DEVELOPMENT OF NOVEL SONOPROCESSING BASED BI- AND TRIPHASIC SYSTEMS FOR THE EXTRACTION OF BIOMOLECULES FROM MICROALGAE

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ABSTRACT

Microalgae have great potentiality to act as reservoirs of viable bioactive compounds due to the attractive composition of high value-added compounds. Besides, lots of advantages have been reported using microalgae compared to plant-based biomolecules as it does not create food competition and has rapid growth rate. The resistant microalgae cell wall has to be overcome through effective yet simple and rapid techniques in separating desired compounds from the biomass for further applications. The conventional processes for processing biomass in the current industries is inefficient and not feasible to be applied on microalgae, at which a cost- and time-saving technique is in desperate need for the efficient production of biomolecules.

This research work reports the development of sonoprocessing-assisted techniques in extracting and purifying biomolecules such as proteins and phycobiliproteins from *Chlorella* sp.: *Chlorella vulgaris* FSP-E, *Chlorella sorokiniana* CY1 and *Spirulina* sp.: *Spirulina platensis*, respectively. The proposed techniques in this research work are liquid biphasic system, three phase partitioning and liquid biphasic flotation integrated with the utilization of sonication waves. The protein extraction is performed using liquid biphasic flotation and three phase partitioning assisted with sonication while the phycobiliproteins extraction is conducted using liquid biphasic system and liquid biphasic flotation. A total protein recovery of 80% and 49% of separation efficiency was obtained using sonication-assisted liquid biphasic flotation while 56.57% of protein recovery and 74.59% of separation efficiency were gained using ultrasonication-assisted three phase partitioning. To investigate the

scalability of the proposed techniques, the study related to up-scaling of system in extracting the biomolecules are presented and discussed as well. This is an important aspect to determine the feasibility of the proposed technique for large scale and commercializing its usage for industrial production. A scale-up study was performed using ultrasonication-assisted three phase partitioning, from total working volume of 10 mL to 150 mL, obtaining around 57% of protein recovery and 71% of separation efficiency in larger scale of system. On top of that, different types of pre-treatment methods, namely freeze-thawing, homogenisation, microwave and sonication were studied in conjunction with the multiphase separation techniques for phycobiliprotein extraction. Phycobiliproteins was recovered at 94.89% with 6.17 of purification factor using liquid biphasic system while 95.10% of recovery and 5.23 of purification factor were gained using liquid biphasic flotation. The conclusions and future work of this research work are further described in the last chapter of the thesis.

LIST OF PUBLICATIONS

Journal Publications

1. **Shir Reen Chia**, Hwai Chyuan Ong*, Kit Wayne Chew, Pau Loke Show, Siew-Moi Phang, Tau Chuan Ling, Dilirani Nagarajan, Duu-Jong Lee, Jo-Shu Chang*. (2018) "Sustainable approaches for algae utilisation in bioenergy production" *Renewable Energy*, 129, 838-852. DOI: 10.1016/j.renene.2017.04.001 [Accepted: 1 April 2017; 2016 IF: 4.357, Q1(18/92)]
2. **Shir Reen Chia**, Kit Wayne Chew, Pau Loke Show*, Yee Jiun Yap, Hwai Chyuan Ong, Tau Chuan Ling, Jo-Shu Chang*. (2018) "Analysis of economic and environmental aspects of microalgae biorefinery for biofuels production: A review" *Biotechnology Journal*, 1-10. DOI: 10.1002/biot.201700618 [Accepted: 22 January 2018; 2017 IF: 3.507, Q2(45/161)]
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8. **Shir Reen Chia**, Kit Wayne Chew, Hayyiratul Fatimah Mohd Zaid, Dinh-Toi Chu, Yang Tao, Pau Loke Show*. (2019) "Microalgal protein extraction from *Chlorella vulgaris* FSP-E using triphasic partitioning technique with sonication" *Frontiers in Bioengineering and*

Biotechnology, 7, 1-13. DOI: 10.3389/fbioe.2019.00396 [Accepted: 21 November 2019; 2018 IF: 5.122, Q1(10/69)]

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1. **Chia, S. R.**, Lam, W. S., Seah, W. H., Show, P. L., 2019. Filtration. *Bioprocess Engineering*. CRC Press, 27-54.
2. Chew, K. W., **Chia, S. R.**, Show, P. L., Ling, T. C., Chang, J.-S., 2018. Biofuels from Microbial Lipids. *Bioreactors for Microbial Biomass and Energy Conversion*. Springer, 359-388.

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TABLE OF CONTENTS

ABSTRACT i

LIST OF PUBLICATIONS i

ACKNOWLEDGEMENTS i

LIST OF FIGURES i

LIST OF TABLES i

LIST OF ABBREVIATIONS i

CHAPTER 1 INTRODUCTION 3

1.1 Background of Research 3

1.2 Problem Statement 5

1.3 Research Objectives 7

1.4 Summary of Research 8

1.5 Outline 10

1.6 Flow Diagram of the Research 11

CHAPTER 2 LITERATURE REVIEW 12

2.1 Algae as Potential Solution 12

2.1.1 Microalgae 15

2.1.2 Cultivation of Microalgae 16

2.2 Bioactive Compounds 21

2.2.1 Proteins 22

2.2.2 Lipids 28

2.2.3 Carbohydrates 34

2.2.4 Pigments 37

2.2.5 Vitamins 46

2.2.6 Polyunsaturated Fatty Acid 46

2.3 Biological Activity 48

2.3.1 Antioxidant Activity 48

2.3.2 Antiproliferative Activity 49

2.3.3 Anti-inflammatory Activity 50

2.3.4 Antimicrobial Activity 51

2.4 Biomass Pre-treatment Techniques 53

2.4.1 Mechanical Pre-treatment 59

2.4.1.1 Freeze-thaw 59

2.4.1.2 Homogenisation 61

2.4.1.3 Size-reduction 63

2.4.1.4 Sonication 65

2.4.1.5 Microwave 68

2.4.2 Chemical Pre-treatment 70

2.4.2.1 Organosolv 70

2.4.2.2 Acid treatment 71

2.4.2.3 Alkali treatment 73

2.4.2.4 Ionic liquid 74

2.4.3 Biological Pre-treatment 76

2.4.3.1 Fungi pre-treatment 76

2.4.3.2 Bacterial pre-treatment 78

2.4.3.3 Microbial consortia pre-treatment 79

2.4.3.4 Enzymatic pre-treatment... 80

2.5 Extraction Techniques 81

2.5.1 Conventional Extraction 82

2.5.1.1 Solvent extraction 82

2.5.1.2 Soxhlet extraction 87

2.5.2 Emerging Extraction Techniques 91

2.5.2.1 Liquid Biphasic System.. 91

2.5.2.2 Liquid Biphasic Flotation 96

2.5.2.3 Three Phase Partitioning... 99

CHAPTER 3 ISOLATION OF PROTEIN FROM *CHLORELLA SOROKINIANA* CY1 USING LIQUID BIPHASIC FLOTATION ASSISTED WITH SONICATION THROUGH SUGARING-OUT EFFECT 105

3.1 Abstract... 106

3.2 Introduction 107

3.3 Materials and Methods 110

3.3.1 Materials... 110

3.3.2 Apparatus 110

3.3.3 Medium Composition and Microalgae Cultivation 111

3.3.4 Determination of Protein Content 112

3.3.5 Determination of Separation Efficiency (E), Total Protein Content and Yield (Y) 113

3.3.6 Cell Disruption with Sonication 114

3.4 Results and Discussion 115

3.4.1 Selection of Sugar 115

3.4.2 Concentration of Glucose 118

3.4.3 Volume Ratio 121

3.4.4 Resting Time 123

3.4.5 Pulse and Continuous Mode 125

3.4.6 Concentration of Microalgae 127

3.4.7 Potential Industrial Application and Up-scaling 129

3.5 Conclusion 131

CHAPTER 4 MICROALGAL PROTEIN EXTRACTION FROM *CHLORELLA VULGARIS* FSP-E USING TRIPHASIC PARTITIONING TECHNIQUE WITH SONICATION 132

4.1 Abstract 133

4.2 Introduction 134

4.3 Materials and Methods 138

4.3.1 Chemicals and Reagents 138

4.3.2 Microalga 138

4.3.3 Determination of Protein Content 139

4.3.4 Determination of Separation Efficiency (*E*), Total Protein Content and Yield (Y) 140

4.3.5 Three Phase Partitioning (TPP)... 141

4.3.6 Ultrasound-assisted Three Phase Partitioning (UATPP) 141

4.3.7 Optimization of Operating Parameters 142

4.3.8 Field Emission Scanning Electron Microscope (FESEM)..... 143

4.3.9 Statistical Analysis 144

4.4 Results and Discussion 144

4.4.1 Comparison Study 144

4.4.2 Optimization of UATPP Process Parameters......................... 145

4.4.2.1 Types of salt... 145

4.4.2.2 Effect of $(NH_4)_2SO_4$ saturation 147

4.4.2.3 Effect of slurry to t-butanol ratio 149

4.4.2.4 Effect of ultrasonic power 152

4.4.2.5 Effect of ultrasonic frequency and irradiation time...... 154

4.4.2.6 Effect of duty cycle... 157

4.4.2.7 Effect of biomass loading 159

4.4.3 Scale Up 161

4.4.4 Field Emission Scanning Electron Microscopy (FESEM) Characteristics of Microalgae Surfaces 164

4.5 Conclusion 166

CHAPTER 5 *SPIRULINA PLATENSIS* BASED BIOREFINERY FOR THE PRODUCTION OF VALUE-ADDED PRODUCTS FOR FOOD AND PHARMACEUTICAL APPLICATIONS 167

5.1 Abstract 168

5.2 Introduction 169

5.3 Materials and Methods 172

5.3.1 Chemicals and Reagents 172

5.3.2 Cell Disruption Methods 172

5.3.2.1 Sonication 173

5.3.2.2 Microwave 174

5.3.2.3 Homogenisation 174

5.3.2.4 Freeze-thawing 174

5.3.3 Preparation of Liquid Biphasic System 175

5.3.4 Determination of C-phycocyanin 175

5.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Analysis (SDS-PAGE) 177

5.4 Results and Discussion 177

5.4.1 Sonication 177

5.4.1.1 Power 177

5.4.1.2 Duty cycle 178

5.4.1.3 Time 180

5.4.2 Microwave 183

5.4.2.1 Duty cycle 183

5.4.2.2 Time 184

5.4.3 Homogenisation 187

5.4.3.1 Time 187

5.4.3.2 Rotor's speed 188

5.4.4 Freeze-thawing 189

5.4.5 Comparison of Cell Disruption Methods 191

5.4.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) 194

5.5 Conclusions 195

CHAPTER 6 SONOPROCESSING-ASSISTED SOLVENT EXTRACTION FOR THE RECOVERY OF PIGMENT-PROTEIN COMPLEX FROM *SPIRULINA PLATENSIS* 196

6.1 Abstract 197

6.2 Introduction 198

6.3 Materials and Methods 202

6.3.1 Materials 202

6.3.2 Ultrasound Treatment 203

6.3.3 Liquid Biphasic Flotation System 204

6.3.4 Determination of the Recoveries of C-PC and APC 205

6.3.5 SDS-PAGE Analysis 206

6.3.6 Statistical Analysis 206

6.4 Results and Discussion 207

6.4.1 Mechanism of Liquid Biphasic Flotation.............................. 207

6.4.2 Effects of Sonication on C-PC and APC 208

6.4.2.1 Ultrasonic amplitude.. 208

6.4.2.2 Sonication time for pulse mode 211

6.4.2.3 Resting time 212

6.4.2.4 Total sonication period 214

6.4.3 Other Effects on the Extraction of C-PC and APC 217

6.4.3.1 Volume ratio 217

6.4.3.2 Air flowrate.. 218

6.4.3.3 Flotation time... 220

6.4.3.4 Biomass loading 222

6.4.4 SDS-PAGE... 226

6.5 Scaling-up and Technical Feasibility of the Process 227

6.6 Conclusions 229

CHAPTER 7 CONCLUSIONS AND FUTURE WORKS 230

7.1 Conclusions 230

7.2 Future Works 233

REFERENCES 235

APPENDIX 303

LIST OF FIGURES

Figure 1.1: Flow diagram of this research work. 11

Figure 2.1: Schematic diagram of solvent extraction (C. Shi et al., 2017)...... 84

Figure 2.2: Conventional Soxhlet extractor (Luque de Castro & Priego-Capote, 2010) 88

Figure 3.1: Schematic of the equipment used in liquid biphasic flotation (LBF). 111

Figure 3.2: Effect of type of sugar on the protein yield and separation efficiency. 117

Figure 3.3: Effect of sugar concentration on the protein yield and separation efficiency. 119

Figure 3.4: Effect of volume ratio on the protein yield and separation efficiency. 122

Figure 3.5: Effect of resting time of ultrasonication on the protein yield and separation efficiency. 124

Figure 3.6: Effect of pulse and continuous modes of ultrasonication on the protein yield and separation efficiency. 126

Figure 3.7: Effect of concentration of microalgae on the protein yield and separation efficiency. 128

Figure 4.1: Effect of types of salt on separation efficiency (E) and yield of protein (Y) where A is Na_2SO_4, B is $MgSO_4$, C is $(NH_4)_2SO_4$ and D is K_2HPO_4. Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). 146

Figure 4.2: Effect of $(NH_4)_2SO_4$ saturation on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). 149

Figure 4.3: Effect of slurry to t-butanol ratio on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test)... 151

Figure 4.4: Effect of sonication power on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test)... 153

Figure 4.5: Effect of sonication time at frequency, 35 kHz on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). Analysis tests were performed with data in **Figure 4.6** (130 kHz frequency). 155

Figure 4.6: Effect of sonication time at frequency, 130 kHz on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). Analysis tests were performed with data in **Figure 4.5** (35 kHz frequency). 157

Figure 4.7: Effect of duty cycle on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test)... 159

Figure 4.8: Effect of biomass loading on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test)... 160

Figure 4.9: FESEM images of microalgae cells (A) before sonication treatment; (B) after ultrasound with water as medium; (C) after ultrasound with UATPP medium. 165

Figure 5.1: Recovery and purification factor of C-phycocyanin using sonication. 182

Figure 5.2: Recovery and purification factor of C-phycocyanin using microwave.. 186

Figure 5.3: Recovery and purification factor of C-phycocyanin using homogenisation... 189

Figure 5.4: Recovery and purification factor of C-phycocyanin using freeze-thawing. 191

Figure 5.5: SDS-Page analysis for C-phycocyanin; Lane 1 is protein marker, Lane 2 is crude extract of C-phycocyanin (pretreated with sonication) and Lane 3 is C-phycocyanin after pretreatment and LBS... 195

Figure 6.1: Schematic of the sonoprocessing-assisted solvent extraction method. 203

Figure 6.2: Effects of sonication parameters towards the purification factor and recovery of c-phycocyanin and allophycocyanin (a) Amplitude (b) Pulse mode (c) Resting time (d) Total sonication period. Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). The indication of black and yellow letters: P_{FC} and R_{CPC}, respectively; red letters: P_{FA} and R_{APC}.. 216

Figure 6.3: Effects of the parameters of liquid biphasic flotation system towards the purification factor and recovery of c-phycocyanin and allophycocyanin (a) Volume ratio (b) Air flowrate (c) Flotation time (d) Biomass concentration.

Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). The indication of black and yellow letters: P_{FC} and R_{CPC}, respectively; red letters: P_{FA} and R_{APC}. 224

Figure 6.4: SDS-PAGE analysis for the obtained C-PC (Lane 1: Molecular marker; Lane 2: Crude extract; Lane 3: C-PC after processing and ultrafiltration). 227

LIST OF TABLES

Table 2.1: Types of algae (Adeniyi et al., 2018; Bailey, 2018; J & Chapman, 1973). 14

Table 2.2: Protein content in microalgae. 26

Table 2.3: Lipid/ fatty acid content in microalgae. 32

Table 2.4: Carbohydrate composition in microalgae. 36

Table 2.5: Pigment content in various microalgae. 44

Table 2.6: Type of pre-treatment methods (Dixon & Wilken, 2018; Günerken et al., 2015; Zabed et al., 2019). 55

Table 2.7: Properties of top phase forming components. 94

Table 2.8: Metabolites extracted using Three Phase Partitioning. 101

Table 3.1: Operating parameters for sonication-assisted LBF through the sugaring-out effect. 115

Table 4.1: Operating parameters for UATPP. 143

Table 4.2: Comparison study between TPP and UATPP. 144

Table 4.3: Comparative evaluation of other studies using TPP and ultrasound-assisted TPP. 162

Table 5.1: Parameters studied in various pre-treatment methods. 173

Table 5.2: Optimum values of pre-treatment methods. 192

Table 6.1: Operating parameters and initial conditions. 204

Table 6.2: Comparison of the current work with results reported from literature. 225

LIST OF ABBREVIATIONS

ALA	α-linolenic acid
AMG	Amyloglucosidase
APC	Allophycocyanins
ARA	Arachidonic acid
ATPS	Aqueous two-phase system
AWW	Aquaculture wastewater
B-PE	B-phycoerythrin
BSA	Bovine serum albumin
CDW	Cell dry weight
CME	Carpet mill effluent
C-PC	C-phycocyanins
C-PE	C- phycoerythrin
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
DPPH	1, 1-diphenyl-2-picryl-hydrazil
DW	Dry weight
E	Separation efficiency
EOPO	Poly(ethylene glycol-ran-propylene glycol)
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FESEM	Field emission scanning electron microscopy
FWH	Food waste hydrolysate
GLA	γ-linolenic acid
HDL	High-density lipoprotein
HW	Hydroponic water
LBF	Liquid biphasic flotation
LBS	Liquid biphasic system
LDL	Low-density lipoprotein

MTT	3 - (4, 5 - dimetiltiazolyl - 2) - 2, 5 - diphenyltetrazolium bromide
N/A	Not available
ODW	Organic dry weight
OFAT	One-factor-at-a-time
PBR	Photo-bioreactor
PC	Phycocyanins
PE	Phycoerythrins
PEC	Phycoerythrocyanins
PEG	Polyethylene glycol
P_F	Purification factor
PUFAs	Polyunsaturated fatty acids
R	Recovery
R-PE	R-phycoerythrin
SDS-PAGE	Sodium deodecyl sulfate-polyacrylamide gel electrophoresis
SS	Solvent sublation
T	Temperature
TAG	Triacylglycerides
TEAC	Trolox equivalent antioxidant capacity
TG	Triglycerides
TPP	Three Phase Partitioning
UATPP	Ultrasonic-assisted Three Phase Partitioning
VLCPUFA	Very Long Chain Poly Unsaturated Fatty Acids
WHO	World Health Organization
WW	Wastewater
Y	Yield

CHAPTER 1 INTRODUCTION

1.1 Background of Research

Up to date, the downstream processing of biological products in industries is utilizing conventional approaches to produce and fulfil the market demands. As the population in worldwide growing significantly over decades, food demand is increasing in rapid rate especially in developing countries (Draaisma et al., 2013; Elferink & Schierhorn, 2016). In addition, the demand of dietary supplements is increasing as well due to more attentions given on health awareness with the improved living standards in most of the countries (Tewfik & Tewfik, 2008). To compromise with the large market demand, the industries have to discover efficient, simple and scalable processing technique in obtaining high recovery and purity of biological products while finding a solution to overcome the high food demand in worldwide.

Despite of efficient processing techniques, a viable natural source to supply bioactive compounds required for dietary supplements that does not compete with food resources is in desperate need to fulfil the market demand. Microalgae are the potential solution to current issue, as they consist of multiple valuable components such as lipid, carbohydrate, protein, vitamins, pigments and more (Rajvanshi et al., 2019). This special trait of microalgae has made them become more significant in the production of vast products across different industries (Dixon & Wilken, 2018). High value-added products like carotenoids, fatty acids, phycobiliproteins and vitamins can be utilized for biopharmaceuticals, nutraceuticals, human health and nutrition industries while

lipid and carbohydrate can be processed for bioenergy production as microalgae also known as third generation biofuels feedstock (Dragone, Fernandes, Vicente, & Teixeira, 2010). High composition of protein is discovered within microalgae biomass and potential to replace the conventional protein source as the microalgae possess high growth rate and able to survive under harsh condition. As compared to terrestrial plant, microalgae seems to be a better option to produce biomolecules for various applications as they do not require land for cultivation and can be cultivated using industrial wastewater and flue gas (Chinnasamy, Bhatnagar, Hunt, & Das, 2010).

To obtain the valuable components within microalgae biomass, efficient and simple techniques such as Liquid Biphasic System and Liquid Biphasic Flotation are developed for maximal recovery yield. These techniques have numerous advantages over conventional separation process, such as low time consumption, scalable, cost and energy saving, high separation efficiency and rapid mass transfer (Leong et al., 2018; Leong, Ooi, Law, Julkifle, & Show, 2019). Most of the studies performed using these techniques have not encountered denaturation of protein or degradation of targeted products due to the high water content of the system (Khoo et al., 2020; Phong et al., 2017).

Sonoprocessing is shown to be an effective and ideal option to break down cell wall of biomass in numerous studies (Lianfu & Zelong, 2008; J.-H. Xie et al., 2012). The sonication waves generate cavitational bubbles in the medium (consists of sample or biomass) and the collapse of these bubbles disrupts the cell wall due to the high shear gradients created (Tian, Xu, Zheng,

& Martin Lo, 2013). The inner components are then released with the rupture of cell wall. High mass transfer from samples to the medium, short processing time and less solvent usage (X. Chen et al., 2010) have lead to great attention of researchers and industrial in extracting and purifying biomolecules. Hence, we have proposed to integrate sonoprocessing with the techniques mentioned in above paragraph to enhance the recovery of targeted compounds from microalgae biomass either in single step operation or as pre-treatment prior to extraction and purification. Single step operation is studied for extraction and purification of protein while two steps operation is investigated for high value-added products, phycocyanins. At last, a scaled-up system of Liquid Biphasic Flotation assisted with sonoprocessing will be demonstrated as compared to a small-scale system of Liquid Biphasic System in extracting phycocyanins to complete this research work.

1.2 Problem Statement

The significant issue faced in worldwide is insufficient supplies of food source and dietary supplement for market demand. As the human population grow rapidly, the increasing demand is difficult to be fulfilled by the industrial supplies and potential food source has to be discovered. Demand of biomolecules for dietary supplement production is increasing as well due to health awareness of modern society. The third generation of renewable feedstock, microalgae are explored and investigated to have key compositions for bioenergy, functional food and biopharmaceutical production. High protein content of microalgae and the comparable quality with conventional protein source have allowed them to be a potential substitution for conventional protein

source while the valuable compound, phycocyanins shows human benefitting properties like antioxidant and anti-inflammatory that can be used as natural colourants and in pharmaceuticals (Becker, 2007; Bhaskar, Gopalaswamy, & Raghu, 2005).

Since all the desired components lie within microalgae biomass, effective and rapid extraction and purification techniques are required to separate these components for further processing. The problems encountered is that the conventional processes are not effective in extracting biomolecules to a high purity, the problem with bi-triphasic systems alone is that there are still not effective to purify compounds, where pretreatment steps are needed but this creates a two-stage operation. Regarding the solution, we have developed a one-stage pre-treatment assisted LBS which can pre-treat and purify the compounds in a one-step operation. Liquid Bi- and Triphasic System are proposed for the efficient bioseparation of biomolecules. The extraction of biomolecules is favourable as no degradation will be observed. The assistance of sonoprocessing with the suggested technique can enhance the recovery of targeted products. This research work aims to develop novel effective bioseparation process with assistance of sonoprocessing techniques for biomolecules extraction from microalgae, followed by examination of various pre-treatment methods in rupturing the resistance microalgae cell wall. Lastly, the feasibility of large scaled system for the production of high value-added products will be investigated too.

1.3 Research Objectives

The research aim for this research study is to discover the potential techniques in extracting bioactive compounds from natural sources for various applications. The main study of this research is to extract valuable compounds mainly from microalgae as they are potential natural source containing various high value-added compounds. The compounds extracted in this research are proteins and C-phycocyanins. This study also aims to explore the potentiality of proposed techniques in extracting bioactive compounds for large scale study. Hence, the extraction of C-phycocyanin using liquid biphasic flotation containing a higher quantity of system which is 500 mL and the protein extraction is upscaled from 10 mL to 150 mL using sonication-assisted three phase partitioning. The research scopes are stated as followed:

1) To extract and evaluate protein content from microalgae using single step bioseparation techniques.
2) To compare the suitable pretreatment methods for high value product extraction from microalgae.
3) To investigate and optimize the proposed pre-treatment method for high value-added product extraction.

1.4 Summary of Research

The proposed research scopes were achieved, and the detail of each scope is described from **CHAPTER 3** to **CHAPTER 6**. Summary of the research contributions are stated as followed:

(1) **Isolation of protein from *Chlorella sorokiniana* CY1 using liquid biphasic flotation assisted with sonication through sugaring-out effect**: An efficient and simple extraction technique, Liquid Biphasic Flotation (LBF) assisted with sonication was developed to rupture and extract protein via sugaring-out effect in a single unit operation. Wet microalgae biomass was subjected to extraction directly without any treatment to obtain protein. Parameters investigated focuses more on system, while determination of irradiation time and mode of sonication was performed too. The total protein yield and separation efficiency from biomass were examined.

(2) **Microalgal protein extraction from *Chlorella vulgaris* FSP-E using triphasic partitioning technique with sonication**: Comparison of conventional three phase partitioning (TPP) and emerging technique, ultrasonic-assisted three phase partitioning (UATPP) in extracting protein from *Chlorella* sp. is reported using similar conditions. UATPP was further investigated and optimized for maximal protein yield through parameters related to phase components, biomass and sonication.

(3) ***Spirulina platensis* based biorefinery for the production of value-added products for food and pharmaceutical applications**: The extraction and purification of pigment-protein complex, also known as C-phycocyanins was reported using pre-treatments techniques and followed by Liquid Biphasic System (LBS). Four types of pre-treatment were investigated and optimized to obtain the most effective cell disruption method. The recovery and purification factor obtained for targeted compounds were measured.

(4) **Sonoprocessing-assisted solvent extraction for the recovery of pigment-protein complex from *Spirulina platensis***: The appropriate cell disruption technique examined from previous scope has been applied with a scalable and feasible bioseparation technique, LBF in larger scale for the extraction of C-phycocyanins and allophycocyanins. Parameters such as amplitude, irradiation time and resting time in pulse mode, total sonication time, volume ratio, flotation rate, air flowrate and biomass loading were studied for maximal recovery and purification factor of C-phycocyanins.

1.5 Outline

The outline of thesis consists of seven chapters and stated as followed. **CHAPTER 1** briefs about the background of research study and the current issues faced in downstream processing. The objectives and contributions of this research are highlighted as well. **CHAPTER 2** covers the literature review on algae as the potential solution for various applications, biomolecules within microalgae and respective biological activities, biomass pre-treatment methods, conventional extraction techniques and emerging extraction techniques for biomolecules. **CHAPTER 3** and **CHAPTER 4** describe about the extraction and purification of protein from microalgae biomass (*Chlorella* sp.) using different emerging techniques. **CHAPTER 3** presents the use of wet microalgae biomass in extracting microalgal protein via single step extraction technique through the sugaring-out effect (published in *Journal of Oceanology and Limnology*), while **CHAPTER 4** outlines the application of triphasic separation system assisted with ultrasound for protein extraction (published in *Frontiers in Bioengineering and Biotechnology*). **CHAPTER 5** and **CHAPTER 6** focus on the extraction and purification of high value-added product from microalgae biomass (*Spirulina* sp.). In **CHAPTER 5**, various pre-treatment techniques are examined with the application of Liquid Biphasic System for phycocyanin purification (published in *Bioresource Technology*), while **CHAPTER 6** reports about the extraction and purification of two type of phycocyanins using sonoprocessing-assisted Liquid Biphasic Flotation (published in *Chemical Engineering Journal*). Lastly, **CHAPTER 7** consists of the conclusion and potential future works of this research.

1.6 Flow Diagram of the Research

The flow diagram of this research is shown in **Figure 1.1**. Microalgae were first cultivated for the valuable components. The protein-rich strain, *Chlorella* sp. (*Chlorella sorokiniana* CY1 and *Chlorella vulgaris* FSP-E) was used for protein extraction and purification and the other strain, *Spirulina* sp. (*Spirulina platensis*) was used for phycocyanin extraction and purification. Novel sonoprocessing-assisted bioseparation techniques were used to process these valuable compounds from microalgae.

