventional extraction method demands for a large amount of solvents and the good efforts in managing the disposal of solvents (Mäki-Arvela et al., 2014, Derwenskus et al., 2019). Emerging technologies such as supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been explored for the extraction of fucoxanthin (Derwenskus et al., 2019, Guler et al., 2020, Raguraman et al., 2018, Zhang et al.,

2018, Khoo et al., 2020b). Unfortunately, these methods suffer from the common limitations in terms of high energy consumption as well as the harsh operating conditions (e.g. temperature and pressure) that could denature sensitive carotenoid (Khoo et al., 2020b). In addition, these energy-intensive processes are commonly deemed to be less economical, especially at the large-scale operation.

The distinguished properties such as negligible vapor pressure, high melting points and high thermal stability make ionic liquids (ILs) a suitable solvent for the processing of biomass processing (Vekariya, 2017, Khoo et al., 2020f). The issues of air pollution and health hazards associated with the volatile organic compounds (VOCs) can be prospectively mitigated by adopting ILs as a solvent candidate. Based on the toxicity evaluation using half maximal effective concentration (EC-50), VOCs including o-xylene, phenol, toluene, methyl isobutyl ketone, benzene, ethylene glycol, chloroform, ethyl acetate, acetone, and methanol exhibit a higher level of toxicity than ILs (Stock et al., 2004, Abramenko et al., 2020). Moreover, some of the ILs are highly compatible with the intrinsic operating conditions of biomass processing (e.g., high temperature and pressure). Nonetheless, the existing conventional ILs composed of imidazolium- and pyridinium-based cations have caused the concerns about their toxicity and biodegradability (Choi et al., 2019, Desai et al., 2016). However, the recycling of these conventional ILs remains a challenge due to the inability of these ILs to be distilled and evaporated from the targeted bioproduct. CO_2-based alkyl carbamate ILs are a type of readily distillable ILs composed of CO_2 gas and secondary dialkylamine, forming the dialkylammonium cation and the dialkylcarbamate anion. They can be easily

distilled at a mild operating temperature within 55 to 200°C, depending on the length of alkyl chain of secondary dialkylamine. The benefits of these CO_2-based ILs alkyl carbamate ILs include the utilization of one of the greenhouse gases, CO_2, and the rapid separation of targeted bioproduct from the ILs. In fact, CO_2-based alkyl carbamate ILs have been successfully applied in electrochemical, biochemicals, organic chemical synthesis, extraction process and catalysis (Li et al., 2011b, Zhou et al., 2007, Feroci et al., 2007).

To the best of the authors' knowledge, the cell permeabilization and extraction of fucoxanthin from *Chaetoceros calcitrans* (*C. calcitrans*) microalgae by CO_2-based alkyl carbamate ILs have not been attempted before. In this study, the extraction of fucoxanthin from *C. calcitrans* was evaluated and optimized using one-factor-at-a-time (OFAT) approach, covering the extraction conditions such as type of CO_2-based alkyl carbamate ILs, concentration of IL, incubation period and temperature. In addition, the surface morphology of the permeabilized cells was analysed by light microscope and Field Emission Scanning Electron Microscope (FESEM). The antioxidant properties of the extracted fucoxanthin, including Trolox equivalent antioxidant capacity (TEAC) and total phenolic content (TPC), were also assessed. Lastly, studies on the recyclability of ILs were conducted to evaluate the performances of ILs for the successive batches of extraction.

7.3 Experimental Section

7.3.1 Materials and methods

Ultrapure water produced from Milli-Q integral water purification system (Merck; Darmstadt, Germany) was used in experiments. Standard fucoxanthin was purchased from Sigma Aldrich (Germany). Dimethylammonium dimethylcarbamate (DIMCARB), dipropylamine, dibutylamine, diallyamine (≥ 99% purity), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, potassium persulfate ($K_2O_8S_2$), Folin-Ciocalteu (F-C) reagent, sodium carbonate (Na_2CO_3) and gallic acid were purchased from Sigma Aldrich (St. Louis, USA). The compositions of cultivation media, including iron (III) chloride ($FeCl_3 \cdot 6H_2O$), manganese chloride ($MnCl_2 \cdot 4H_2O$), boric acid (H_3BO_3), ethylene diamine tetraacetic acid disodium (Na-EDTA), sodium hydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), sodium nitrate ($NaNO_3$), zinc chloride ($ZnCl_2$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), ammonium molybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$], copper sulphate ($CuSO_4 \cdot 5H_2O$), silica ($Na_2SiO_3 \cdot 9H_2O$), cobalamin (vitamin B_{12}) and ammonium formate (NH_4HCO_2) were purchased from Sigma Aldrich (St. Louis, USA). Methanol (99.9% purity, HPLC grade) and acetone (99.5% purity, HPLC grade) were purchased from Fisher Chemical (Selangor, Malaysia). All above-mentioned chemicals were of analytical grade, unless mentioned otherwise.

7.3.2 Cultivation and collection of microalgae biomass

C. calcitrans (UPMC-A0010) was mass cultured in a 120-L annular photobioreactors under UV-sterilized seawater supplemented with Conway culture

medium [1.3 g L^{-1} of $FeCl_3 \cdot 6H_2O$, 0.36 g L^{-1} of $MnCl_2 \cdot 4H_2O$, 33.6 g L^{-1} of H_3BO_3, 45 g L^{-1} of Na-EDTA, 20 g L^{-1} of $NaH_2PO_4 \cdot 2H_2O$, 100 g L^{-1} of $NaNO_3$, 2.1 g L^{-1} of $ZnCl_2$, 2.0 g L^{-1} of $CoCl_2 \cdot 6H_2O$, 0.9 g L^{-1} of $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 2.0 g L^{-1} of $CuSO_4 \cdot 5H_2O$, 46.5 g L^{-1} of $Na_2SiO_3 \cdot 9H_2O$ and 1 mL of vitamin B_{12}]; the culture was exposed to the continuous light intensity of 150 µmol $m^{-2}s^{-1}$ (light/dark 12:12 cycle), room temperature and within pH 8.0 – 8.5 for 14 days (Foo et al., 2015). Subsequently, the microalgae were harvested with a tubular separator (model J-1250, Hanil Science, Industrial Co. Ltd., Korea). The harvested biomass was collected and rinsed with 1 mol/L of ammonium formate to remove the excess salt crystal. Then, the microalgae biomass was lyophilized and was stored in the autoclaved sample bottles at -80°C. The lyophilized microalgae biomass was sieved through a 250-micron sized sieved prior to analysis.

7.3.3 Synthesis and characterization of CO_2-based alkyl carbamate ILs

CO_2-based alkyl carbamate ILs were synthesized based on previous study conducted by Khoo et al. (2021) and Song et al. (2018a) (see **Figure 7.1**). The successfully synthesized CO_2-based alkyl carbamate ILs namely dimethylammonium dimethylcarbamate (DIMCARB), dipropylammonium dipropylcarbamate (DPCARB), diallylammonium diallylcarbamate (DACARB) and dibutylammonium dibutylcarbamate were subjected to characterization using Fourier Transform Infra-Red (FTIR) Spectrophotometer, (Frontier Perkin Elmer) with an average scan of 32 scans over 4000 – 400 cm^{-1} (see **Figure Appendix 1** to **5**). The functional group found in the CO_2-based alkyl carbamate ILs such as

symmetric carbamate and carbamate C-O stretching peak can be verified at the wavelengths approximately at ≈1408 cm^{-1} and ≈1622 cm^{-1}, respectively.

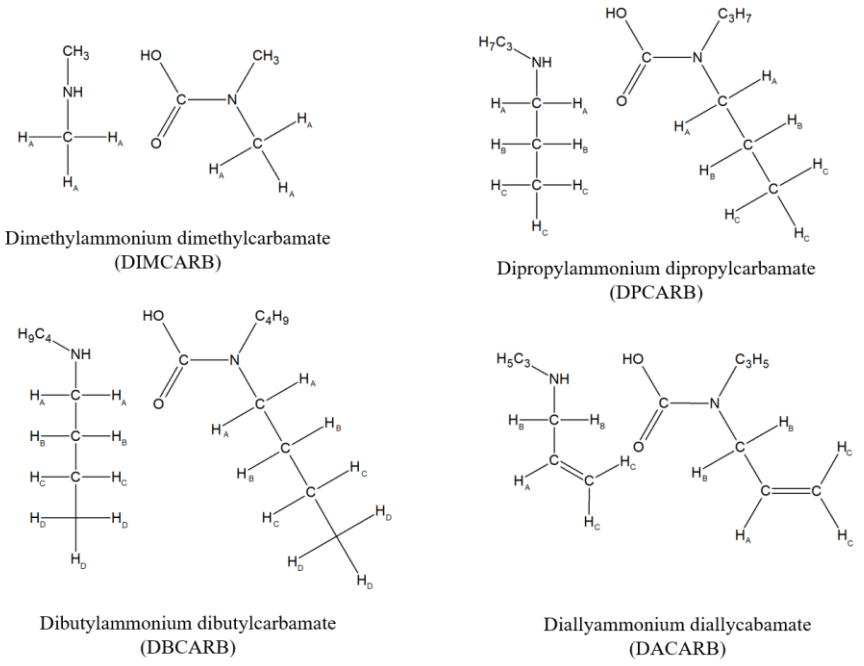

Figure 7.1: Chemical structure of CO_2-based alkyl carbamate ILs

7.3.4 Extraction of fucoxanthin from *C. calcitrans* by CO_2-based alkyl carbamate ILs

The operating parameters in IL-based solvent extraction systems, namely types of CO_2-based alkyl carbamate (DIMCARB, DPCARB, DBCARB and DACARB), concentrations of IL (60-100%, v/v), extraction period (1-5 min) and temperature (25-55°C), were studied systematically, and their effects on the performances of cell permeabilization and fucoxanthin extraction were evaluated based on the yield of fucoxanthin obtained. OFAT approach was used to optimize these operating parameters. In brief, approximately 25 mg of lyophilized microalgae

biomass was incubated in a solution made of ILs and diluent. The mixture was then incubated at a present temperature in an incubator for a predefined duration. The biomass fraction was then separated from the mixture by centrifugation at 7500 rpm for 10 min. The supernatant containing ILs and the extracted fucoxanthin was distilled using a rotary evaporator (Rotavapor R-210, Buchi, Postfach, Flawil, Switzerland). The fucoxanthin was collected as the solid residue from the round bottom flask. The solid fraction was resuspended and diluted in 3 mL of methanol. The sample was then filtered using a 0.45-μm PTFE disposable syringe-filter (Terumo, Malaysia) prior to HPLC analysis.