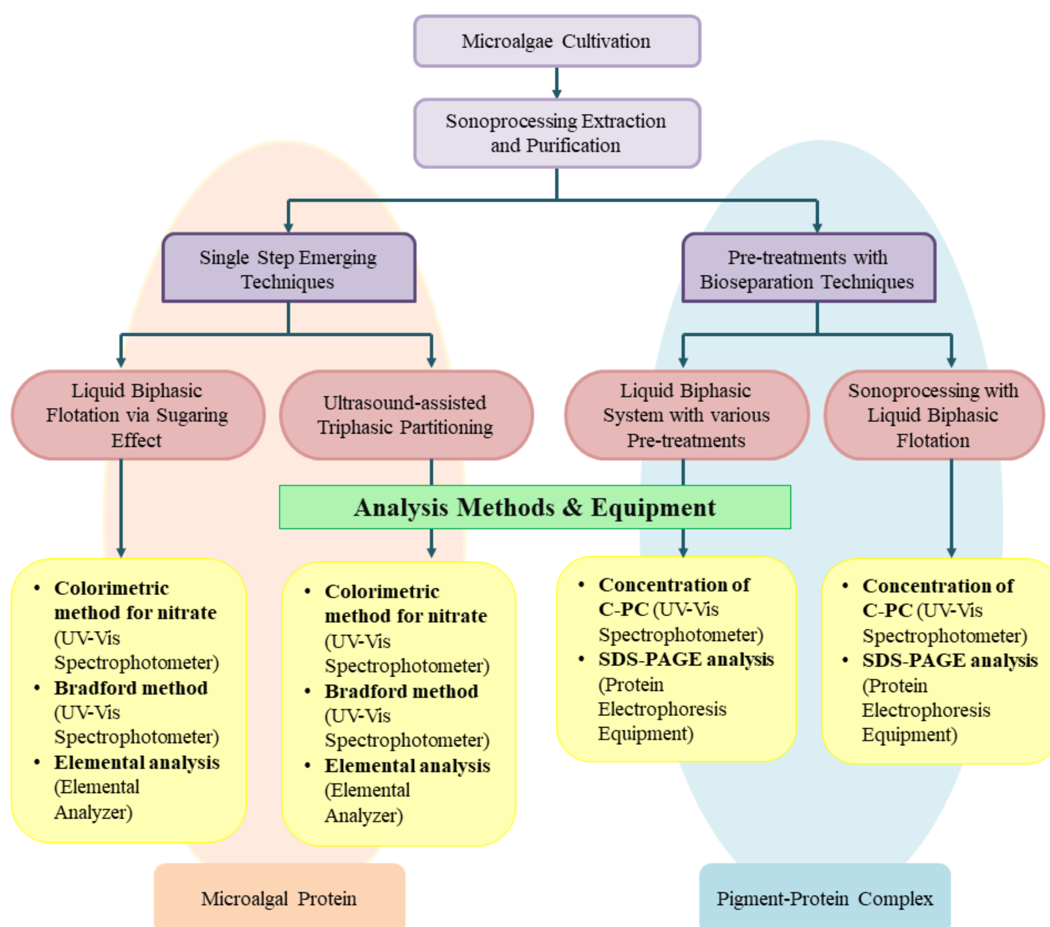


Figure 1.1: Flow diagram of this research work.

CHAPTER 2 LITERATURE REVIEW

2.1 Algae as Potential Solution

Over decades, the tremendous increased of energy demand due to higher populations are leading to the exploration of alternative renewable source in replacing the primary energy source, petroleum. Conventional fossil fuels are not sustainable, fast depleting, and cause a significant increment in atmospheric carbon dioxide concentrations. A sustainable source with lower carbon footprint is required to replace the conventional fossil fuels. Biofuels such as bioethanol and biodiesels are produced using virgin and waste lignocellulosic biomass, and used oils, grease and oil-based crops, respectively (Chia et al., 2018). However, the plantation of oil-based crops has occupied the agricultural space and created threat to both food industrial and forest as the cultivation of crops for biofuels required vast amount of lands. The monoculture performed for biofuel production can substantially deprives the nutrients of soil in the same field used every year. The high population across the global not only leads to high energy demand, the requirement of nutritional supplements and functional food are increasing as the consumers are becoming more health-conscious and aware of the diet intake. The functional food often consists of one or more than one type of functional ingredients which provides health benefit (Plaza et al., 2010). As the development and manufacturing of functional food is uprising, the functional ingredients in the products are preferable to be natural rather than synthetic compounds.

Algae are one of the alternative resources for both biofuels and functional ingredients production. Microalgae, the algae in microscopic size, are recognized as third-generation feedstock for biofuel production due to their rapid growth rate, existence in abundance, ability to survive in harsh environments (high salinity) and short cycle of cultivation period. The cultivation of algae does not require land, thus improving the land use efficiency compared to the oil-based crops. Cultivation of algae can also reduce the environmental footprint through utilization of wastewater as the culture medium to supply the nutrients required for algae growth. In addition, viable components with biological activities are found in algae, such as vitamins, pigments and polyunsaturated fatty acids, which are beneficial to human health. The compositions of essential bioactive compounds required for production of functional foods, pharmaceuticls and cosmeceuticals, are varied according to the type of algae strain and the cultivation conditions. However, their high amount of oil fractions and carbohydrates make algae as a valuable option for biodiesel, bioethanol and bio-gasoline production (Saad, Dosoky, Zoromba, & Shafik, 2019; Suali & Sarbatly, 2012). Algae are commercially used as animal feed and biofertilizer as they are rich in nutrients and organic matters in the form of algae cake. Besides, algae can be utilized as food additives such as stabiliser to avoid the separation of a mixture. Wide range of application has showed great possibilities in algae to be the source of bioactive compounds, renewable fuels and pharmaceuticals.

Algae are a diverse group of aquatic organisms that capable to perform photosynthesis and often exist in two forms, unicellular and multicellular. Unicellular algae are known as microalgae and the latter is macroalgae, which

commonly named as seaweed. Both have chlorophyll as the key photosynthetic pigment to fix atmospheric CO_2 via photosynthesis, like terrestrial plants. However, microalgae do not have stems, roots or leaves like terrestrial plants while macroalgae does. The size of algae is ranging from microscopic up to hundred meters in length. Algae are categorized into eight main groups that possess distinct functions and colours, namely Euglenophyceae (euglenoids), Chrysophyceae (golden-brown algae), Pyrrophyceae (fire algae), Rhodophyceae (red algae), Chlorophyceae (green algae), Phaeophyceae (brown algae), Xanthophyceae (yellow-green algae) and Cyanophyceae (blue-green algae) (Adeniyi, Azimov, & Burluka, 2018). The details of each alga are tabulated in **Table 2.1** and more details about microalgae will be further discussed in **Section 2.1.1** and **Section 2.1.2**.

Table 2.1: Types of algae (Adeniyi et al., 2018; Bailey, 2018; J & Chapman, 1973).

Type of cell	Algae type	Structure	Habitat	Characteristics
Procaryota	Cyano-phyceae	Unicellular and colonial algae	Marine and freshwater	No distinct mitochondria and nuclei
Eucaryota	Eugleno-phyceae	Unicellular	Freshwater	Some are autotrophic and others heterotrophic
	Chryso-phyceae	Unicellular and diatoms	Marine and freshwater	Most abundant type of unicellular algae

Table 2.1 (Continue)

Type of cell	Algae type	Structure	Habitat	Characteristics
Eucaryota	Pyrro-phyceae	Unicellular	Marine and freshwater	Move around with flagella
	Rhodo-phyceae	Unicellular and multicellular	Marine and tropical water	Do not have flagella and centrioles
	Phaeo-phyceae	Multicellular	Marine and cool water	Largest algae species
	Chloro-phyceae	Unicellular and multicellular	Marine, freshwater and brackish water	Cell walls made of cellulose
	Xantho-phyceae	Unicellular and small colonial algae	Freshwater	Cell walls made of cellulose and silica

2.1.1 Microalgae

Microalgae are algae in microscopic size which only can be observed under the microscope. Most of the algae types consist of microalgae, except Phaeophyceae as shown in **Table 2.1**. Microalgae are simple structure organisms yet possesses with incredible chemical and physicochemical composition as a natural source for various biomolecules in wide range of applications. The study of German-Báez et al. (2017) have proved that even the waste biomass after utilized for biofuels production, microalgae biomass still contain considerable high antioxidant activity and high dietary fibre content (German-Báez et al., 2017). The growth of microalgae requires sufficient amount of sunlight, water (fresh water or seawater) and nutrients such as carbon dioxide, nitrogen,

phosphorus, trace metals and minerals. Light and nutrient sources in optimal amounts are required by microalgae to generate carbohydrate, protein and lipid via photosynthesis. In most cases, environmental stress results in the enhancement of carbohydrate and lipid accumulation in algae but affects the growth or productivity of algal biomass simultaneously (C.-Y. Chen, Zhao, et al., 2013). Hence, some improvements have to be applied to the current cultivation techniques in producing high quality microalgal biomass and boosting up the productivity of microalgae.

2.1.2 Cultivation of Microalgae

The productivity and biochemical composition of microalgae are influenced by environmental and physiological factors, such as temperature, pH, light intensity, nutrient availability, and carbon dioxide level supplied to the culture. The temperature of the habitat or environment where algae are cultivated affects the algae growth and biochemical composition. Temperature ranging from 4 to 35°C has been tested and the effect on the composition and growth of algae has been studied. It has been observed that the composition of algae is easily affected by the cultivation temperature. The lipid content of *Nannochloropsis oculata* increased when the temperature was in between 20 to 25°C, while the lipid content of *Chlorella vulgaris* decreased (Converti, Casazza, Ortiz, Perego, & Del Borghi, 2009), indicating that temperature affects the algae composition significantly (differed around 7~9%). Besides, the growth rate of microalgae is influenced by the environment's temperature as well. *C. vulgaris* is found to have maximum growth rate at 15°C and able to survive in lower temperature which is around 4°C in longer periods compared to

Scenedesmus subspicatus (Bartosh & Banks, 2007). This is of significant important as seasonal variations in temperature will affect outdoor cultures and algal strains that could withstand alteration from optimal temperature remain unaffected in terms of biomass productivity. Some microalgae like *Scenedesmus subspicatus*, *Chlamydomonas reinhardtii* and *Scenedesmus acuminatus* has higher growth rate at temperature around 20 to 35 ^{o}C (Lürling, Eshetu, Faassen, Kosten, & Huszar, 2013) and able to survive through a wide range of temperature from 10 to 30 ^{o}C (Xin, Hong-ying, & Yu-ping, 2011). The effect of temperature on algae cultivation is also known to vary depending on the algal strain.

The pH of cultivation medium is one of the important factors as it influences not only the composition, but even the lifespan of algae. Many essential cellular functions of microalgae like uptake of CO_2 (pH determines the availability and solubility of CO_2), uptake of other ions and nutrients and the function of intracellular and cell wall-associated enzymes (mainly for photosynthesis) are affected by variations in the extracellular pH or medium pH (Juneja, Ceballos, & Murthy, 2013). Acidic pH alters nutrient uptake capabilities and interferes with cellular processes, while alkaline pH lowers the affinity for CO_2 and delays the completion of cell cycle (Juneja et al., 2013). The optimal pH for microalgae cultivation is dependent on the strain isolated, but usually the pH range, 6 to 10 is known to suit most of the algae. The pH value ranging from 8 to 9 are considered as most favourable pH in increasing algae biomass production and cell density, and it also known to lower the competitors *Nannochloropsis salina* in outdoor cultures (Bartley, Boeing, Dungan, Holguin, & Schaub, 2014). *Chlorella* sp. tends to have higher lipid content when grown

in slightly alkaline pH, although a lower biomass production is obtained. Cyanobacteria or blue-green algae are found to be inhibited at acidic pH (pH 5.5 to 6.0) by CO_2 acidification where cell death occurs at pH 5.5 and 6.0, indicating that acidic pH can be applied for preventing algal blooms (X. Wang, Hao, Zhang, Feng, & Yang, 2011). In general, the functionality of pH values includes boosting up algae production, enhancing content of algae and decreasing the number of invading organisms.

The effect of light intensity on algae growth has been investigated by several researchers as light source directly affects the photosynthesis of microalgae. Light intensity can affect the rate of photosynthesis in microalgae, and the effect of light can be light imitation, light saturation or light inhibition. Below light saturation conditions, the rate of photosynthesis and biomass growth increases with increasing light intensity and at a certain point light saturation occurs where the photon absorption is equal to the electron turnover by photosynthesis. Beyond saturation, light inhibition occur causing irreversible damage to the photosynthetic apparatus and results in a decline in biomass productivity (Juneja et al., 2013). It should also be noted that optimum light intensity is required for efficient photosynthesis and biomass production varies depending on the strain used. Studies have shown that better productivity and nutrient utilisation in *Tetraselmis chui* (PLY429) was observed using higher light intensities (Meseck, Alix, & Wikfors, 2005; Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 2008). However, microalgae such as *Botryococcus braunii* KMITL 2 and *Chlorella vulgaris* ESP-31 had higher biomass production at lower light intensity of 87.5 $\mu E\ m^{-2}\ s^{-1}$ and 9 W/m^2 (41.13

μE m^{-2} s^{-1}), respectively (Ruangsomboon, 2012; Yeh, Chang, & Chen, 2010). At optimal light intensity, *Chlorella* sp. was found to contain highest lipid content. Compared to *Chlorella* sp., *Botryococcus* sp. required higher light intensity (538 μE m^{-2} s^{-1}) to produce higher lipid content. Other than light intensity, some studies investigated the photoperiod or the light-dark cycle of the algae cultivation system. It was observed that the higher the ratio of light to dark cycles, the higher the biomass production for autotrophic growth of algae (Meseck et al., 2005; Rai, Gautom, & Sharma, 2015).

Carbon is supplied for the microalgal culture in the form of CO_2 gas or soluble carbonates for photoautotrophic cultivation. The atmospheric air has very low content of CO_2 which is not sufficient for effective photosynthesis. Hence, air mixed with pure CO_2 is usually supplied under laboratory conditions to cultivate microalgae. As an alternative CO_2 source, industrial exhaust gas or flue gas rich in CO_2 can be used as carbon source. A marine algal strain, *Nannochloropsis* sp. was cultivated in a mixture of seawater and municipal wastewater with 15% CO_2 from flue gas as a carbon source. The algal strain was found to have good tolerance towards a maximum limit of 15% of carbon dioxide aeration in the culture medium, enhancing the biomass productivity. In the second growth phase with nitrogen deprivation and high light intensity, the biomass and lipid content increased from 0.71 to 2.23 g L^{-1} and 33.8 to 59.9%, respectively (L. Jiang, Luo, Fan, Yang, & Guo, 2011). However, some studies have shown that the growth rate of algae may be inhibited by high concentration of carbon dioxide supplement (Chiu et al., 2008; Chiu et al., 2009). Hence, the aeration percentage of carbon dioxide and cell concentration used as inoculum

is believed to be closely related, as higher amount of cell concentration is required to cope with higher concentration of carbon dioxide supplied.

Reuse and recycling of media constituents can lower the carbon footprint of the plants and production costs to some levels. Use of inorganic fertilizers is unsustainable as they are derived from fossil fuel resources. This can overcome by substituting inorganic fertilisers with wastewater. Wastewater contains significant amount of phosphate and nitrate which are not removed in the wastewater treatment process. Additional processes are needed to remove the impurities completely which could add up to 60% to 80% of the total cost. By using wastewater as the nutrient source for large scale microalgae cultivation, it serves as a secondary treatment process for wastewater which is more economical and reduce the need of chemical fertiliser (Arenas, Palacio, Juantorena, Fernando, & Sebastian, 2017). Energy analysis of biodiesel production for growing *Chlorella* sp. in wastewater revealed that it is energetically favourable when the microalgae are grown in open ponds. Even without an energy credit for nutrient removal, energy required for lipid extraction and biodiesel production can be covered by the net energy content of the microalgal biomass obtained (Sturm & Lamer, 2011). Sturm and Lamer (2011) indicated that the nitrogen content of 19.5 mg/L in the tested wastewater with a total wastewater effluent of 25,800 MG/day could potentially yield an estimated 31,800 tonnes of algae per day, with an energy content of 21.4 kJ/g, which could produce 62.7×10^9 kW h yr^{-1} (Sturm & Lamer, 2011). Many reports have suggested that wastewater can be an efficient nutrient source for the

cultivation of microalgae (Chinnasamy et al., 2010; Cho, Luong, Lee, Oh, & Lee, 2011).

Microalgae can be cultivated either in open ponds (circular ponds or raceway ponds) or in closed systems like photo-bioreactors (PBRs). The production costs of PBRs are considerably higher compared to raceway ponds due to the associated installation and operational costs to suit the process needs (Chia et al., 2018). PBRs can help in maintaining the axenic status of the culture and are more suitable to produce high value-low volume compounds like pharmaceuticals and bioactive compounds. It is observed that in both open and closed systems, economical concern remains the main priority in making the microalgal biorefinery a reality. Further steps should be considered to minimize the production cost such as reducing the high cost of PBR for closed systems by using cheaper materials such as polyethylene (Wijffels & Barbosa, 2010). Besides, the light dilution principle could be performed as well in closed system by applying self-shading panels in order to give higher photosynthetic efficiency to the culture.

2.2 Bioactive Compounds

The microalgal biomass cultivated in either open ponds or bioreactors contain numerous bioactive compounds. Microalgae are being selected as the potential feedstock for natural bioproducts. They have many advantages compared to other feedstocks as discussed previously in **Section 2**, the important ones being the avoidance of land use changes and clash with food crops. The major constituents of microalgae include proteins, lipids and carbohydrates, at

the same time rich in high value-added components such as pigments, vitamins and polyunsaturated fatty acid. The spent microalgal biomass after extraction can also be used for biofuel production or processed in other ways to be used as feed or fertilizer. Main components of algal biomass are discussed in the following sub-sections.

2.2.1 Proteins

Proteins are one of the major components from microalgae and considered to be an essential protein source. Microalgal proteins are claimed to have higher compositions of hydrophilic and hydrophobic amino acids than soybean flour (Samarakoon & Jeon, 2012). The good qualities and quantities of proteins can be found in most of the microalgae strains especially *Chlorella* sp., *Spirulina (Arthrospira)* sp., and *Dunaliella* sp. with the protein content ranged from 55% to 65% (Buono, Langellotti, Martello, Rinna, & Fogliano, 2014). The amino acid profiles of two species, *Spirulina* sp. and *Chlorella*, are claimed to have balanced profiles equivalent to conventional protein sources such as soybean and eggs (Becker, 2007). Some essential amino acids such as leucine (Leu), valine (Val), lysine (Lys) and phenylalanine (Phe) in total protein of microalgae are comparable to the amount reference of WHO/FAO as shown in the study of Buono et al. (2014), revealing the potentiality of microalgal protein to be commercialized for human consumption and animal feed (Buono et al., 2014). A study by Chen et al. (2016) has reported the microalgal protein with high percentage of essential amino acid, 60.3%, was obtained using outdoor photobioreactor under semi-batch operation (C.-Y. Chen, Chang, & Chang, 2016). Indoor and outdoor cultivation performed using PBR have showed that

the productivity of biomass and the protein content was both affected by the environment of cultivation. Light source and temperature are often varied in outdoor cultivation compared to the indoor ones, where the light intensity can be controlled in indoor and there are possibilities to contaminate the microalgae culture in outdoor cultivation. Furthermore, the availability of sunlight has to be considered for outdoor cultivation at which tropical countries are more suitable to cultivate microalgae at outdoor compared to the countries in temperate or polar regions. However, the protein content and productivity obtained using outdoor cultivation during summer (7~8 h of daylight) are comparable to the results obtained using indoor cultivation (24 h illumination of low light intensity) (C.-Y. Chen et al., 2016).

Different types of culture medium have impacts on the specific protein content of microalgae. Use of food waste hydrolysate as culture medium have showed a three-fold of protein content (259.8 mg g^{-1}) compared to the biomass composition obtained grown using conventional medium (82.1 mg g^{-1}) (Pleissner, Lam, Sun, & Lin, 2013). However, the lipid and carbohydrate content of the *Schizochytrium mangrovei* were similar using both culture mediums. The other species, *Chlorella pyrenoidosa*, favoured the conventional medium compared to the food waste hydrolysate as higher protein content was obtained while high concentrations of dominant fatty acids such as, oleic acids, palmitic and α-linolenic acid (ALA) were gained using food waste hydrolysate with the addition of phosphate (Pleissner et al., 2013). Slightly higher protein content was found at the exponential growth phase of *Chlorella vulgaris*, around 300 mg g^{-1} with excess nutrients presented in medium under heterotrophic cultivation (Lau,

Pleissner, & Lin, 2014). The excess nutrients will be consumed to develop and accumulate the lipids and carbohydrate contents for synthesizing daughter cells. Therefore, the protein contents are decreased as the biomass reaching the stationary phase of cultivation. Another case study was performed using aquaculture wastewater and synthetic medium (BG11) to the culture microalgae. It is reported that comparable proteins productivity was obtained with the sufficient nutrient from aquaculture wastewater and potential to be a promising method for integrated biomass generation and nutrient removal (Ansari, Singh, Guldhe, & Bux, 2017). This indicates the culture medium has to be optimized to suit the specific microalgae strain in obtaining a high concentration of targeted components including protein content. Besides, the cultivation using continuous flow culture with nutrient limitation (three dilution rates) has resulted in lower protein contents, ranging from 159 to 179 mg g^{-1} compared to the batch culture system (281 mg g^{-1}). The limitation of nutrient supply has improved the cellular response of biomass, causing the increment of protein content with decreasing dilution rate of the flow culture (Pleissner, Lau, & Ki Lin, 2017). The observed results from Ansari et al. (2017) were in agreement the study of Pleissner et al. (2017), at which limiting the supply of nutrient causing the low composition of protein content in biomass harvested (Ansari et al., 2017). This phenomenon is observed due to the synthesis of carbohydrate is preferred compared to synthesis of protein in the carbon fixation during photosynthesis (Hena, Fatimah, & Tabassum, 2015). The nitrogen concentration of culture medium is closely related to the nitrogen concentration incorporated in biomass as protein. High nitrogen content of biomass is achieved with the medium contains high amount of ammonium, leading to high protein content as well as inhibit the anaerobic

digestion process of microalgae (Samorì, Samorì, Guerrini, & Pistocchi, 2013). In the cultivation of a microalgae consortium, the biochemical composition of the cell culture is observed to have higher protein content compared to both lipid and carbohydrate contents. Chinnasamy et al. (2010) have reported that the biochemical composition of microalgae consortium may influence by the dominant microalgae strain (Chinnasamy et al., 2010), while Samorì et al. (2013) have stated that a lower C/N ratio leads to generation of higher protein content in the microalgae consortium despite the type of medium used (Chinnasamy et al., 2010; Samorì et al., 2013). Various factors such as nutrient supply, cultivation system and culture medium are concluded to manipulate the composition of microalgal protein. Selection of a protein-rich strain with appropriate cultivation medium is the crucial step for the commercialization of microalgal protein by considering the sustainability and economy of whole process.

Table 2.2: Protein content in microalgae.

Microalgae species	Medium	Protein content	References
Porphyridium cruentum	Hemerick	88.52% hydro-soluble proteins	(Carl Safi, Charton, et al., 2014)
Arthrospira platensis	Zarrouk	76.02%	(Carl Safi, Charton, et al., 2014)
Chlorella vulgaris	Sueoka	51.68%	(Carl Safi, Charton, et al., 2014)
Nannochloropsis gaditana	-	49% w/w total proteins	(C. Safi et al., 2017)
Chlorella vulgaris FSP-E	Modified Basal	52.2%	(C.-Y. Chen et al., 2016)
Chlorogloeopsis fritschii	BG11	50%	(Biller et al., 2012)
Scenedesmus dimorphous	3N-BBM + V	43%	(Biller et al., 2012)
Algal consortium (14 species, major species: *Scenedesmus* sp.)	CME	54.5%	(Chinnasamy et al., 2010)
Schizochytrium mangrovei	FWH	82.1 mg g^{-1}	(Pleissner et al., 2013)
Chlorella pyrenoidosa	FWH	69.4 mg g^{-1}	(Pleissner et al., 2013)
Chlorella vulgaris	FWH	200 mg g^{-1}	(Lau et al., 2014)
Chlorella pyrenoidosa	FWH	281 mg g^{-1}	(Pleissner et al., 2017)
S. obliquus	BG11	26.16%	(Ansari et al., 2017)
Chlorella sorokiniana	BG11	33.26%	(Ansari et al., 2017)

Table 2.2 (Continue)

Microalgae species	Medium	Protein content	References
Adferfe falcatus	BG11	36.94%	(Ansari et al., 2017)
S. obliquus	AWW	19.52%	(Ansari et al., 2017)
Chlorella sorokiniana	AWW	28.81%	(Ansari et al., 2017)
Adferfe falcatus	AWW	30.59%	(Ansari et al., 2017)
Microalgae consortium (12 species, major species: green microalgae)	Treated WW	45.09%	(Hena et al., 2015)
Microalgae consortium (*Scenedesmus*, *Desmodesmus* and *Monoraphidium)*	WW effluent	39.2% (w/w)	(Samorì et al., 2013)

*AWW: aquaculture wastewater; CME: carpet mill effluent; FWH: food waste hydrolysate; WW: wastewater

2.2.2 Lipids

Lipid content of various microalgae strains are summarized in **Table 2.3** and it is observed that the lipid content of 50 to 70% can be achieved using high lipid accumulating microalgal strains and application of various cultivation strategies. The lipids extracted can be used for the production of biofuels and in some cases the use of wastewater and flue gases for the cultivation of lipid-rich microalgae greatly improves the economics of the process and provide cost competitive biofuels. Lipids in microalgae are present in two forms which is similar to carbohydrates: (a) the structural or polar lipids that form the structural parts of cell and can be used as food supplement and (b) the non-polar or neutral lipids which are the energy reserves, mainly are Triacylglycerides (TAG).

Microalgae, especially marine eukaryotic microalgae are rich in Very Long Chain Poly Unsaturated Fatty Acids (VLCPUFA) that have a double bond at the C-3 position and hence named as ω3 fatty acids. γ-linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are some of the important PUFA that can be extracted from microalgae. *Crypthecodinium, Schizochytrium, Nannochloropsis, Nitzschia* and *Phaeodactylum* are some of the microalgal genera that can commercially exploit for the production of EPA and DHA (Harwood & Guschina, 2009). The second group of lipids that are extensively exploited by the industry for biofuel production is the neutral storage lipids or TAGs. The choice of funnelling the fixed carbon into carbohydrates or lipids is purely strain dependent and several environmental stress factors can be applied to trigger lipid accumulation.

In marine microalgae, salinity or salt stress is an important stress factor for enhancing lipid accumulation. In *Dunaliella teraticola* with the presence of 0.5M NaCl (sea water level), the lipid content was 60% by weight and increasing the salt concentration to 1.0M increased lipid content to 67%. Addition of high initial NaCl concentration of 1.0M and the addition of 1.0M NaCl during culture resulted in high intracellular lipid content of 70 to 71% and high percentage of triacylglyceride in the lipid (Mutsumi Takagi, Karseno, & Yoshida, 2006). It has been demonstrated that iron is an important nutritional factor to control marine algal biomass in oligotrophic and high nitrogen waters. The effect of different concentrations of added iron on the biomass and lipid accumulation of *Chlorella vulgaris* was studied. It was shown that addition of iron at a concentration of $1.2x10^{-5}$ mol L^{-1} $FeCl_3$ in the lipid accumulation phase enhanced the lipid content of algae to 56.6%, which was 3 to 7-fold higher than that obtained in low nitrogen medium (Z.-Y. Liu, Wang, & Zhou, 2008). Nitrogen depletion is the most widely accepted and used method to accumulate lipids. The effect of nitrogen as a nutrient source in accumulating fatty acids of three types of microalgae strains, *Tetraselmis subcordiformis*, *Nannochloropsis oculata* and *Pavlova viridis* was studied by using different amounts of nitrogen for cultivation (X. Huang, Huang, Wen, & Yan, 2013). It was shown that growth and biomass production increased with higher nitrogen content of the medium, while total lipids and the proportion of neutral lipids of microalgae decreased significantly. It was also observed that the fatty acid profile was species specific, such that *Nannochloropsis* had higher EPA content, while the other two type of microalgae were rich in lipids with chain length greater than C16. Nitrogen deprivation triggers lipid accumulation in both marine and freshwater algae,

leading to very high lipid content in cells. *Nannochloropsis* sp. was cultivated in seawater with 50% municipal wastewater, under high CO_2 aeration of 15% supplied by flue gas and high light intensity. In the lipid accumulation phase, nitrogen deprivation and high light intensity increased the lipid content of cells from 33.8% to 59.9% (L. Jiang et al., 2011). In a similar study, growth of *Chlorella vulgaris* ESP-31 in a tubular photobioreactor using single stage method with nitrogen deprivation increased the lipid accumulation to 55.9% (Yeh & Chang, 2011). *B. braunii* FACHB 357, when cultivated in a specially designed attached cultivation system under nitrogen deprivation has attained higher lipid content of over 50% (P. Cheng et al., 2013). Conversely, it was also shown for *Botyrococcus braunii* that consuming and maintaining nitrogen at a constant level is essential for biomass and lipid productivity. Fed-batch cultivation in an air-lift photobioreactor with nitrogen and phosphorus maintained at 128.7 and 5.025 mg/L, enhanced the lipid accumulation of *B. braunii* IPE 001 to 64.3%, while 58.7% was achieved in batch cultivation (Xu, Wang, Guo, & Liu, 2012). *Scenedesmus obliquus* CNW-N, when cultivated under nitrogen stress for 5 days, accumulated a total lipid content of 22.4%, rich in C16/C18 fatty acids that suitable for the conversion to biodiesel (Ho, Chen, & Chang, 2012). *Nannochloropsis oculata* and *Chlorella vulgaris* when grown under nitrogen deprived conditions, their lipid content have increased from 7.90 to 15.31% and from 5.90 to 16.41%, respectively (Converti et al., 2009). In *Chlamydomonas reinhardtii*, it was shown that sulphur deprivation is more effective in increasing lipid content and maintaining cell biomass than nitrogen deprivation (Cakmak et al., 2012). Various cultivation conditions like temperature (Converti et al., 2009), light intensity (Ho et al., 2012) and CO_2

concentration were known to enhance lipid accumulation in various microalgal strains. Detailed reviews are available about the effect of various culture parameters on lipid accumulation in microalgae and interested readers are referred to (C.-Y. Chen, Yeh, Aisyah, Lee, & Chang, 2011; K. K. Sharma, Schuhmann, & Schenk, 2012). Species of the genera *Botyrococcus, Chlorella, Nannochloropsis, Scenedesmus, Neochloris, Phaeodactylum* and *Dunaliella* are well known lipid producers that can be commercially exploited for biofuels production. Isolation of strains genetically capable of high lipid accumulation combined with cultivation strategies is the major goal in microalgal lipid production for commercial success.