7.3.5 Quantification of extracted fucoxanthin using HPLC analysis

The fucoxanthin standard as well as the samples were analysed using a High-Performance Liquid Chromatography, HPLC system (Agilent Technologies, 1200 Infinity) coupled with a variable-wavelength detector and an autosampler. A C_{18} column (ZORBAX Eclipse Plus, 4.6 ×100 mm, 3.5 μm) was installed in the HPLC system. The injection volume and flow rate were set at 20 μL and 0.7 mL/min, respectively. A mixture made of methanol and water (97:3) was used as the mobile phase. Prior to the HPLC operation, the mobile phase was filtered through a 0.45-μm membrane filter and was degassed (Khoo et al., 2021). At 445 nm, the fucoxanthin peak was recorded at about 3.5 min of retention time (see **Figure Appendix 13**) (Foo et al., 2015). The standard curve was constructed by using the fucoxanthin standard solutions prepared in methanol at six different concentrations (250, 125, 62.5, 31.25, 15.62 and 7.81 μg/mL). All the extracted fucoxanthin

samples were analysed in triplicate, and the results were expressed in milligram of fucoxanthin per gram microalgae biomass (mg/g).

7.3.6 Spectrophotometric determination of chlorophyll *a* and *b* content

Concentrations of chlorophyll *a* and *b* in the extracts were determined according to Equation (1), and (2) (Lichtenthaler and Buschmann, 2001). The absorbances of chlorophyll *a* and *b* were determined at 665.2 nm and 652.4 nm, respectively, via a spectrophotometer (UV-1800, Shimadzu, Japan).

$$Chl\ a\ (mg\ g^{-1}) = 16.72\ OD_{665.2} - 9.16\ OD_{652.4} \quad (1)$$

$$Chl\ b\ (mg\ g^{-1}) = 34.09\ OD_{652.4} - 15.28 OD_{665.2} \quad (2)$$

where *Chl a* and *b* represent the concentrations of chlorophyll *a* and chlorophyll *b* in methanol, respectively.

7.3.7 Evaluation on antioxidant activities

7.3.7.1 TEAC assay

The antioxidant properties of fucoxanthin extracted from *C. calcitrans* were analysed using the TEAC assay as reported in the previous work by Khoo et al. (2021) with minor modification. In brief, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical ($ABTS^{\bullet}$) stock solution was freshly prepared by mixing 7 mmol/L (0.18 g) of $ABTS^{\bullet}$ solution and 2.45 mmol/L potassium persulfate, $K_2O_8S_2$ solution at 1:1 of volume ratio, then the stock solution was incubated for 24 hr at room temperature under dark condition. $ABTS^{\bullet}$ stock solution was further diluted with methanol until an absorbance value of 0.70 ± 0.05 at 734 nm was

achieved. The stock solution was stored at room temperature prior to usage. TEAC assay was carried out by mixing 100 µL of sample or methanol (as control) with 3.8 mL of diluted ABTS$^{\bullet}$ stock solution. The sample was incubated for 5 min at room temperature and was analysed using a UV-Vis spectrophotometer (UV-1800, Shimadzu) at 735 nm. The results were recorded in triplicated and were expressed as µmol of Trolox equivalents per g of crude extract (TEs/g CE). Trolox solutions in the concentration range of 31.25 - 2000 µM/L were used as the standard. The percentage of scavenging activity was calculated using Equation (3):

$$Percentage\ scavenging\ (\%) = \frac{Control\ (Diluted\ ABTS) - Sample\ (Extracted\ fucoxanthin\ or\ Trolox\ standard\ solution)}{Control\ (Diluted\ ABTS)} \times 100\% \qquad (3)$$

7.3.7.2 TPC assay

The TPC of fucoxanthin was quantified using Folin-Ciocalteu (F-C) method as reported elsewhere (Hajimahmoodi et al., 2013). Firstly, the F-C reagent was diluted 10 folds with the purified water. 100 µL of samples was then mixed with 500 µL of the diluted F-C reagent. The sample was incubated for 5 min at room temperature under dark condition. Then, 2 mL of 75 g/L Na_2CO_3 solution was added into the mixture, which was then left settling for 90 min at room temperature under dark condition. The absorbance of the sample was then measured at 760 nm using a UV-vis spectrophotometer. Gallic acid (7.81 – 500 mg/L) was used as the standard in this assay. The results were expressed as mg of gallic acid equivalents per 100 g of crude extract (GAEs/g CE).

7.3.8 Microscopic and surface morphology analysis of *C. calcitrans*

To evaluate the impact of CO_2-based alkyl carbamate ILs on the degree of cell permeabilization, the surface morphology of *C. calcitrans* was analyzed before and after the treatment by ILs. The surface morphology of *C. calcitrans* was analysed by a compound microscope (CX21, Olympus) equipped with an eyepiece power of 10× and two objective lenses with 40× and 100× (oil immersion) magnification. The surface morphology of permeabilized cells was also scrutinized under a higher magnification by a Field Emission Scanning Electron Microscope (Quanta 400, FEI, USA) equipped with an Environmental Scanning Electron Microscope (ESEM) detector.

7.3.9 Recyclability of DACARB

DACARB was distilled from the extracted fucoxanthin samples via rotary evaporation, and the recovered IL was reused for the subsequent round of extraction. In this study, the extraction system was scaled up by 5 folds because a larger volume of ILs could avoid the loss of ILs that were trapped in the condenser. The samples collected from the extraction systems were first heated to 50°C in a rotary evaporator. The evaporated DACARB was condensed at 1°C and was collected in the round bottom flask. The DACARB recovered from the rotary evaporator was used in the treatment of a new batch of microalgal biomass. To compensate the loss of IL fraction during evaporation, the concentration of DACARB used in the new batch of extraction system was adjusted accordingly. The successive extraction of fucoxanthin by the recycled ILs was conducted three times.

7.3.10 Statistical analysis

The statistical analysis was conducted by calculating the average of triplicate readings collected from the experiments. The concentration of fucoxanthin extracted from *C. calcitrans* was expressed as mean ± standard deviation (SD) mg per gram of biomass (mg/g). All the collected data was subjected to one-way ANOVA using Microsoft Excel to identify the significant differences of $p \leq 0.05$.

7.4 Result and discussion

7.4.1 Effect of types of CO_2-based alkyl carbamate ILs on extraction of fucoxanthin from *C. calcitrans*

Four types of CO_2-based alkyl carbamate ILs, namely DIMCARB, DPCARB, DBCARB and DACARB, were investigated for their performance in the cell permeabilization of *C. calcitrans* and the extraction of fucoxanthin. Based on **Table 7.1**, DACARB system gave the highest yield of fucoxanthin (14.98 ± 0.11 mg/g), followed by DPCARB, DIMCARB and DBCARB systems yielding 14.31 ± 0.14 mg/g, 12.04 ± 0.01 mg/g, and 5.42 ± 0.31 mg/g of fucoxanthin, respectively. The results showed that the performance of CO_2-based alkyl carbamate ILs in permeabilization of the microalgal cell wall was governed by the structure, functional group and chemical properties of the cationic and anionic counterparts of IL. The mechanism of cell wall permeabilization was mainly attributed by the hydrogen bonding interaction (hydrogen-bonding acceptor) between the anions of IL, [R_2N-COO^-], and the hydroxyl groups of cellulose from the cell wall of *C. calcitrans*. This finding was supported by Endo et al. (2016) reporting a gradual

downshift from 4.5 ppm to 6.8 ppm in the ^{1}H NMR spectrum of hydroxyl group (-OH) of cellulose, which indicates that the deconstructed crystalline structure of cellulose was caused by the anion bridging phenomenon induced by the hydrogen bonding. On the other hand, Swatloski et al. (2002) claimed that the hydrophilic IL has a higher tendency to disrupt the hydrogen bond network of cellulose in the cell wall. This finding was in an agreement with the results reported here, where the hydrophobic nature of DBCARB resulted in a lower extraction yield of fucoxanthin. In fact, a similar trend of results was demonstrated in a previous study conducted by Desai et al. (2016), who reported that a longer alkyl chain of anions in imidazole-based ILs is important in dissolving certain components in the encysted cell wall of *Haematococcus* microalgae. The excellent performance of DACARB in the extraction of fucoxanthin from *C. calcitrans* may be contributed by the long anion chain as well as the hydrophilic nature of IL. In consideration of the highest yield of fucoxanthin obtained, DACARB was selected for the studies of other operating conditions.

Table 7.1: Yields of fucoxanthin, chlorophyll *a* and *b* extracted from *C. calcitrans* by various types of CO_2-based alkyl carbamate ILs

Type of CO_2-based ILs [a]	**Fucoxanthin content (mg. g^{-1})**	**Chlorophyll *a* (mg. g^{-1})**	**Chlorophyll *b* (mg. g^{-1})**
DIMCARB	12.04 ± 0.11	2.45 ± 0.01	2.04 ± 0.03
DPCARB	14.31 ± 0.13	3.88 ± 0.05	2.19 ± 0.05
DBCARB	5.42 ± 0.31	3.43 ± 0.01	5.34 ± 0.09
DACARB	14.98 ± 0.11	2.71 ± 0.01	1.69 ± 0.02

[a] Experiment conditions: 100% (v/v) of ILs; 3 min of incubation; 25°C

7.4.2 Effect of concentrations of DACARB on extraction of fucoxanthin from *C. calcitrans*

For comparative purposes, the control studies were conducted using conventional organic solvents (i.e., 100% of methanol and acetone) for the extraction of fucoxanthin from *C. calcitrans*. Conventional solvents have been widely applied in the extraction of bioactive compounds due to their characteristic polarity indices (i.e., chloroform (4.1), methanol (5.1) = acetone (5.1), ethanol (5.2) and water (9.0) (Foo et al., 2015). To evaluate the effect of DACARB concentration on the performance of fucoxanthin extraction, the diluted solutions of DACARB diluted with purified water (diluent) ranging from 60% (v/v) to 90% (v/v) was used. Based on **Figure 7.2**, as the concentration of IL increased from 60% (v/v) to 90% (v/v), the yield of extracted fucoxanthin increased from 11.99 ± 0.17 mg/g, 13.70 ± 0.14, 15.06 ± 0.22 mg/g and 17.51 ± 0.10 mg/g, respectively. The results showed that a lower concentration of DACARB does not promote a good permeabilization of microalgal cells for the extraction of fucoxanthin from *C. calcitrans*. Similarly, 100% (v/v) of DACARB was not favourable for the extraction, which could be due to the absence of diluent (purified water) for aiding the cell permeabilization. It is postulated that the presence of diluent facilitates the release of fucoxanthin from microalgae, while a definite amount of ILs is necessary for permeabilizing the cells in the first place. The effect of concentration of DACARB on fucoxanthin yield was subjected to statistical analysis, which shows that the yield of fucoxanthin was significantly affected ($p<0.05$) by the concentration of DACARB. As a result, 90% (v/v) of DACARB was selected as a constant in the following extraction studies.