Table 2.3: Lipid/ fatty acid content in microalgae.

Microalgae species	Content (%)	References
Dunaliella tertiolecta ATCC 30929	70.6-71.4	(Mutsumi Takagi et al., 2006)
B. braunii IPE 001	64.3	(Xu et al., 2012)
B. braunii UK 807-2	65-70	(Yan Li & Qin, 2005)
B. braunii FACHB 357	51.6	(P. Cheng et al., 2013)
B. braunii Showa	30-39	(Yoshimura, Okada, & Honda, 2013)
Nannochloropsis sp.	59.9	(L. Jiang et al., 2011)
Chlorella vulgaris C7	56.6	(Z.-Y. Liu et al., 2008)
Chlorella vulgaris ESP-31	55.9	(Yeh & Chang, 2011)
Isochrysis zhangjiangensis	53	(D. Feng, Chen, Xue, & Zhang, 2011)
Scenedesmus sp. LX1	53	(Xin, Hong-ying, Ke, & Ying-xue, 2010)
Neochloris oleabundans UTEX 1185	56	(Gouveia, Marques, da Silva, & Reis, 2009)
Monoraphidium sp. FXY-10	51.72	(Zhao, Yu, Li, Tang, & Huang, 2014)
Nannochloris sp. UTEX LB1999	50.9	(M. Takagi, Watanabe, Yamaberi, & Yoshida, 2000)
C. vulgaris FACHB1068	42	(Y. Feng, Li, & Zhang, 2011)
N. oleoabundans	40	(Yanqun Li, Horsman, Wang, Wu, & Lan, 2008)

Table 2.3 (Continue)

Microalgae species	Content (%)	References
Botryococcus sp.	28.6	(J.-Y. Lee, Yoo, Jun, Ahn, & Oh, 2010)
Chlorella vulgaris	10	(J.-Y. Lee et al., 2010)
Chlorella vulgaris	16.41	(Converti et al., 2009)
Chlorella vulgaris P12	11	(Anjos, Fernandes, Vicente, Teixeira, & Dragone, 2013)
Nannochloropsis oculata	15.31	(Converti et al., 2009)
Porphyridium cruentum	8	(Biller & Ross, 2011)
Scenedesmus obliquus CNW-N	22.4	(Ho et al., 2012)
Scenedesmus sp.	10	(J.-Y. Lee et al., 2010)
Tetraselmis subcordiformis	29.77	(X. Huang et al., 2013)

2.2.3 Carbohydrates

In carbohydrate accumulating microalgae, light intensity and CO_2 concentration which determines the photosynthetic efficiency of the strain can highly influence the final carbohydrate content of the strain (C.-Y. Chen, Zhao, et al., 2013). Carbohydrate accumulation in microalgae is represented in **Table 2.4**, and it is observed that the highest carbohydrate content is presented in the alga *Chlorococcum littorale*. The highest cell density was obtained by growing in PBR and with optimized light intensity and CO_2 contents, high carbohydrate content of 70% was achieved. In the case of *Chlamydomonas reinhardtii* UTEX 90, fed batch cultivation with the additional use of acetic acid as an organic carbon source seems to enhance carbon fixation and carbohydrate accumulation, with a carbohydrate content of 59.7% (S. P. Choi, Nguyen, & Sim, 2010). Comparable carbohydrate composition is observed in *Chlorella* sp., *Chlorococcum littorale*, *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*. Particularly, *Chlorella* sp. able to accumulate carbohydrate content from 18% to 51%, which is a considerably high amount for microalgae strain. Several nutritional stresses like nitrogen depletion, sulphur depletion and phosphorus depletion are known to induce carbohydrate accumulation. For example, the effect of nitrogen starvation has increased the carbohydrate content in *Chlorella vulgaris* P12 from 5% to 41% of dry cell weight, which is around 8-fold higher than the control sample after 24 hours. Besides that, a 2-day nitrogen starvation resulted in total accumulation of 51% carbohydrate in *Chlorella vulgaris* FSP-E (Ho et al., 2013). *Scenedesmus obliquus* CNW-N has produced higher amount of carbohydrate with only one day of nitrogen starvation (Ho et al., 2012). *Tetraselmis subcordiformis* has produced 62.1% of

carbohydrate on a dry weight basis which corresponds to 0.62 g L^{-1} d^{-1} of carbohydrate under sulfur-deprived conditions (Yao, Ai, Cao, Xue, & Zhang, 2012). Therefore, microalgae such as *Chlorella* sp., *Chlamydomonas* sp., *Tetraselmis* sp., can be considered as potential feedstock in alcoholic fermentations that requires high amount of carbohydrates.

Table 2.4: Carbohydrate composition in microalgae.

Microalgae species	Carbohydrate/ Starch content (%)	Reference
Chlorella vulgaris FSP-E	51	(Ho et al., 2013)
Chlorella vulgaris P12	41	(Dragone, Fernandes, Abreu, Vicente, & Teixeira, 2011)
Chlorella. sorokiniana	18	(Hernández, Riaño, Coca, & García-González, 2015)
Chlamydomonas reinhardtii UTEX 90	57 (Starch)	(S. P. Choi et al., 2010)
Chlorococcum littorale	70	(Q. Hu, Kurano, Kawachi, Iwasaki, & Miyachi, 1998)
Gracilaria sordida	12 (ODW)	(Ekman, Yu, & Pedersen, 1991)
Nostoc muscurum TISTR 8871	32.97	(Rodjaroen, Juntawong, Mahakhant, & Miyamoto, 2007)
Porphyridium cruentum	40	(Biller & Ross, 2011)
Scenedesmus obliquus CNW-N	46.65	(Ho et al., 2012)
Tetraselmis subcordiformis (marine)	62.1	(Yao et al., 2012)

2.2.4 Pigments

Pigments are considered as high value-added compound of microalgae compared to the three major components mentioned in the previous sections. Three major classes of pigments in microalgae are chlorophylls, carotenoids and phycobilins that exhibit beneficial biological activities to human health. Pigments are compounds that pigmented microalgae and play a role in the photosynthesis of microalgae. The composition of these pigments is varied according to the type of microalgae and some microalgae just consist particular type of pigment. It is claimed that pigments can promote human health include enhancing immune system, acts as anti-inflammatory agents, and vitamin precursors (Christaki, Bonos, & Florou-Paneri, 2015). The functionality and the rareness of pigments have resulted in their high market price compared to the synthetic ones. These unique features have made microalgae as a viable natural source in field of pharmaceutical, nutraceutical, cosmeceutical and functional food development (Ambati et al., 2019).

The attachment of phycobilins to polypeptides covalently causes the formation of phycobiliproteins. Phycobiliproteins is a pigment-protein complex that is water-soluble, unlike other pigments, which are organic solvent-soluble. It can be often found in blue-green algae and red algae. Phycobiliproteins are caterogized into three groups, namely phycoerythrins (PE) or phycoerythrocyanins (PECs), phycocyanins (PCs) and allophycocyanins (APCs). They are classified according to their phycobilin energy from the highest energy (PECs) can be detected at absorbance of 480-580 nm, intermediate energy (PCs) can be detected at absorbance of 600-640 nm to the

lowest energy (APCs) can be detected at the absorbance of 620-660 nm (Yen et al., 2013). Properties of phycobiliproteins are high fluorescence quantum, high extinction coefficient, having a broad absorption in visible light spectrum and less fluorescence quenching (Yen et al., 2013). Hence, phycobiliproteins are widely utilized as fluorescent labelling reagents in biomedical researches and fluorescence immunoassay (Matamala, Almonacid, Figueroa, Martínez - oyanedel, & Bunster, 2007). Deep blue phycobiliproteins like C-phycocyanin (C-PC) and green-bluish phycobiliproteins like APC are producible from the blue-green algae, *Spirulina* sp. while the red phycobiliproteins such as R-phycoerythrin (R-PE), B-phycoerythrin (B-PE) and C- phycoerythrin (C-PE) are existed in red microalgae, *Porphyridium* sp., *Rhodomonas* sp., and blue-green microalgae, *Anabaena* sp. (Bermejo Román, Alvárez-Pez, Acién Fernández, & Molina Grima, 2002; Chaloub, Motta, de Araujo, de Aguiar, & da Silva, 2015; Rodriguez, Rivas, Guerrero, & Losada, 1989). In the study of Rodriguez et al. (1989), it is stated that the variation of light irradiance and the cell density of chlorophyll have impacted the pigment content under the autotrophic cultivation and nitrogen-fixing mode (Rodriguez et al., 1989). A remarkable increment from 16.5% to 21.9% was observed by adding 9 mg more of chlorophyll liter^{-1} into the culture system, which enhanced the PEs content. Past studies have stated that light-induced pigment like PCs is more favourable to be synthesized with the mixotrophic fed-batch culture on glucose (F. Chen & Zhang, 1997). The light irradiance is one of the factors to manipulate the pigment accumulation. Low light intensity is required to increase the accumulation of phycobiliproteins (F. Chen & Zhang, 1997; Y. Xie et al., 2015). C-PC functions as the collecting light pigments for photosynthesis at wavelength where chlorophylls poorly absorb,

and transfer the energy to chlorophylls a with high efficiency (Kuddus, Singh, Thomas, & Al-Hazimi, 2013). The C-PC was observed to decrease around 16% from the light intensity of 75 to 450 µmol/m^2/s, which caused by the light absorption by the growing microalgae cells (Y. Xie et al., 2015). Hence, the cellular of C-PC are not required and result in lower production of C-PC. A light intensity, 150 µmol/m^2/s with a moderate temperature, 36 ^oC was claimed to achieve 23% of C-PC and 12% of APC per dry algal biomass (Chaneva, Furnadzhieva, Minkova, & Lukavsky, 2007). A low light intensity, 90 µmol/m^2/s of white light used is able to produce 90 mg/l of C-PC and 23.28 mg/l of APC as reported by Mihova et al. (1996), which showed phycobiliproteins favours a light intensity ranged from 90 to 150 µmol/m^2/s (Mihova, Georgiev, Minkova, & Tchernov, 1996). The type of light source influences the accumulation of phycobiliproteins too. It is reported that the phycobiliproteins of red algae, *Rhodella reticulate*, tend to have higher accumulation under red light compared to white and blue light (Mihova et al., 1996). The obtained result was controversial with the results observed by Khatoon et al. (2018) using blue-green algae (*Pseudanabaena mucicola*) as main strain, which APC favours white light and phycocyanin favours blue light, uses wastewater media and bold basal medium as culture medium, respectively (Khatoon et al., 2018). The criteria for accumulation of PE also similar with PC and APC, where low light intensity (15 µmol/m^2/s) is required (Chaloub et al., 2015). By studying the effect of temperature, phycobiliproteins content reduces if the temperature is extremely low or high temperature. Phycobiliproteins tend to accumulate from 20 to 26 ^oC and starts to decrease at 32 ^oC due to the intolerance of microalgae, *Rhodomonus* sp. to the high temperature (Chaloub et al., 2015). Other microalgae strain, blue-

green microalgae, can tolerate higher temperature compared to *Rhodomonus* sp., produces maximal phycobiliproteins at 36 ^{o}C (Chaneva et al., 2007).

Carotenoids are fat-soluble and usually yellow, orange and red in colour. It can be classified into two classes, namely carotenes and xanthophylls (Gong & Bassi, 2016). The oxygenated carotenes are xanthophylls, which are hydrophilic compounds. There are difficulties to handle and store carotenoids since they are antioxidants and sensitive to light, oxygen as well as heat. The primary carotenoid, lutein has the similar function with phycobiliproteins; while the secondary carotenoids such as astaxanthin and canthaxanthin are involved in cell protective mechanisms of microalgae. A formation of protective layer will be carried out when the cells are stressed by the environment conditions, leading to appearance of pink or red in the microalgae (Begum, Yusoff, Banerjee, Khatoon, & Shariff, 2016). Due to their strong antioxidant properties, carotenoids are not only utilized in feed additives, food colorants and cosmetics, they are prone to have a bigger market as functional food and pharmaceuticals as more demands of natural products from consumers nowadays. Carotenoids such as lutein, astaxanthin, and lycopene are proved to have both anti-oxidant and anti-cancer properties; while fucoxanthin has anti-obesity properties and β-carotene can prevent night blindness and liver fibrosis, respectively (Gong & Bassi, 2016). The major carotenoid market is taken up by astaxanthin and β-carotene according to the report of BCC Research study (März, 2008). Numerous health-benefits are possessed by astaxanthin, such as sun-proofing, enhancing the antibody production, anti-inflammatory and anti-aging properties, while β-carotene can prevent the build up of toxin in liver and improves the immune

system. However, long term consumption may lead to cancer. Carotenoids are commonly found in green microalgae, for example *Dunaliella* sp., *Haematococcus* sp., and *Chlorella* sp., which have potential to use for the mass production of carotenoids (J. Zhang, Sun, Sun, Chen, & Chen, 2014). The production of carotenoids can be enhanced through higher light intensity, lower nitrogen supplies or salt stress. The exposure of environmental stress has produced carotenoids when the green microalgae have turns to red or pink. Comparing with phycobiliproteins mentioned above, carotenoids are not sensitive toward the temperature change at low light intensity (<100 μmol/m^2/s) and the production is slightly increased at higher light intensity (150 and 300 μmol/m^2/s) (Chaneva et al., 2007). High light intensity has reduced the chloroplast photosynthetic activities and boosted the protective mechanisms of microalgae by carotenoids, causing the carotenoids content to be two fold by increasing the light intensity ten times higher (Q. He, Yang, Wu, & Hu, 2015). The addition of sodium chloride and nitrate has shown positive effect to the cantaxanthin production, doubling up the yield of cantaxanthin compared to the cultivation without sodium chloride and nitrate (Grama, Chader, Khelifi, Agathos, & Jeffryes, 2014). As for culture medium, the higher dilution of hydroponic wastewater slightly reduces the total production of carotenoids while lowest carotenoid content was obtained using Bold Basal Medium. This indicates the hydrophonic wastewater is more suitable to accumulate carotenoids compared to that conventional medium (Bertoldi, Sant'Anna, da Costa Braga, & Oliveira, 2006).