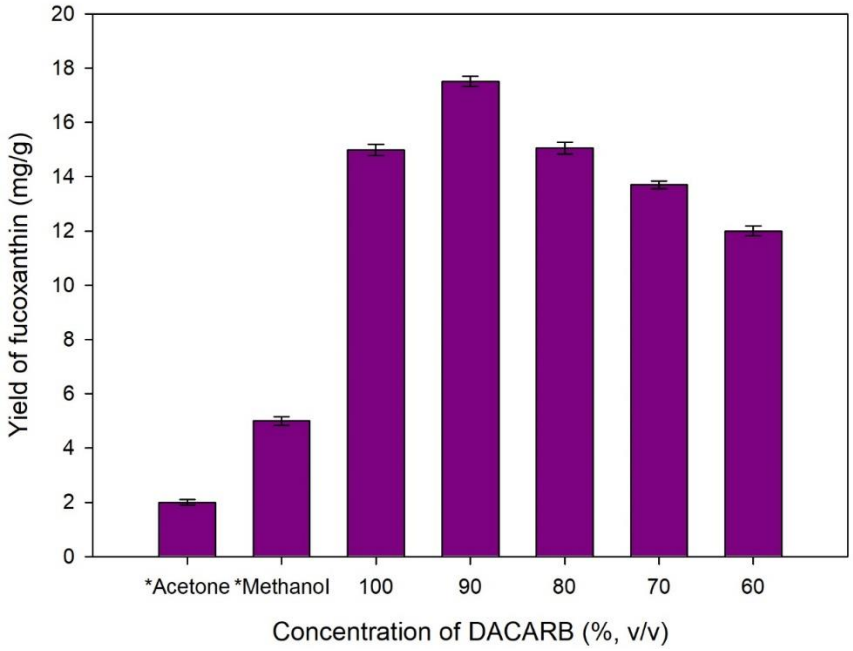

Figure 7.2: Yields of fucoxanthin from the extraction systems conducted at different concentrations of DACARB. Control studies were performed by using acetone and methanol at 100% (v/v). Experiment conditions: 3 min of incubation; 25°C.

7.4.3 Effect of extraction time on the extraction of fucoxanthin from *C. calcitrans*

Duration of extraction can have a significant effect ($p < 0.05$) on fucoxanthin extraction because a sufficient contact period ensures the complete for permeabilization of *C. calcitrans* by DACARB and the dissolution of fucoxanthin by the ILs from biomass. **Figure 7.3** shows the yield of fucoxanthin obtained at different extraction periods. When the duration of extraction increased from 1 min

to 3 min, the yield of fucoxanthin increased marginally from 14.28 ± 0.30 mg/g to 17.51 ± 0.10 mg/g. In general, a longer incubation period allows a greater degree of cell permeabilization by DACARB, thereby enhancing the permeation and disintegration of cellular structure of *C. calcitrans*. However, there was no significant increase in the yield beyond 3 min of extraction process; this phenomenon may indicate the complete diffusion of fucoxanthin from *C. calcitrans*, reaching the maximal mass transfer of target compound between the inner and outer regions of cells (Ruen-ngam et al., 2012). Therefore, 3 min of extraction time was selected as the optimum incubation period for the extraction of fucoxanthin from *C. calcitrans*.

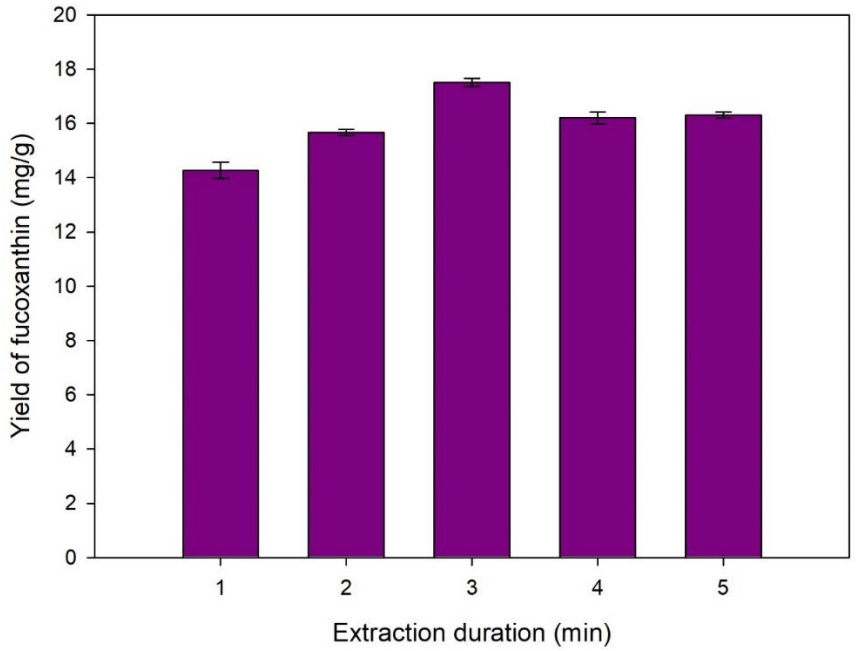

Figure 7.3: Yields of fucoxanthin from the extraction systems incubated for different durations. Experiment conditions: 90% (v/v) of DACARB; 25°C.

7.4.5 Effect of temperature on the extraction of fucoxanthin from *C. calcitrans*

The temperature of extraction condition may influence the diffusibility of bioactive compounds from microalgae as well as solubility of IL during cell permeabilization. An optimal temperature used in the extraction is critical because a high temperature may degrade the temperature-sensitive compounds, especially carotenoids (i.e., lutein, astaxanthin, fucoxanthin and β-carotene). As shown in **Figure 7.4**, an increase in the temperature of extraction from 25°C to 55°C causes a gradual reduction in fucoxanthin yield from 17.51 ± 0.10 mg/g to 13.85 ± 0.47 mg/g. Based on the statistical analysis, the yield of fucoxanthin was significantly affected ($p < 0.05$) by the temperature used in the extraction process. Hence, 25°C is considered as an optimal temperature for the extraction of fucoxanthin from *C. calcitrans*. This temperature condition is in an agreement with a previous extraction study conducted by Viera et al. (2018) at optimum temperature of 25°C, who showed the maximal yield of carotenoid ranging from 2.57 ± 0.26 mg/g to 3.31 ± 0.02 mg/g, depending on the batch of algae stock used (Vieira et al., 2018). In addition, a high temperature condition is also not desirable because of the low boiling point of DACARB (59°C); the loss of DACARB during the extraction will affect the maximal extraction yield of fucoxanthin. Hence, the ideal temperature used in the extraction of fucoxanthin from *C. calcitrans* is at 25°C, which can be easily achieved without requiring excessive heating and cooling of the extraction system.

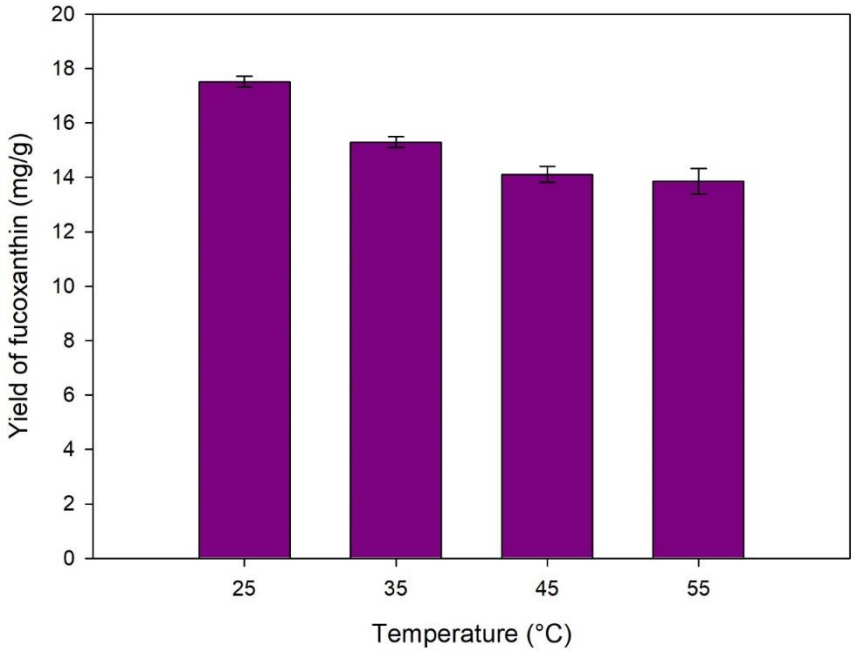

Figure 7.4: Yields of fucoxanthin from the extraction systems conducted at different temperatures. Experiment conditions: 90% (v/v) of DACARB; 3 min of incubation.

7.4.6 Comparison studies and assessment of environmental sustainability of DACARB-based extraction system

The DACARB-based solvent extraction system was compared with other reported extraction strategies in terms of yield of fucoxanthin and operation conditions. Conventional solvent extraction has been successfully used in the extraction of fucoxanthin from microalgal source; however, their yields of fucoxanthin were considered significantly lower than the DACARB-based extraction system. For example, the yields of fucoxanthin obtained from the solvent

extraction systems comprising 100% (v/v) of methanol, ethanol, acetone or ethyl acetate were in the range of 1.73 – 2.23 mg/g (Foo et al., 2015, Kim et al., 2012a). Extraction of fucoxanthin from *Phaeodactylum tricornutum* microalgae has also been attempted by using various extraction methods including maceration extraction (conditions: 25°C, 30 min; yield: 15.71 mg/g), Soxhlet extraction (conditions: 100% ethanol, 80°C; yield: 15.42 mg/g), ultrasound-assisted extraction (conditions: 70 KHz, 25°C, 30 min; yield: 15.96 mg/g) and pressurized liquid extraction (conditions: 100°C, 1500 psi at 30 min; yield: 16.51 mg/g) (Kim et al., 2012a). The performances of these extraction systems are comparable to the DACARB-based solvent extraction system, but most of these extraction systems rely of energy-intensive instrumentation (e.g., high temperature and pressure), which may also easily denature the labile carotenoids after the processing (Desai et al., 2016, Liu et al., 2019b).

ILs have been claimed to be an expensive solvent due to their costly starting materials for synthesis; therefore, it is important to adopt strategies that allow the recovery of ILs after their usage and ensure the practicability of ILs at the commercial scale (Abu-Eishah, 2011). The recovery of IL can be attained by approaches like distillation, extraction, adsorption, chromatography, membrane-based separation and crystallization. The selection of recovery strategies must consider the characteristics of ILs such as viscosity, chain length, volatility, stability, toxicity and biodegradability. In this study, DACARB used in the optimized extraction conditions was recovered and reused in multiple cycles of extraction. The yield of fucoxanthin as well as the recovery percentage of ILs are reported in **Figure**

7.5. The yields of fucoxanthin slightly decreases after multiple rounds of extraction using the recycled DACARB. This might be due to the presence of water residues generated during the distillation of DACARB. As stated in **Section 7.4.2**, a drop in the DACARB concentration below the optimal level will reduce the yield of fucoxanthin because the diluent concentration (i.e., distillate water) could impact the performance of cell permeabilization. Besides, the loss of DACARB could be caused by the residues of IL trapped in the algal biomass after the extraction process. Based on the findings from Zhou et al. (2019) reported that the poor performance of IL-based solvent extraction may also be due to the presence of components (i.e., impurity and residual) from microalgae. Therefore, it is important to maintain the composition of recovered ILs before employing them for the multiple rounds of extraction.

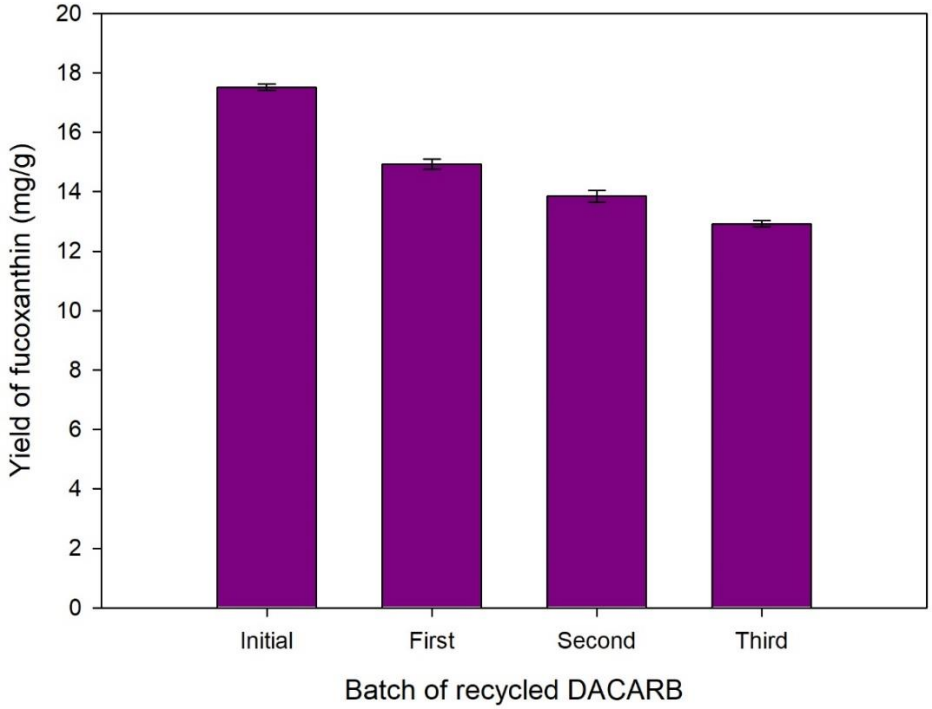

Figure 7.5: Yields of fucoxanthin from the extraction systems conducted using different batches of recycled DACARB. Experiment conditions: 90% (v/v) of DACARB; 3 min of incubation; 25°C.

7.4.7 Cellular and surface morphology studies of DACARB-treated *C. calcitrans*

The degree of cell permeabilization by DACARB can be assessed by evaluating the surface morphology of *C. calcitrans* after the extraction process. The microscopic images of *C. calcitrans* before and after the treatment by DACARB under the optimized extraction conditions are shown in **Figures 7.6**. Based on **Figure 7.6 (A1)** and **(A2)**, the *C. calcitrans* microalgae are irregular-like spherical in shape and composed with yellowish fucoxanthin-rich pigments encapsulated

inside the cells by cell walls. After the treatment of microalgae by DACARB, the yellowish fucoxanthin-rich pigments were absent, leaving the colourless cells (see **Figure 7.6 (B1)** and **(B2)**). This revealed the release of fucoxanthin pigments to the surrounding through the permeabilized cell wall of *C. calcitrans*. The similar findings were also reported in previous studies adopting IL in the extraction studies of biomass processing (Choi et al., 2019, Desai et al., 2016, Vijayaraghavan and Macfarlane, 2014). Since the cavities formed on the cell surface is difficult to be observed, the microalgal cells were subjected to FESEM (see **Figure 7.7**). From the FESEM images, the freeze-dried *C. calcitrans* before DACARB treatment had a smoother surface that is free from cavities shown in **Figure 7.7 (A1) to (A4**). Conversely, the cell surface of the treated *C. calcitrans* appeared to be wrinkled and rough (see **Figure 7.7 (B1) to (B4)**). Moreover, the treated *C. calcitrans* cells have more cavities on the surface, and this indicates the compromised structure of cell wall that permits the diffusion of fucoxanthin-rich pigment through these tiny cavities. These findings prove the capability of DACARB in permeabilizing the cell wall of *C. calcitrans*.

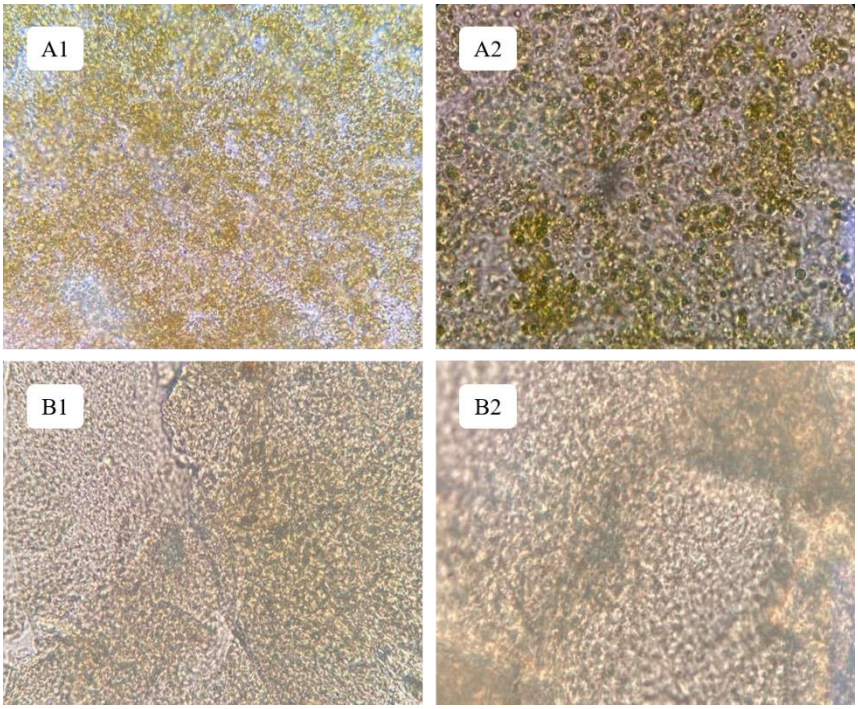

Figure 7.6: Microscopic images of cellular morphology of *C. calcitrans* microalgae **(A)** before, and **(B)** after cell permeabilization by DACARB-optimized extraction conditions under magnification of **(1)** 400× and **(2)** 1000×, respectively.

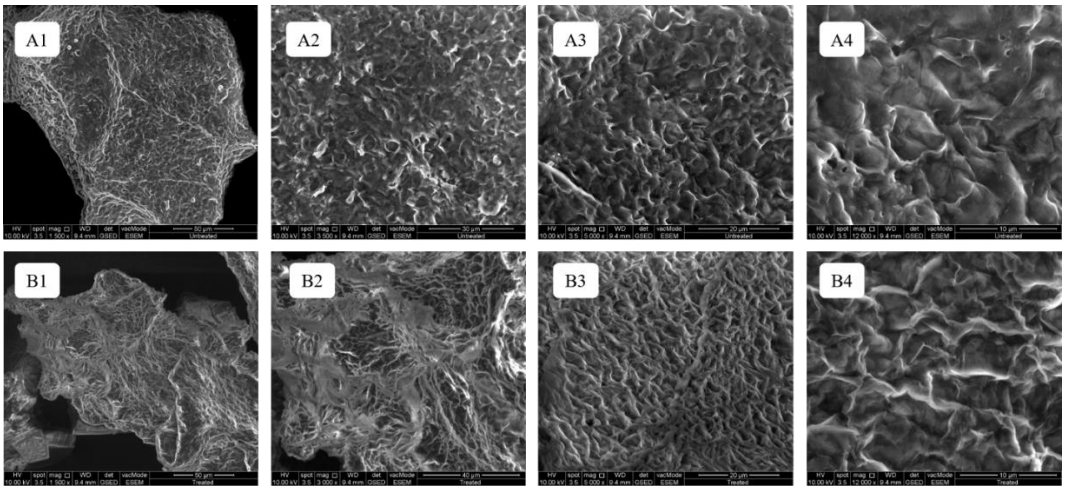

Figure 7.7: SEM of *C. calcitrans* microalgae **(A)** before, and **(B)** after cell permeabilization by DACARB-optimized extraction conditions under magnification, where **1-4** refers to the magnification at 1500×, 3500×, 5000× and 12000×, respectively.

7.4.8 Antioxidant activities of fucoxanthin extracted from *C. calcitrans*

The fucoxanthin extracted from *C. calcitrans* was subjected to antioxidant analyses to further confirm its bioactive properties. TEAC assay determined the fucoxanthin's antioxidant capacity, while the TPC assay confirms the presence of polyphenolic compounds in the samples. **Table 7.2** shows the antioxidant properties of fucoxanthin extracted from *C. calcitrans* using different CO_2-based alkyl carbamate ILs. The fucoxanthin extracted by DACARB showed the highest antioxidant value (934.45 ± 0.08 μM TE/g CE; standard curve: y = 0.0996x + 2.5374; R^2 = 0.9962). As compared to the previous findings, the scavenging activity of the extracted fucoxanthin was higher than that extracted by the conventional organic solvents (Goiris et al., 2012, Cha et al., 2010, Foo et al., 2017). On the other hand, the fucoxanthin extracted by DACARB system exhibited a TPC value of 6.55 ± 0.07 mg GAEs/g CE [standard curve: OD_{760} = 0.0032(Gallic acid concentration) + 0.0519; R^2=0.9997).