On the other hand, chlorophylls are fat-soluble, green in colour and widely distributed in terrestrial plants as well as micoalgae. It functions as the compound to capture the solar energy and convert them into chemical energy through photosynthesis. Types of chlorophylls include chlorophyll *a*, *b*, c_1, c_2, c_3, *d*, and *f*, that able to absorb light at various wavelengths (Kume, Akitsu, & Nasahara, 2018). Chlorophyll *a* and *b* are the most abundant chlorophylls with chlorophyll *a* functions as primary photosynthetic pigment and chlorophyll *b* functions as the accessory pigments. These chlorophylls absorb light in two regions, blue (450 nm) and red (650-700 nm) regions (Boyer, 1990). The high growth rate of microalgae and available throughout the year has made microalgae a promising source for chlorophylls. The production of chlorophylls in market is well-established compared to carotenoids and phycobiliproteins, as they have been applied as natural food colourant, additives to products like deodorants and shampoos as well as utilized in dermatology (Ferreira, Pinto, & Sant'Anna, 2016). The cultivation condition with low light intensity and nitrogen deprived can modulate the chlorophyll content. Ferreira et al. (2016) have concluded that reducing the light intensity has increased the chlorophyll content but removing the nitrogen supplies could change the productivity of chlorophyll *a* and *b* (Ferreira et al., 2016). Chlorophyll *a* decreased around 8% while chlorophyll *b* remained unchanged in the absence of nitrogen compared to the control group, indicating different cultivation conditions may vary the proportions of chlorophylls in microalgae. Similar with phycobiliproteins, chlorophylls experience photoinhibition when it exposes to high light intensity. The types of light source also influence the production of chlorophylls same as other pigments. It is reported that the chlorophylls from *Chlorella* sp. (green

microalgae) have the optimum accumulation using red light as light source while comparable yield of chlorophylls from *Gloeothece* sp. (blue-green microalgae) is obtained using violet light (Mohsenpour, Richards, & Willoughby, 2012). In conclusion, the production of maximal pigments yield relies closely on the cultivation conditions and type of strain used.

Table 2.5: Pigment content in various microalgae.

Pigments	Microalgae strain	Cultivation mode	Contents	References
Phycocyanins	*Spirulina platensis*	Mixotrophic fed-batch; 100 g L^{-1} glucose; Light: 80-160 µmol $m^{-2}s^{-1}$ illuminated 24 h; Zarrouk medium	795 $mg.L^{-1}$	(F. Chen & Zhang, 1997)
Phycocyanins	*Spirulina platensis*	Phototrophic fed-batch; Light: 300 µmol $m^{-2}s^{-1}$ illuminated 24 h; Zarrouk medium; 5 mM medium feeding	16.1%	(Y. Xie et al., 2015)
Phycoerythins	*Anabaena* sp.	Autotrophic semi-continuous; Light: 40 Wm^{-2} with 12 h light and dark cycles; addition of 10 mM $NaHCO_3$ and 30 mM NACl	8.3% CDW	(Rodriguez et al., 1989)
Phyco-biliproteins	*Porphyridium* sp.	Batch; Chemostat culture: 0.049 h^{-1} dilution; Hemerick's medium; T: 20 ºC	2% DW	(Bermejo Román et al., 2002)
Phyco-biliproteins	*Rhodella reticulate*	Batch; Red light of 90 µmol $m^{-2}s^{-1}$ illuminated 24 h; Modified Brody and Emerson's medium	213.02 $mg.L^{-1}$	(Mihova et al., 1996)
Phyco-biliproteins	*Pseudanabaena* sp.	White light of 180 µmol $m^{-2}s^{-1}$ illuminated 24 h; WW medium; T: 25 ºC	237 $mg.g^{-1}$	(Khatoon et al., 2018)
Carotene	*Dactylococcus* sp.	Batch; LED light: 100 µmol $m^{-2}s^{-1}$ illuminated 24 h; 3N-BBM+V stress medium with 0.75 g L^{-1} $NaNO_3^-$ and 0.025 g L^{-1} NaCl	0.45 $mg.L^{-1}$	(Grama et al., 2014)

Table 2.5 (Continue)

Pigments	Microalgae strain	Cultivation mode	Contents	References
Carotenoids	*Porphyridium* sp.	Batch; Chemostat culture: 0.049 h^{-1} dilution; Hemerick's medium; T: 20 °C	0.10% DW	(Bermejo Román et al., 2002)
Carotenoids	*Chlorella vulgaris*	Batch; Fluorescent light: 150 µmol $m^{-2}s^{-1}$ illuminated 24 h; HW with 50% dilution; T: 25 °C	0.071 pg $cell^{-1}$	(Bertoldi et al., 2006)
Chlorophyll *a*	*Chlorella* sp. L1	Batch; Light: 40 µmol $m^{-2}s^{-1}$ illuminated 24 h; Modified BG-11; T: 25 °C	14 $mg.L^{-1}$	(Q. He et al., 2015)
Chlorophyll *a*	*M. dybowskii* Y2	Batch; Light: 40 µmol $m^{-2}s^{-1}$ illuminated 24 h; Modified BG-11; T: 25 °C	11 $mg.L^{-1}$	(Q. He et al., 2015)
Chlorophyll *a*	*Chlorella vulgaris*	Batch; Xenon spectrum red light: 250 µmol $m^{-2}s^{-1}$ with 12 h light and dark; 3N-BBM+V medium; T: 23 °C	1.29% g Chl g^{-1}	(Mohsenpour et al., 2012)
Chlorophyll *a*	*G. membranacea*	Batch; Xenon spectrum green light: 150 µmol $m^{-2}s^{-1}$ with 12 h light and dark; BG-11 medium; T: 23 °C	1.23% g Chl g^{-1}	(Mohsenpour et al., 2012)

*HW: Hydroponic water WW: Wastewater; DW: Dry weight; CDW: Cell dry weight; T: Temperature

2.2.5 Vitamins

Microalgae are tremendous source of vitamins with antioxidants properties, such as provitamin A, B1, B6, B12, C (ascorbic acid), D, E (α-tocopherol), biotin, riboflavin, nicotinic acid, pantothenate, folic acid. High concentration of vitamin C and riboflavin, 1-16 mg g^{-1} and 20-40 μg g^{-1}, respectively, are observed in 40 species of microalgae from seven algal classes (Brown, Jeffrey, Volkman, & Dunstan, 1997). Raposo and de Morais (2015) have hypothesized that ingestion of microalgae consist of these vitamins have the potential to prevent the oxidative damage and occurrence of cancers (Raposo & de Morais, 2015). Several studies were performed to examine the effect of light towards vitamins accumulation in microalgae. Barbosa et al. (2005) has reported a total production of 3.48 mg g^{-1} vitamin C from *Dunaliella tertiolecta* was obtained using average light intensity under acceleration-stat cultivation (continuous change of dilution rate was performed under constant acceleration rate) (Barbosa et al., 2005). The production of vitamin E is inversely proportional to the light availability (Carballo-Cárdenas, Tuan, Janssen, & Wijffels, 2003). However, higher contents of vitamin E are produced under light compared to dark condition, relating the differentiation of chloroplasts to vitamin E productivity (Tani & Tsumura, 1989). These reported results show that light intensity is the crucial factor in optimizing vitamins production.

2.2.6 Polyunsaturated Fatty Acid

Polyunsaturated fatty acids (PUFAs) are long chain fatty acids having more than 18 carbons, which can be synthesized by microalgae. The examples of PUFAs include docosahexaenoic acid, DHA (C22:6), eicosapentaenoic acid,

EPA (C20:5), arachidonic acid, ARA (C20:4) and γ-linolenic acid, GLA (C18:3) (I. Sousa, Gouveia, Batista, Raymundo, & Bandarra, 2008). DHA and EPA are often required for daily diet, especially during childhood and elderly life due to low conversion efficiency of ALA to both DHA and EPA. In addition, EPA and DHA are capable to reduce cholesterol level and acts as anti-inflammatory agents through decreasing triglycerides (TG) and increasing the high-density lipoprotein (HDL) content. These PUFAs are found synthesized abundance in microalgae strains, such as *Spirulina* sp., *Porphyridium* sp., *Odontella* sp., *Isochrysis* sp., and *Pavlova* sp. (Babadzhanov et al., 2004; Raposo & de Morais, 2015). The importance of PUFAs in neutraceutical and pharmaceutical applications is well-recognized as the consumption of PUFAs is proved to prevent various cardiac disorders (Chew et al., 2017). High culture temperature will reduce the production of PUFAs in microalgae biomass. It is reported that the PUFAs from *Chlorella pyrenoidosa* favour low temperature of winter compared to other seasons with high culture temperatures (Chu et al., 2015), which is in agreement with the findings by Jiang and Gao (2004) using *Phaedactylum tricornutum* as the source of PUFAs (H. Jiang & Gao, 2004). Besides culture temperature, the type of culture medium affects the production of PUFAs. The enriched seawater medium, GPM able to accumulate more PUFAs compared to the synthetic medium, ASW as stated by Carvalho and Malcata (2000) (Carvalho & Malcata, 2000). Lastly, the utilization of glucose as carbon source also enhance the production of PUFAs compared to fructose as carbon source in the culture medium (S.-T. Wu, Yu, & Lin, 2005).

2.3 Biological Activity

2.3.1 Antioxidant Activity

Antioxidant activity is the biological activity that able to defend the living cells from oxidation damage and inhibit lipid deterioration for food preservation. The substances contain antioxidant activity are proposed in potentially treating and curing multiple types of degenerative diseases by blocking the generation of free radical chain reaction caused by lipid peroxidation and reduced the reactive species (Murthy et al., 2005). Without antioxidant activity, a series of deteriorative changes will be occurred in the biological system and leads to cell inactivation in the end. Therefore, increasing interests to natural sources containing antioxidant activity are observed in recent years (Maadane et al., 2015). Several types of components within microalgae are possessed with antioxidant activity, such as vitamins, phycobiliproteins, β-carotene, astaxanthin and sulphated polysaccharides (Raposo & de Morais, 2015).

The antioxidant activity of the compounds can be assessed by DPPH (1, 1-diphenyl-2-picryl-hydrazil) radical scavenging assay, Trolox equivalent antioxidant capacity (TEAC) assay, reducing power of the compounds and determining the scavenging ability on superoxide radicals, hydroxyl radical, and hydrogen peroxide (Yingying Sun, Wang, Guo, Pu, & Yan, 2014). For the analysis of reducing power, greater reducing capacity is indicated by higher absorbance value for the reaction mixture. In the study of Sun et al. (2014), polysaccharides from *Isochrysis* sp. have showed moderate scavenging activity against superoxide and hydroxyl radicals while demonstrated effective reducing

power through potassium ferricyanide reduction method (Yingying Sun et al., 2014). In the analysis of DPPH radical scavenging activity, the lower IC_{50} is desired for strong antioxidant activity. The ethanol extracts of *Tetraselmis* sp. is found to possess highest antioxidant activites (247 µg/mL of IC_{50}) compared to the ethanol extracts of *Dunaliella* sp. and *Navicula* sp. as well as ethanol/water extracts of other microalgae species (Maadane et al., 2015). Similar results are obtained by Jaime et al. (2010) as ethanol extracts showed better antioxidant activities than other type of extracts, by investigating the antioxidant activity through TEAC assay (Jaime et al., 2010).

2.3.2 Antiproliferative Activity

Antiproliferative activity is an ability to inhibit the cell growth especially malignant cells, into the surrounding tissues. The natural products with antiproliferative activity are high in demand as it is hard to engineer a chemically derived drug specific for the cytotoxicity of cancer cells without harming the normal cells. Besides, the current treatment of cancer such as chemotherapy may stress the patient and further harm their health. Hence, a replacement of synthetic drugs from natural resource is recommended. Polysaccharides, carotenoids and sulfoglycolipidic fraction from microalgae are claimed to have antiproliferative activity through past studies (Bergé, Debiton, Dumay, Durand, & Barthomeuf, 2002; Pasquet et al., 2011; Trabelsi, Chaieb, Mnari, Abid-Essafi, & Aleya, 2016).

The antiproliferative activity can be determined using MTT assay (3 - (4, 5 - dimetiltiazolyl - 2) - 2, 5 - diphenyltetrazolium bromide) against human

hepatocellular carcinoma (HepG2 cells), colon (Caco-2 cells), cervical (HeLa), lung (A549), prostate (22Rv-1) and breast (HCC38 and MDA-MB-231) cancer cell lines (Garcia-Galaz et al., 2014). MTT assay is a colorimetric assay, commonly used to measure cell metabolic activity and the absorbance is quantified around wavelength of 500 to 600 nm using spectrophotometer (Kuete, Karaosmanoğlu, & Sivas, 2017). The extract of red microalgae, *Rhodosorus marinus* obtained through methanol extraction, shows antiproliferative activity against five types of cancer cell lines with an IC_{50} of 0.5 (colon), 0.8 (cervical), 0.4 (prostate) and 0.9 (breast) mg/mL (Garcia-Galaz et al., 2014). Antiproliferative activity also observed in the extract of green microalgae, *Desmococcus olivaceous* and *Chlorococcum humicola*, with IC_{50} value of 1.56 μg/mL and 0.625 μg/mL, respectively, against Hep-2 cell line (Uma, Sivasubramanian, & Devaraj, 2011). Different methods used to extract the microalgae biomass affects the examination of antiproliferative activity of the sample. The extract obtained through ammonium sulphate precipitation showed no antiproliferative activity towards the cancer cell line while the extract obtained through methanol extraction has showed significant activity towards the same cancer cell line, indicating that the antiproliferative activity presented in each extracts is influenced by the bioactive compounds extracted through different methods (Garcia-Galaz et al., 2014).

2.3.3 Anti-inflammatory Activity

Inflammation often occurs when the tissues are injured, which the chemicals released by the injured cells cause the blood vessel to leak fluid into the tissue and lead to swelling. This is because the immune system has identified

the harmful stimuli, removed it and started to heal the wounds. There are two types of inflammation, acute and chronic inflammation. The former usually occurs within hours or day and the later usually continued presence for a long period (sometimes over many years) (Freund, Orjalo, Desprez, & Campisi, 2010). Diseases such as heart disorders, diabetes, chronic respiratory diseases and stroke are chronic inflammatory diseases caused significant death across worldwide (Deepak, Axelrad, & Ananthakrishnan, 2019).