Table 7.2: Antioxidant properties of fucoxanthin extracted by different CO_2-based alkyl carbamate ILs.

CO_2-based alkyl carbamate ILs	**Percentage scavenging (%)**	**TEAC (μM TE/g CE) [a]**	**TPC (mg GAEs/g CE) [a]**
DIMCARB	95.28 ± 0.04	931.19 ± 0.09	5.88 ± 0.06
DPCARB	95.55 ± 0.01	933.87 ± 0.08	5.30 ± 0.06
DBCARB	95.55 ± 0.04	933.86 ± 0.07	5.75 ± 0.08
DACARB	95.61 ± 0.02	934.45 ± 0.08	6.55 ± 0.07

[a] TE is Trolox equivalents, CE is crude extract, and GAE is gallic acid equivalent.

7.5 Conclusion

This work unveiles the potential of cell permeabilization and extraction of fucoxanthin from *C. calcitrans* using CO_2-based alkyl carbamate ILs. DACARB system shows the best extraction performance, under which the optimized conditions [90%, (v/v) of DACARB, 3 min, 25°C)] gave the highest yield of fucoxanthin (17.51 mg/g). From the analysis of surface morphology, fucoxanthin was released through the tiny cavities present on the permeabilized cell walls of *C. calcitrans*. The satisfactory antioxidant activities of the extracted fucoxanthin was

validated by TEAC and TPC assays. The recyclability of DACARB was proven by the successive cycles of fucoxanthin extraction from *C. calcitrans*.

CHAPTER 8 CONLUSIONS AND FUTURE WORKS

This thesis project established the green alternative extraction and separation approach for the recovery of pigments from microalgae. In this chapter, the research findings and achievements conducted throughout the study will be summarized. This section also demonstrates the recommendation and possible future work of this project that can be explored in the future.

8.1 Research Achievements

This thesis has accomplished the sustainable and green alternative extraction approaches for the recovery of pigments from microalgae. The development of liquid biphasic system with recent advances technologies using ultrasound- and electropermeabilization-assisted has been developed in this research work as an effective pretreatment method or cell disruption technique. The implementation of ionic liquids technology as a greener alternative solvent for the cell permeabilization of astaxanthin and fucoxanthin has been successfully performed. In regard to the society impact of the research, the work presented in the thesis has addressed the limitation of current microalgae biorefinery process (e.g. time-consuming, multistep process, high temperature and pressure). The incorporation of liquid biphasic system with assisted technologies has provide a one-step pretreatment and extraction process to overcome the prolonged multistep process of conventional biorefinery. As for the ionic liquid technology, the proposed ILs used has showed a milder permeabilization approach and the treated solvent used can be easily separated and

recycled for the subsequent extraction process. Thereby, the society impact has been addressed in which the overall cost of the extraction solvent used has been reduced and concerns such as disposing hazardous waste has been overcome by recycling the extraction solvent after the process. The comprehensive description of each research achievement is stated as followed:

(1) **Extraction of natural astaxanthin from *Haematococcus pluvialis* using liquid biphasic flotation system:** Liquid biphasic flotation (LBF) system has been developed for the extraction of astaxanthin from *Haematococcus pluvialis* microalgae. The optimized conditions for the LBF system composed of 100% (w/w) of 2 propanol, 350 g/L of ammonium sulphate salt, volume ratio of 1:1, flotation period of 15 min and biomass loading of 10 mg has successfully achieved astaxanthin recovery yield of $95.11 \pm 1.35\%$ and extraction efficiency of $99.86 \pm 0.05\%$, respectively. A scaled up 300 mL LBF system was also performed, indicating the feasibility of extracting astaxanthin from microalgae with recovery yield of $78.38 \pm 0.93\%$ at a larger scale. The bubbling effect in the LBF system has effectively improved the recovery yield and extraction efficiency of astaxanthin and it is considered a cost effective and shorter processing time for the downstream processing of various carotenoids.

(2) **Integrated ultrasound-assisted liquid biphasic flotation for efficient extraction of astaxanthin from *Haematococcus pluvialis:*** The development of an effective one-step pretreatment and extraction of astaxanthin from *Haematococcus pluvialis* microalgae using integrated

ultrasound-assisted liquid biphasic flotation was performed. The maximal astaxanthin recovery and extraction efficiency of 95.08 ± 3.02% and 99.75 ± 0.06% is achieved under previous optimized LBF parameters and additional ultrasound-assisted parameters as shown: ultrasound horn at interface, pulse mode of 30 s ON/5 s OFF, 40% of amplitude, flowrate of 100 cc/min, flotation period of 15 min and biomass loading of 100 mg. The cellular and surface morphologies after ultrasonication treatment was assessed using compound microscope and Field Electron Scanning Electron Microscope (FESEM). The mechanism studies of ultrasonication in the cell disruption of *Haematococcus pluvialis* microalgae was investigated. The results obtained from the scaled up 1.5L LBF system has shown a promising recovery of astaxanthin of 83.73 ± 0.70%. This indicate the efficiency and feasibility of the integrated ultrasound technology with LBF system in producing astaxanthin in industrial scale.

(3) Permeabilization of *Haematococcus pluvialis* and solid-liquid extraction of astaxanthin by CO_2-based alkyl carbamate ionic liquids: Sustainable and green extraction approaches of astaxanthin from *Haematococcus pluvialis* microalgae by CO_2-based alkyl carbamate ionic liquids was proposed. This proposed CO_2-based carbamate ionic liquids are readily distillable after the permeabilization process compared to conventional imidazole- and pyridinium-based ILs. Among the characterization and tested CO_2-based ILs, dimethylammonium dimethylcarbamate, DIMCARB achieved the best performance of astaxanthin extraction of 27.99 mg/g under optimized condition of 100% (w/w) of DIMCARB, extraction period of 75

min at extraction temperature of 45°C. The DIMCARB-treated cell was subjected to cellular and surface morphology analysis by a compound microscope and Field Emission Scanning Electron Microscope (FESEM). The recyclability performance of DIMCARB extraction system achieved three successive rounds of astaxanthin extraction with the recycled DIMCARB solution. Nonetheless, the extracted astaxanthin exhibit excellent antioxidant properties verified using TEAC and TPC assay. The implementation of CO_2-based alkyl carbamate has shown great potential green solvent used in the extraction of astaxanthin.

(4) Electropermeabilization-assisted liquid biphasic flotation for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae: The implementation of electropermeabilization-assisted liquid biphasic flotation system for the recovery of fucoxanthin from *Chaetoceros calcitrans* microalgae was evaluated. The maximal extracted fucoxanthin achieved 16.09 ± 0.27 mg/g with extraction efficiency of 99.80 ± 0.05% and partitioning coefficient of 236.72 ± 0.88 under optimized electropermeabilization-assisted liquid biphasic flotation system: 90% (w/w) of 2-propanol, 250 g/L of $(NH_4)_2SO_4$, flotation time of 10 min, flotation rate of 125 cc/min, electropermeabilization period of 5 min, interphase position of copper electrode, 15 V of voltage applied and biomass concentration of 50 mg. The extracted fucoxanthin from liquid biphasic flotation system and electropermeabilization-assisted liquid biphasic flotation system exhibited satisfactory antioxidant properties verified by TEAC and TPC assay. The incorporation of electropermeabilization-assisted

shows a greener and non-thermal disintegration approach for the permeabilization of cell membrane which is favourable for temperature-sensitive carotenoids.

(5) **Bioprocessing of *Chaetoceros calcitrans* for the recovery of fucoxanthin using CO_2-based alkyl carbamate ionic liquids**: The potential of cell permeabilization of *Chaetoceros calcitrans* microalgae using CO_2-based alkyl carbamate ionic liquids for the recovery of fucoxanthin was assessed. Diallylammonium diallylcarbamate, DACARB extraction system achieved a maximal yield of fucoxanthin at 17.51 mg/g under optimized condition of 90% (v/v), extraction time of 3 min at extraction temperature of 25°C. The DACARB-treated cell was subjected to cellular and surface morphology studies to validate the degree of cell permeabilization process. The recyclability performance of DACARB extraction system has achieved three successive rounds of fucoxanthin extraction. The extracted fucoxanthin provide satisfactory antioxidant activities verified using TEAC and TPC. These findings indicated that CO_2-based alkyl carbamate can prospectively substitute conventional organic solvents as green solvent in the downstream processing of carotenoids from microalgae.

8.2 Future Works

This section focuses on the current research gaps and future work are recommended to improve the downstream processing towards commercialization in

a greener and sustainable approach. The future works and opportunities to further improve and extend are as followed:

(1) Recyclability of phase component and scale-up studies from liquid biphasic system and ionic liquid technologies. After the extraction process, it is highly suggested to recycle the phase components from the liquid biphasic system technologies such as alcohols, salts and ionic liquids for the subsequent extraction process. This approach will mitigate the operating cost by reducing the usage of fresh chemicals for every extraction process. Besides that, the recycled phase components must be evaluated in terms of its quality and recovery of the targeted compound compared to the initial extraction process. High recovery yield, separation efficiency and partition coefficient were obtained throughout the utilization of LBF, ultrasound-assisted LBF, electropermeabilization-assisted LBF and ionic liquid technologies. However, it is important to consider the feasibility of adapting these techniques for commercialization application by developing a larger scale system that fit the liquid biphasic system and ionic liquid technologies. For instance, design and modelling of a recovery column that can accommodate the condition required after the extraction process. Furthermore, future work in applying real-time monitoring using biosensors and internet of things technologies with liquid biphasic system and ionic liquid technologies to examine and avoid any possibilities of errors during the extraction process.

(2) Purification of carotenoids after the extraction process. The extraction of carotenoids from microalgae consists of chlorophyl a, b and c in the extracted product. It is suggested to improvise a purification step after the extraction process

to purify these carotenoids before the encapsulation step. Besides that, conventional purification techniques such as membrane filtration and chromatography are not economic feasible in large-scale processes. It is proposed as a future work to incorporate activated carbon or silica gels into liquid biphasic system and ionic liquid technologies for a simultaneous extraction and purification process.

(3) Life cycle assessment of extracted astaxanthin and fucoxanthin. The extraction of astaxanthin and fucoxanthin from this research work suggest considering their respective life cycle assessment of the overall extraction process. This evaluation would help researchers to further investigate the sustainability from cradle to grave. In addition, life cycle economical assessment of the liquid biphasic system and ionic liquid technologies would provide insights on the costing distinguish from the upstream and downstream processing of the final product. Lastly, the energy assessment of the extraction process needs to be investigated to ensure these merits as energy output are sustainable in the large-scale system.

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APPENDIX

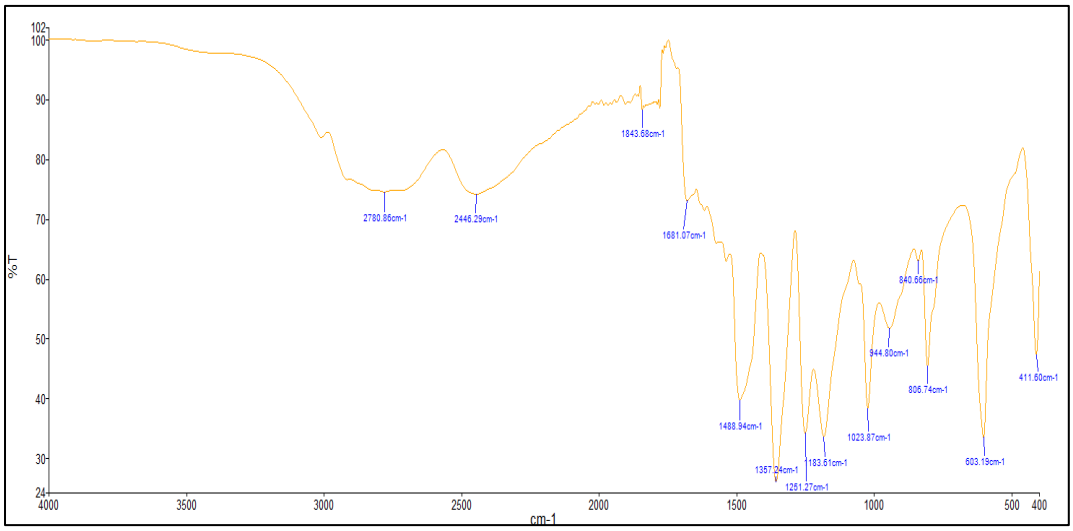

Figure A1: FTIR spectrum of DIMCARB

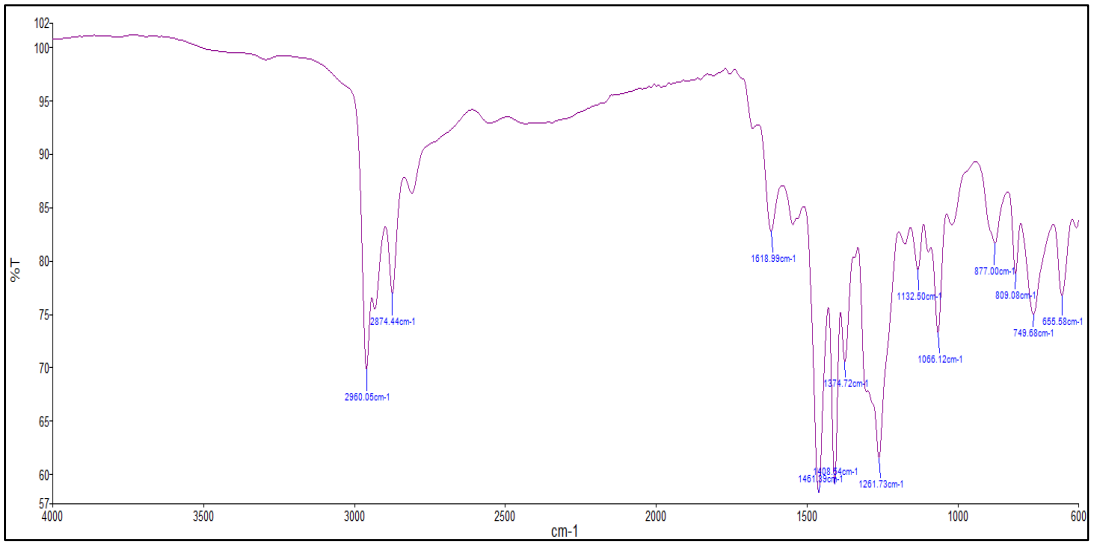

Figure A2: FTIR spectrum of DPCARB

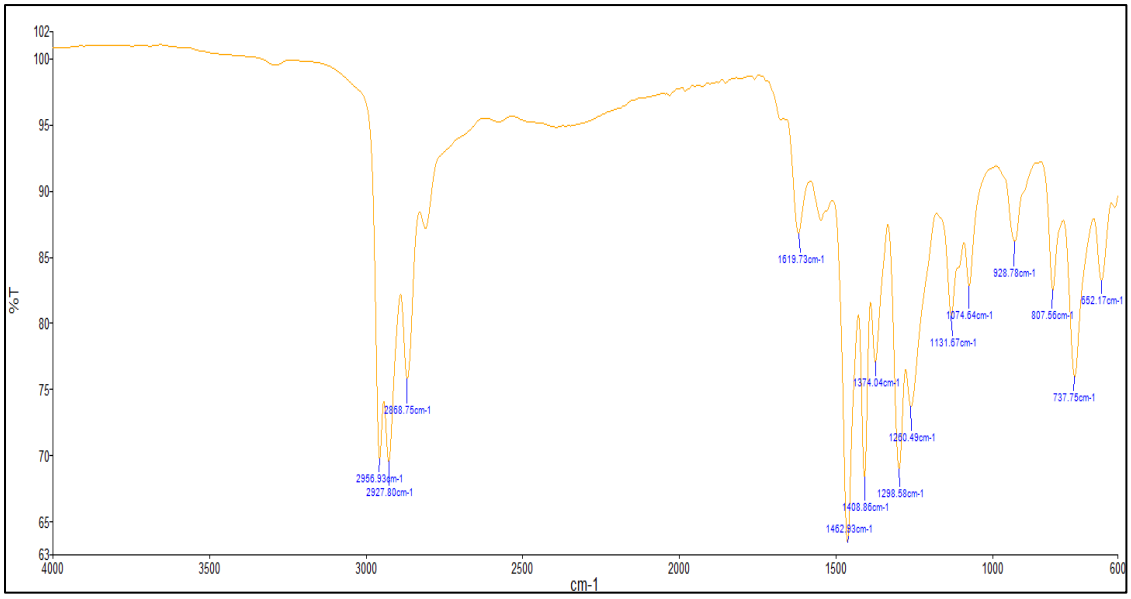

Figure A3: FTIR spectrum of DBCARB

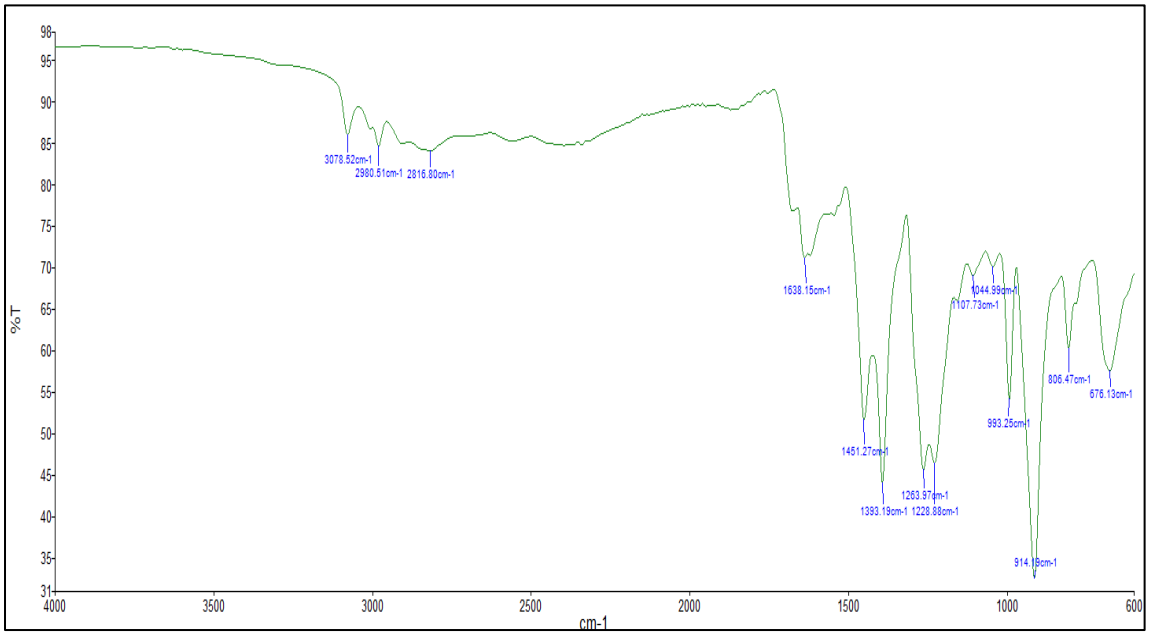

Figure A4: FTIR spectrum of DACARB

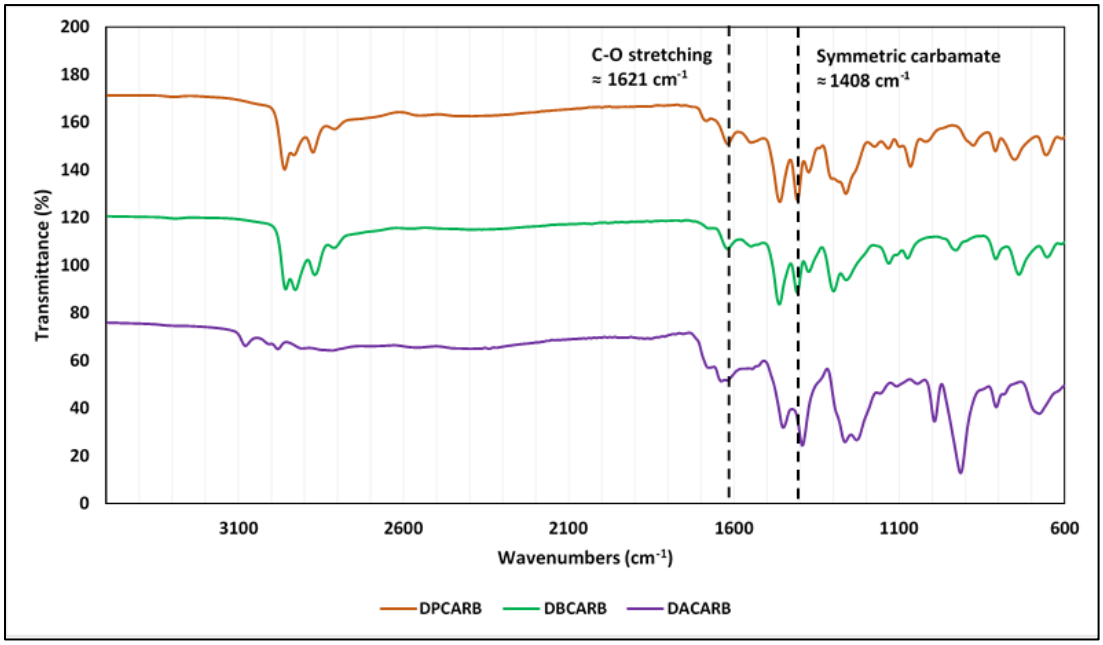