Several studies have showed that the extracts from marine algae such as *Nannochloropsis oculata* and *Tetraselmis suecica* exhibit strong anti-inflammatory effect (Jo et al., 2010; Sanjeewa et al., 2016). PUFAs such as EPA and DHA, carotenoids especially astaxanthin and β-carotene, phycobiliproteins like C-PC and polysaccharides in microalgae are claimed to have anti-inflammatory effects. It is reported that polysaccharides of *Porphyridium* sp. have exhibited anti-inflammatory effects in improving inflammatory state, and successfully inhibited the tumor necrosis factor (TNF)-α-induced oxidative stress in human coronary artery endothelial cells (Levy-Ontman, Huleihel, Hamias, Wolak, & Paran, 2017).

2.3.4 Antimicrobial Activity

The inhibition of bacteria growth, prevention of the microbial colonies' formation and possibly devastate microorganisms can be achieved through antimicrobial activity of active agents (El Mogahzy, 2009). This activity is required for food preservatives, especially to replace the synthetic preservatives with natural ones as the demand of functional food is increasing in recent years.

In addition, the exploration of novel antimicrobial compounds is important for clinical application to treat against the resistant pathogenic bacteria (Salem, Hoballah, Ghazi, & Hanna, 2014).

The antimicrobial activity of extracts can be determined using agar well diffusion method and disc diffusion method (Ghasemi, Moradian, Mohagheghzadeh, Shokravi, & Morowvat, 2007; Salem et al., 2014). It can be measured against Gram positive bacteria (*Staphyloccocus aureus*, *Streptococcus pyogenes*), Gram negative bacteria (*Proteus vulgaris*) and pathogen yeast (*Candida albicans*). Microalgae extracts and active constituents from numerous strains have shown effective antimicrobial activity towards pathogens. In the study of Mendiola et al. (2006), the antimicrobial activity of *Chaetoceros muelleri* is induced by their lipidic content, in which the total triglycerides and docosapentaenoic acid (DPA) content are the major compounds to control the antimicrobial capacity of the supercritical CO_2 extracts (Mendiola et al., 2007). The supernatants of 21 types of microalgae strains isolated from the paddy fields in Iran have showed significant antimicrobial activity, especially *Chroococcus disperses*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, which emitted a wide spectrum of antimicrobial activity (Ghasemi et al., 2007). However, no antimicrobial activity was detected using the hexane extracts of the same species of microalgae due to the active compounds are hydrophilic compounds. In the study of Thillairajasekar et al. (2009), hexane and the ethyl acetate extracts of *Trichodesmium erythraeum* showed antimicrobial activity towards the bacteria strains studied, which is not in agreement with the findings of Ghasemi et al. 2007 (Thillairajasekar, Duraipandiyan, Perumal, & Ignacimuthu, 2009). The

extracts' compositions are consisted of Myristic acid, palmitic acid, linoleic acid and oleic acid that active against bacteria. Hence, the type of solvents used to extract from various type of strains is crucial in determining the antimicrobial activity of the microalgae extracts too.

2.4 Biomass Pre-treatment Techniques

In order to extract the compounds within microalgae, the pre-treatment to the biomass can always be performed to maximize the recovery yield of the targeted product prior to extraction. The rupturing of microalgae cell wall can be achieved with the applied pre-treatment methods. There are three types of pre-treatment to be discussed in this chapter, mechanical, chemical and biological pre-treatments. The advantages and disadvantages of various pre-treatment methods are tabulated in **Table 2.6**. In this study, we are focusing more on the mechanical pre-treatment compared to chemical and biological pre-treatment prior to the extraction of valuable components. The mechanical pre-treatments are studied in extraction of biomolecules such as proteins and C-phycocyanin as they are efficient in rupturing microalgae cell wall with no requirement of hazardous or caustic chemicals. Several studies have reported that mechanical pre-treatments able to disintegrate the microalgae cell wall and extract considerable high amount of targeted compounds (Kwak, Roh, Yang, Lee, & Chang, 2019; Neto et al., 2013; Parniakov et al., 2015; Carl Safi, Ursu, et al., 2014). The short processing time is one of advantages using mechanical pre-treatment for cell disruption because longer processing time will result in lower productivity and possibly lead to loss of profit. Besides, the chemical pre-treatments such as acid, alkaline and organosolv and biological pre-treatments

require separation of solvents or enzymes after pre-treatment and prior to extraction which leads to extra processing step that are not favourable for industrial application. Therefore, this study is focusing on mechanical pre-treatment techniques rather than other types of pre-treatment methods.

Table 2.6: Type of pre-treatment methods (Dixon & Wilken, 2018; Günerken et al., 2015; Zabed et al., 2019).

Type	Pre-treatment method	Approaches	Advantage	Disadvantage
Mechanical	Freeze-thaw	Exposure of biomass to low temperature (complete frozen) followed by thawing biomass at room temperature	• Not degrading compounds or biomolecules • Low reagent requirement • Simple to perform	• Long processing time (hours to several days) • Increased energy consumption
	Homogenisation	Shear force is applied to reduce the size of biomass in suspension through homogeniser	• Fast and effective • Effective in homogenising wide range of samples • No hazardous chemical required	• Energy intensive • Temperature increases in suspension during processing • Expensive capital cost
	Size reduction	Size of biomass is reduced using miller, grinder, crusher or pestle and mortar	• Relatively inexpensive (pestle and mortar) • Applicable to dry, wet and frozen samples • Small particles can be obtained • No hazardous chemical required	• Possible contamination • Low throughput • Possible lost of samples on surface of mortar
	Sonication	Exposure of biomass to ultrasound irradiation via sonicator or ultrasonic bath	• High energy transfer • Rapid disruption • Effective cell disruption • No hazardous chemical required • Low maintenance cost	• Heat generated must be dissipated • High noise level • Limited area (sonicator) • High cost and energy input

Table 2.6 (Continue)

Type	Pre-treatment method	Approaches	Advantage	Disadvantage
Mechanical	Microwave	Exposure of biomass to microwave irradiation	• Rapid extraction time • Low energy input • No hazardous chemical required • Effective cell disruption	• Generates heat • High maintenance cost
Chemical	Organosolv	Exposure of biomass to mixture of organic solvent and water (mineral acid may used to lower operating temperature	• Able to operate under mild conditions (neutral pH, low temperature and pressure) • Delignify biomass effectively	• Requirement to remove solvent • High cost required • Generate fermentation inhibitor (during delignification)
	Acid	Pre-treatment using diluted or concentrated acid, made from inorganic acid (mostly use H_2SO_4)	• High reaction rate • Well-studied method	• Degrades pigments and decolourise pigments • Some may require tedious condition (for delignification) • Corrosion of reactor • Involvement of caustic acid
	Alkaline	Pre-treatment using salts (commonly use: NaOH and KOH)	• Mild operating condition • Delignify low lignin biomass	• Denatures biomolecules like proteins • Require longer reaction time • Hard to neutralize

Table 2.6 (Continue)

Type	Pre-treatment method	Approaches	Advantage	Disadvantage
Chemical	Ionic liquid	Exposure of biomass to ionic liquids which are salts in liquid form at room temperature/ with low boiling point	• Ionic liquid is recyclable and reusable • Low energy requirement • Properties can be fine tuned	• High cost for ionic liquid • Recovery of ionic liquid is needed
Biological	Fungal	Using single fungal species via submerged or solid-state culture	• No release of toxic compounds • Low recovery cost • Low energy requirement	• Long processing time (weeks to months) • Carbohydrate lost during treatment
	Bacterial	Application of lignin degrading bacteria or hydrolytic bacteria	• Higher growth rate than fungi (few days) • Cost-effective than fungi • High adaptability	• Unable to degrade lignin as efficient as fungi
	Microbial consortia	Exposure of biomass to co-culture of fungal-bacteria, bacteria-bacteria or fungal-fungal system	• Enhanced productivity • Acceleration of organic molecules breakdown • Shorter processing time compared to fungal and bacterial pre-treatments (need few days)	• Difficulty for some individual strains to adapt into co-culture environment • Repeat experimental for suitable strains for co-culture system

Table 2.6 (Continue)

Type	Pre-treatment method	Approaches	Advantage	Disadvantage
Biological	Enzymatic pre-treatment	Application of purified enzymes or crude enzymes to biomass	• High specificity • No severe condition requirements • Ease to perform in industrial scale • Less processing time compared to fungal and bacterial pre-treatments (few hours to days)	• High cost of enzyme required • Poor stability of ligninolytic enzymes (industrial processes)

2.4.1 Mechanical Pre-treatment

2.4.1.1 Freeze-thaw

Freeze-thaw consists of freezing the extracts completely (approximately -15°C to -80 ^{o}C) for a period of time or freezing it quickly using liquid nitrogen, and thaw it at room temperature (Dixon & Wilken, 2018). It is often considered as mild "homogenisation" and hence, homogenisation using pestle will be performed after 1 freeze-thaw cycle (Gagné, 2014). Freeze-thaw process is well-established and commercialized in the industries to test the stability of drug as well as used in the shipping of drugs from a location to another. The company that provide delivering services is Meissner Filtration Products in USA (Meissner Filtration Products Inc, 2007), and the example of company providing feeze-thaw stability testing is Microchem Laboratory in Texas (Microchem Laboratory, 2018). Freeze-thawing can be utilized for rupturing the cell, deoxyribonucleic acid (DNA) extraction, and agrobacterium transformation (Bellete, Flori, Hafid, Raberin, & Tran Manh Sung, 2003; Weigel & Glazebrook, 2006). In the freeze-thaw process, the cells in suspension swell as ice crystals formed in the freezing process and contract during thawing process. The ice crystals will ultimately break and result in cell lysis in the repeating cycles. This method is widely used to rupture plant tissue, insect cells, microalgae, and more (Ezure et al., 2006; Minocha, Shortle, Long, & Minocha, 1994). It can disrupt the cell effectively and does not degrade the compounds during the process.

A direct DNA extraction with freeze-thaw approach can be performed using three cycles to extract the DNA of microbial cells from soil samples (Tsai & Olson, 1991). Polysomes also can be extracted from the bacterial, *Escherichia*

coli, through freeze-thawing with lysozyme, followed by treating mixture with sodium deoxycholate to achieve high yield (Ron, Kohler, & Davis, 1966). Besides, the DNA concentration of sample does increased the stability of DNA during repeated 18 freeze-thaw cycles but the adjustment of freeze-thaw parameters did not impact DNA stability significantly (W. Shao, Khin, & Kopp, 2012). Bellete et al. (2003) also stated that DNA stability was depended on the volume of extracts subjected to the repeated freeze-thaw cycles (Bellete et al., 2003). However, the study of Ross et al. (1990) has stated around 25% of DNA yield in blood samples was reduced with only frozen once (Ross, Haites, & Kelly, 1990). Apart from DNA extraction, an interesting study showed that the total petroleum hydrocarbon content in the recovered oil from solvent extraction can be enhanced through the freeze-thaw process to dewater the recovered oil and increased the content from 40% to 60% (G. Hu, Li, & Hou, 2015).

For microalgae extraction, freeze-thaw can be applied to disrupt the resistant cell wall and release the intracellular compounds from the biomass. Chen et al. have concluded that maximal purity and higher yield of B-PE from *Porphyridium* sp. and *Rhodella* sp. were obtained using freeze-thaw compared to the treatment using beading and calcium chloride (W.-p. Chen, Chen, & Zhang, 2008). PC is extracted through repeated freeze-thaw cycles of -25°C and 4 ^{o}C to release the phycobiliproteins through temperature shocks (Soni, Kalavadia, Trivedi, & Madamwar, 2006). The synergistic effect of maceration in combination with freeze-thaw and freeze-thaw alone also investigated for phycobiliproteins extraction. With maceration, the extraction efficiency of R-PE and R-PC were increased significantly, around 36% and 25%, respectively. The

findings showed that freeze-thaw alone is not capable to rupture the resistant microalgae cell wall that consists of polysaccharide complex matrix (Mittal, Tavanandi, Mantri, & Raghavarao, 2017). The results obtained by Horváth et al. (2013) is in agreement with the findings reported by Mittal et al. (2017), where freeze-thaw has to be applied with combination of other extraction method to gain higher yield of phycobiliproteins (Horváth, Kovács, Riddick, & Présing, 2013; Mittal et al., 2017). However, a study stated that three complete cycles of freeze-thaw using liquid nitrogen has reduced the yield of esterified fatty acid (biodiesel) by 17.5% compared to the control, which might resulted by the loss of esterified fatty acid during the performance of multiple steps (Abomohra, Jin, & El-Sheekh, 2016). Freeze-thaw also can be applied to fractionate the microalgae extracts after alkaline extraction and enhance algal degradability (Kinnunen, Craggs, & Rintala, 2014; Machado, Rodrigues, Moro, Duarte, & Noseda, 2020).

2.4.1.2 Homogenisation

Homogenisation can be performed using various type of homogeniser, such as rotor-stator homogeniser, high pressure homogeniser, and blade type homogenisers. These homogenisers have their own functions and properties in rupturing the cells or treating biomass. For rotor-stator homogeniser, it often serves as an effective equipment to homogenise plant, animal tissue and microalgal biomass suspension in small volume of liquid medium. It is also known as high speed homogeniser. This type of homogeniser is better than blade type homogeniser as less foam is found and can accommodate for small biomass volume. The suction created by the rotor draws the algal biomass into the

apparatus and the homogenised endproduct exits from the slots at the tip of stator. The rotor in high speed recycles the product through the equipment and reduces the tissues' size by the liquid shear force and mechanical shearing at the tip of stator (K.-Y. Show, Lee, Tay, Lee, & Chang, 2015). It is only applicable for low processing volume and only required short contact time. Variables such as rotor speed, concentration of samples, feed viscosity and the design of rotor-stator probes can affect the cell disruption efficiency. The application of rotor-stator homogenisers in lipid extraction from wet microalgae has enhanced the lipid yield significantly compared to conventional stirrer (Kwak et al., 2019). The recovery of other high value product like phycobiliproteins can be performed using rotor-stator homogeniser too, but the combination of homogenisation with sonication have improved the recovery yield around 9% compared to homogenisation alone (Mittal et al., 2017). Kannah et al. (2020) have concluded the biohydrogen production from seagrass through ozone coupled rotor-stator homogeniser has reduced the energy input around 58% and achieving higher cell lysis efficiency than using rotor-stator homogeniser alone, suggesting an energetically efficient technique for bioenergy production (Kannah et al., 2020).