Figure A5: FTIR spectra of the synthesized ILs

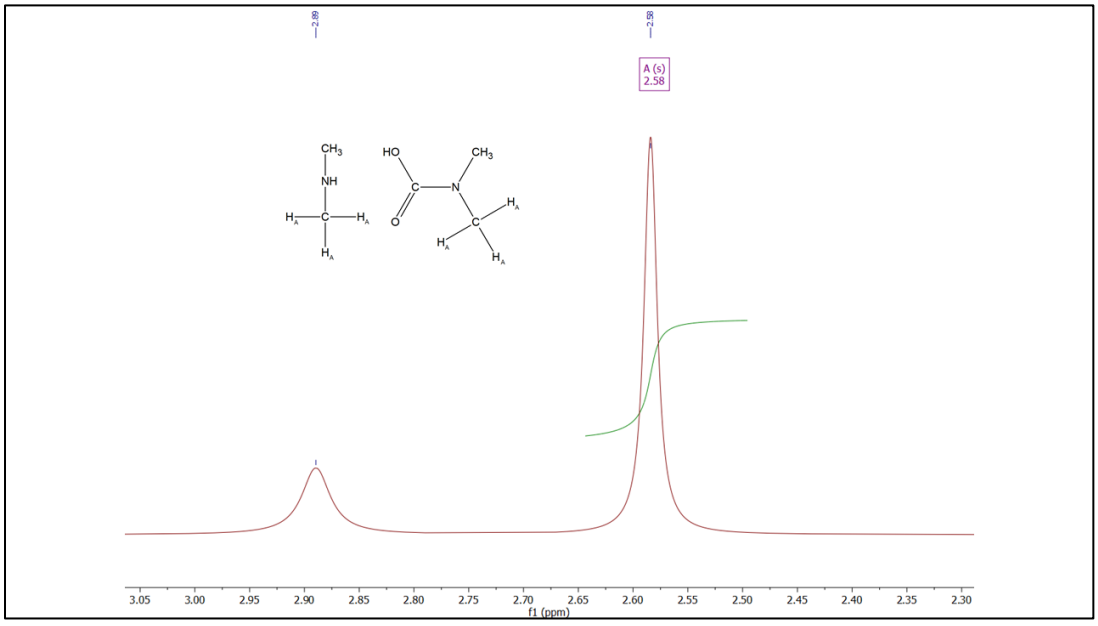

Figure A6: ^{1}H NMR spectrum of DIMCARB

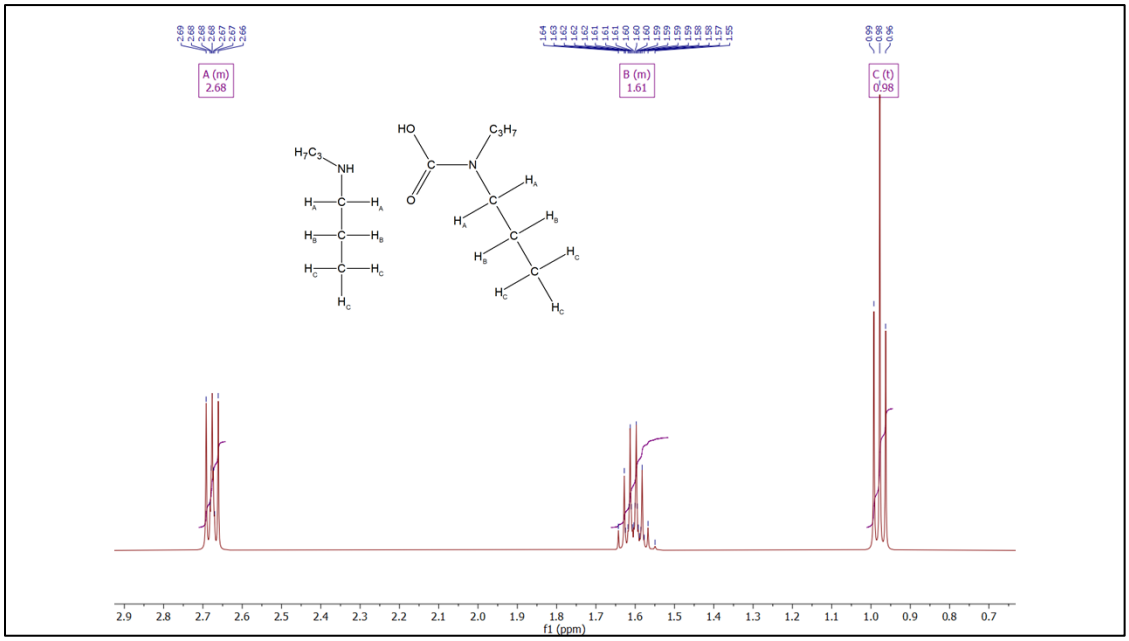

Figure A7: ^{1}H NMR spectrum of DPCARB

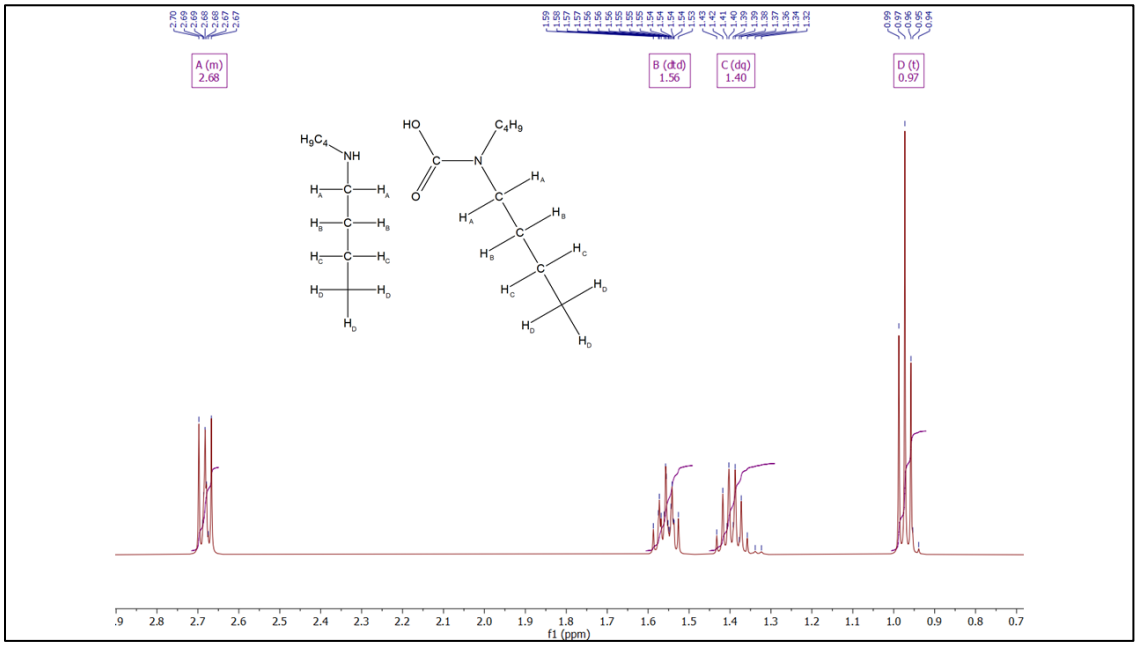

Figure A8: ^{1}H NMR spectrum of DBCARB

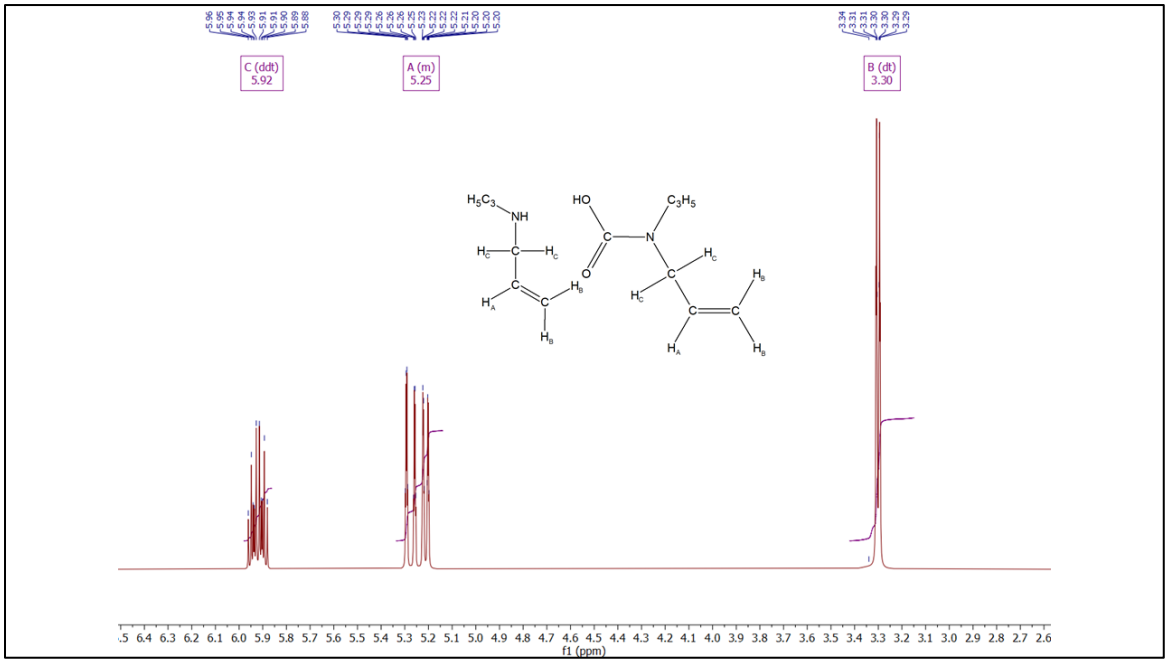

Figure A9: ^{1}H NMR spectrum of DACARB

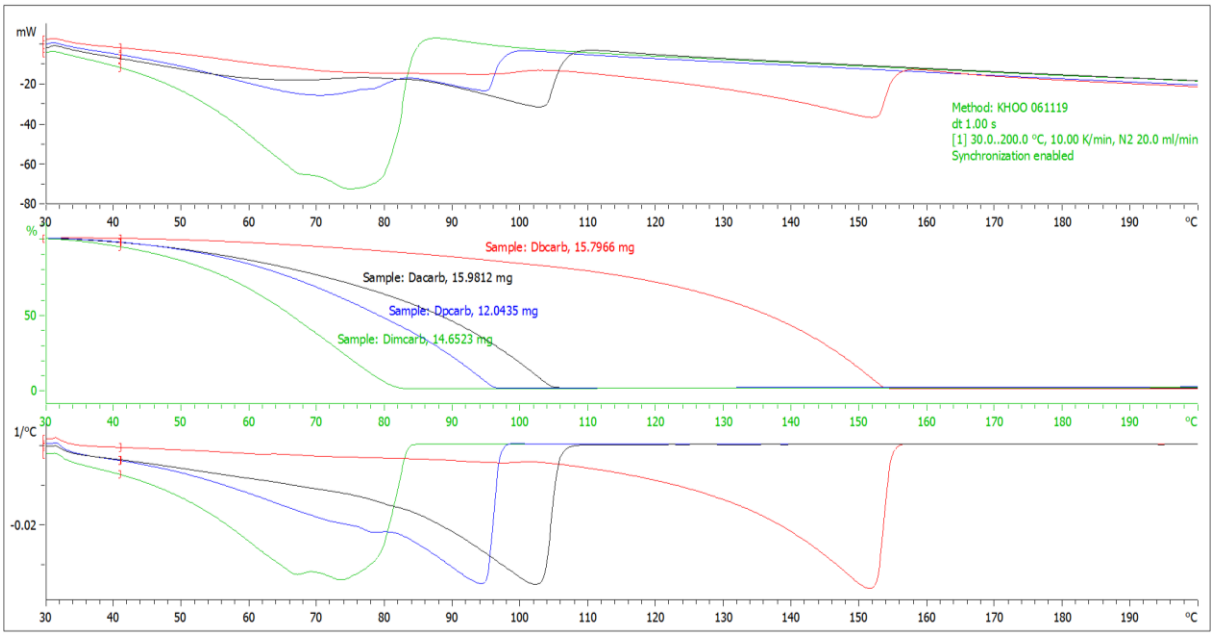

Figure A10: Thermal analysis (DSC) of CO_2-based alkyl carbamate ILs

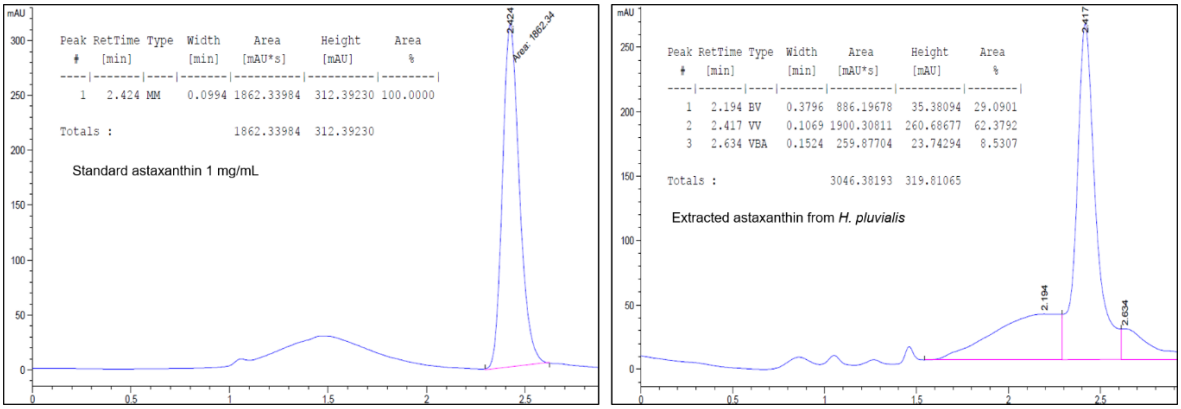

Figure A11: HPLC chromatograms of standard and extracted astaxanthin

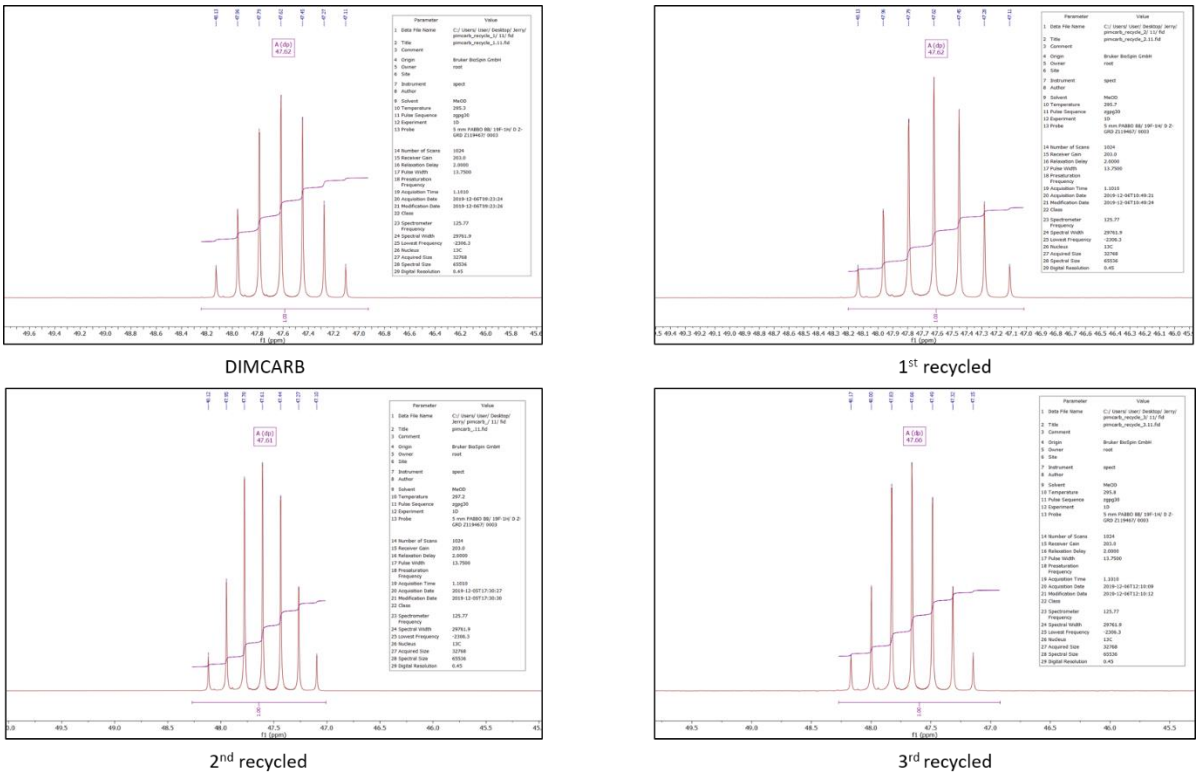

Figure A12: ^{13}C NMR spectra of DIMCARB

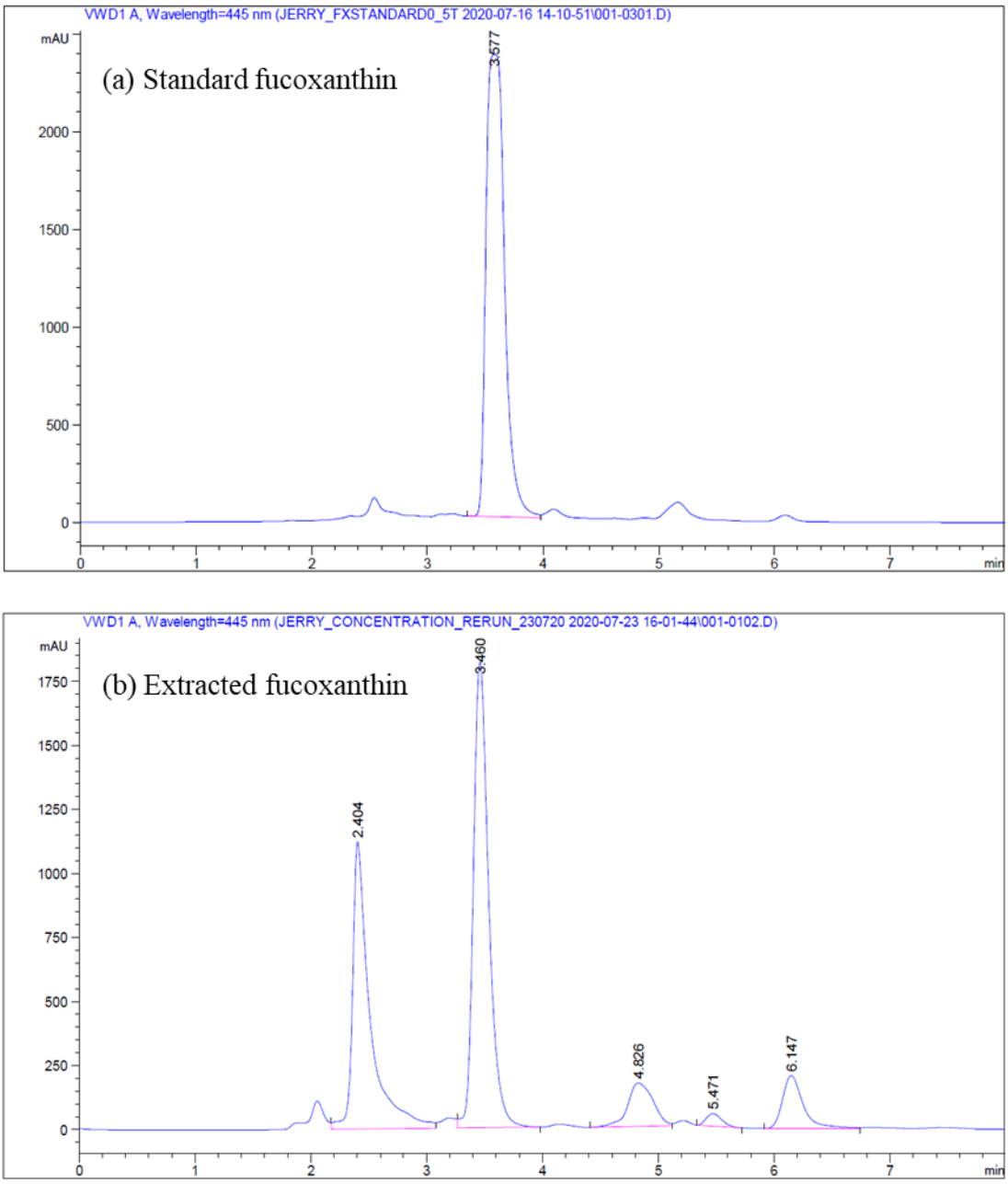

Figure A13: HPLC analysis of (a) standard fucoxanthin and (b) extracted fucoxanthin