Blade homogenisers are less efficient than rotor-stator homogenisers, but they are suitable to use for fine extracts as final product. A consistent particle size can be obtained using the blade homogeniser with high speed (K.-Y. Show et al., 2015). The users need to be extra careful while handling the homogenisation with flammable solvents using blade homogeniser, because the brush motors used in this type of homogeniser may generate sparks in high speed

operation. Hence, some accessories such as cooling jacket and insulated vessels are required for use with cryogenic solvents. In compare with rotor-stator and blade homogenisers, high pressure homogeniser is proven to be applicable for large-scale applications. The high shear force is generated through the pump in accelerating the liquid flow to high velocity in order to achieve the disruption of cell walls (Krisnangkura, 1986). This equipment involved high usage of energy; hence, it is proposed to be applied for high-value product recovery (Doucha & Lívanský, 2008). It is widely used in rupturing the *Haematococcus* sp. for fish feed production as reported by Chisti and Moo-Young, 1986 (Chisti & Moo-Young, 1986). The processing capacity and cell disruption efficiency were claimed to be independent of the feed concentration (with determined microalgae, *Nannochloropsis* sp. concentration up to 25% w/w) and only depends on the homogenising pressure. This finding is only applicable for weak microalgae if concentrated paste is feed into the equipment (Yap, Dumsday, Scales, & Martin, 2015). It is reported that high pressure homogeniser able to disrupt the microalgal cell wall completely and release all of the intracellular compounds, but the concern about finely sized cell debris in the extract may complicates the downstream processing (Carullo et al., 2018).

2.4.1.3 Size-reduction

Size-reduction of biomass can be achieved through various processes such as milling, grinding, crushing and cutting. Reducing the size of samples not only making the biomass easier to process, it also helps to pre-treat the biomass for releasing the intracellular products from the biomass. This type of pre-treatment process is widely used to process lignocellulosic biomass for biofuel

production, especially grinding as the proven preliminary pretreatment technique (Che Kamarludin, Jainal, Azizan, Safaai, & Mohamad Daud, 2014). It is stated that the pre-treating the biomass can increase the enzymatic hydrolysis rate by 3 to 10-fold, showing the importance of pre-treatment to biomass influencing the product yield in subsequent process (Aftab, Iqbal, Riaz, Karadag, & Tabatabaei, 2019). It is noticed that grinding is mostly applied to rupture the microalgae cell for lipid extraction too. Zheng et al. (2011) have reported that grinding the biomass with different type of medium has varied results obtained, where grinding the biomass with quartz sand under dehydrated condition has higher lipid concentrations than grinding with quartz sand under wet condition and grinding with liquid nitrogen (Zheng et al., 2011). In this case, grinding biomass with liquid nitrogen has proven to be effective method even compared to sonication, microwave and enzyme lysis, with the suitable fatty acid profile obtained for biodiesel. However, the lipid extraction from *Schizochytrium* sp. S31 strains using osmotic shock for pre-treatment has higher lipid yields which is not studied in the work of Zheng et al. (2011) (Byreddy, Gupta, Barrow, & Puri, 2015). Yet, the lipid yield obtained using grinding with liquid nitrogen was comparable with the yield obtained using osmotic shock, proving the effectiveness of grinding with liquid nitrogen in rupturing the cell. The manual grinding of dry microalgae biomass using mortar for protein extraction is investigated too, but this technique has the lowest protein recovery compared to other cell disruption methods (Carl Safi, Ursu, et al., 2014).

On the other hand, milling is another method in reducing size of biomass. Milling can be performed for both dry and wet material. Different types of

milling are utilized depending on the type of material. For example, colloid mill, dissolver and fibrillator are suitable for wet materials; hammer mill, extruder, cryogenic mill and roller mill can utilize for dry materials whereas ball mill can be used for both type of materials. Most of the microalgae biomass is pre-treated through bead milling for lipid extraction as bead milling are a mild disintegration technology. The original purpose of bead milling is for the application of reducing size of paint or lacquer particles, which then found to be suitable for algae disintegration in controlling the temperature of algae suspension (Postma et al., 2015). Types and compositions of bead, speed of the agitator, contact time as well as the properties of feed are the parameters influencing the disruption efficiency of bead milling (A. K. Lee, Lewis, & Ashman, 2012). In the work of Montalescot et al. (2015), the disruption efficiency of bead milling is varied according to the type of microalgae strain, which *Nannochloropsis* sp. is more resistant then *Porphyridium* sp. (Montalescot et al., 2015). Zheng et al. (2011) has reported that the application of bead milling can only recover 7% of lipid from microalgae while the grinding microalgae with liquid nitrogen can achieved 29% of lipid recovery (Zheng et al., 2011). In conclusion, the cell disruption through size reduction is very effective but the method has to be chosen very much depending on the type of feed.

2.4.1.4 Sonication

Sonication is a type of pre-treatment utilizes the sound energy or ultrasonic irradiation to breakdown the cellular membranes or cell walls for releasing the inner cell contents, which also referred as sonoporation. The vibration or the sound waves were converted from electrical signal and able to

perform agitation in a suspension and generates cavitational bubbles. These bubbles collapse when they are not able to absorb any more energy (Ohl, Klaseboer, & Khoo, 2015). The implosion of cavitational bubbles produces shock wave to rupture the cells in suspension. Various studies have investigated the performance of sonication in different applications, such as pre-treatments for biomass and biological tissues, enhancement for the kinetics of gelatine hydrolysis as well as lyses of bacteria to release DNA, showing that sonication can be applied in a variety of fields (Fykse, Olsen, & Skogan, 2003; La Calle, Costas, Cabaleiro, Lavilla, & Bendicho, 2012; Yu, Zeng, Zhang, Liao, & Shi, 2016). Sonication can be performed either with ultrasonic bath or sonicator, which the former often irradiates to the samples indirectly and the latter provides irradiation directly to the samples. Ultrasonic cleaning is a technology performed using ultrasonic bath to remove contaminants in the areas that could not be cleaned effectively. The functionality of ultrasonic bath enables it to penetrate and clean surfaces that can be achieved by a sound-conducting liquid (Fuchs, 2015). In the other words, the biomass submerged in any solvents can be irradiated and reached by the sound-conducting liquid in the ultrasonic bath as well. However, if the biomass is freeze-dried, sonication using ultrasonic bath does not inflict more cell disruption as the cells were probably destroyed during freeze-drying process (Boyd et al., 2012). The microalgae pre-treatment using ultrasonic bath for lipid extraction is proven to enhance the lipid extraction yield compared to the conventional extraction, using hexane as solvent. The potentiality of sonication-assisted solvent extraction is discovered for the microalgae strains, especially *Thalassiosira* sp., which around 40% of lipid content is extracted (Neto et al., 2013).

Alternatively, sonicator often induces higher energy input compared ultrasonic bath. The irradiation distance of ultrasound from sonicator is limited to the area around the probe tip as the probe is directly submerged into the samples. Hence, this method usually applied for smaller volumes of sample preparation. As for sonicator, the cleaning of probe has to be performed every time after usage due to the probe direct contacts with the samples tested. Before any test is performed, the correct type of sonicator probe has to be selected to cater for the tested samples volume. Sonication performed using sonicator often applied for biomass pre-treatments in a short period of time. Therefore, this method has gained lot of interest from expertise in various fields. In the study of Prabakaran and Ravindran (2011), only 15 min of sonication using 50 Hz of resonance was applied and the microalgal cells were ruptured effectively (Prabakaran & Ravindran, 2011). The above statement is further supported by the work of Guldhe et al. (2014), which showed that high lipid yield was obtained through 2 min sonication with 15 kHz applied (Guldhe, Singh, Rawat, Ramluckan, & Bux, 2014). Several parameters of sonication are found to affect the yield or recovery of the endproducts, such as amplitude, pulse mode, continuous mode, irradiation time and power. The condition of sonication needs to be optimized to prevent excessive sonication towards the biomass and cause degradation/denaturation of the endproduct. In the case of Gerde et al. (2012), excessive sonication induces the formation of lipid hydroperoxides subsequently affect the quality of lipid obtained (Gerde, Montalbo-Lomboy, Yao, Grewell, & Wang, 2012). For heat sensitive compounds, a cooling jacket is required to control the temperature of samples. It is because the heat generated through

sonication possibly degrades the samples or targeted product during the experiment.

2.4.1.5 Microwave

The application of microwave includes drying, heating and cell disruption. The interaction of microwaves with polar molecules of the suspension, which is water, has led to local heating. The expansion of water molecules with high pressure induces damage to the cell membranes or cell walls, releasing the intracellular metabolites from the biomass (Günerken et al., 2015). It is stated that most of the radiation energy is absorbed by the medium rather than the water inside the cells, has resulted in the protein aggregation and denaturation (Woo, Rhee, & Park, 2000). Microwave has been widely applied for sludge treatment in recent years and claimed to be environmental friendly due to its fast heating, energy efficient and elimination of hazardous product formation as well as emissions (Tyagi & Lo, 2013). The period for anaerobic digestion of sludge is shorten if the sludge is subjected to microwave treatment beforehand, saving the long retention time and upgrading the degradation efficiency from 49% to 53% (Pino-Jelcic, Hong, & Park, 2006). The pre-treated sludge also shows higher bioavailability for biogas production compared to the controls (Eskicioglu, Droste, & Kennedy, 2007).

Factors that influence the disruption efficiency of microwave are quite similar to sonication, especially temperature is the significant factor for heat sensible components. For heat sensitive components, prolonged microwave irradiation period often not encourage to be performed to prevent denaturing of

the components. The extraction also can be performed with lower irradiation power to achieve such target. The fast heating mechanism of microwave has led to short irradiation period required to breakdown the cell wall of microalgae. Most of the optimized condition does not exceed 30 min, with lowest 1.2 kW power is applied (Balasubramanian, Allen, Kanitkar, & Boldor, 2011; Sheng, Vannela, & Rittmann, 2012). Sheng et al. (2012) have reported that 1.13-fold more of lipid was extracted compared to untreated biomass by using only 1 min of microwave irradiation and 1.4 kW (Sheng et al., 2012). The lipid extracted via pulse mode in Sheng et al. (2012) is slightly lower than the continuous mode, explaining 30s is insufficient to completely breakdown the microalgae cell wall. Similar optimized conditions for extracting lipid from *Chlorella* sp. are observed from the studies of Prabakaran and Ravindran (2011), and Zheng et al. (2011), which 5 min of irradiation with 2450 MHz at temperature of 100 ^{o}C is required to obtain higher lipid yield (Prabakaran & Ravindran, 2011; Zheng et al., 2011). Higher temperature of microwave leads to higher disruption level. Cheng et al. (2013) have showed that the wet microalgae biomass are completely disrupted (no integrated cells) around 100 ^{o}C while fine cell debris in hundred nanometers are found using higher temperature like 120 ^{o}C (J. Cheng et al., 2013). As microwave and water bath are both consist of heating element, a study by Wahidin et al. (2014) have investigated the capability of these technique in producing biodiesel through transesterification (Wahidin, Idris, & Shaleh, 2014). Even the temperature of microwave and conventional water bath-assisted solvent extraction are the same, the production of fatty acid methyl esters (FAME) using microwave is higher due to the energy carrier of microwave generate heat directly within the cells while the heat is transferred to the substrate

via convection and conduction in the conventional method (Wahidin et al., 2014).

2.4.2 Chemical Pre-treatment

2.4.2.1 Organosolv

Organosolv pre-treatment is commonly used for solubilising lignin without altering the structure of lignin and recover it as byproduct. It can be carried out by exposing the biomass to organic solvent mixtures or organic solvent and water mixtures, for example ethanol, methanol, chloroform, and acetone (Merklein, Fong, & Deng, 2016). The solvents involved in this method are required to be removed prior to bioethanol production as the solvents may limit the yield of endproducts during fermentation or enzymatic hydrolysis. Therefore, ethanol and methanol are more favourable in organosolv due to low boiling points. Mineral acid can be added as catalyst to further enhance lignin removal and reduce the process temperature during organosolv (Zabed et al., 2019). Feedstocks for biofuel production are often treated with organosolv especially for lignocellulosic biomass or woody crops. Organosolv is extremely effective for high lignin biomass such as corn stover, wheat straw and bamboo (Y.-H. Lee, Robinson, & Moo-Young, 1987; Mou & Wu, 2017; F. F. Sun et al., 2015). As most of the studies have investigated organosolv performed using alcohols, there is an interesting study performed by Sun et al. (2015) using glycerol as the organic solvent to treat wheat straw. It is reported that substrates after treated with glycerol can perform comparable hydrolyzability with substrates treated with ethanol, across various type of feedstocks (F. F. Sun et al., 2015). The major effects that can be observed through organosolv are,

hemicelluloses and lignin of biomass are solubilised and the cellulose of biomass is removed in solid form (Zabed et al., 2019). Besides, organosolv also proved to be better technique compared to autoclave for pre-treatment of microalgae biomass (Á. D. González-Delgado & Kafarov, 2012). The organosolv treated microalgae biomass is found to have positive correlated with fermentation to produce a total theorectical bioethanol yield of more than 90% (Chng, Lee, & Chan, 2017). Furthermore, the lipid extraction efficiency using organosolv is higher compared to acid hydrolysis as the microalgae biomass was disrupted at higher degree (Á. González-Delgado, García-Martínez, & Peralta-Ruíz, 2017). However, organosolv is not commercialized for large-scale production and further studies have to be performed, optimized and enhanced to treat large volume of biomass in a more economic and feasible way.

2.4.2.2 Acid treatment

In acid treatment, inorganic acids such as H_2SO_4 (commonly used), HCl, H_3PO_4, HNO_3, and organic acids like fumaric acid and maleic acid are utilized to treat the samples (Zabed et al., 2019). It can be done with either diluted acid treatment or concentrated acid treatment, with acid concentration $< 4\%$ and 70 to 77%, respectively. One of the applications is to remove chlorophyll from microalgal extract through acid precipitation. The analysis of pigments such as carotenoids can be problematic if the extract consists of another type of pigment (in a major portion), for example chlorophyll. The absorbance of the extract will be influenced by the pigments in the extract; therefore, the removal of chlorophyll is required in some cases. Fujii (2012) has studied several types of acids at different concentrations in removing chlorophyll. He has concluded that

the large portion of chlorophyll in vegetative green microalgae was successfully removed using 0.01 to 0.1 N of sulphuric acid but the target carotenoid, astaxanthin, is lost using acid concentration ranges from 0.02 to 0.1 N (Fujii, 2012). This indicates that astaxanthin is removed by the sulphuric acid in that range of concentration. Optimization of the acid concentration is hence important to achieve both chlorophyll removal and maintaining the astaxanthin in extract. The weak acid like phosphoric acid and acetic acid are not able to remove chlorophyll as the strong acids, sulphuric acid and hydrochloric acid do. In the case of cell disintegration, higher concentration of acid treatment is preferable. Acid treatment often utilized in sugar extraction for the bioethanol production using microalgae. As for the microalgae with resistant cell wall (*Scenedesmus* sp.), Miranda et al. (2012) have concluded that 2 N of sulphuric acid is required to lysis the cell at a temperature of 120 ^{o}C over 30 min. Higher concentration of sulphuric acid than 2 N leads to degradation of sugar and therefore lower sugar is yielded (Miranda, Passarinho, & Gouveia, 2012). Halim et al. (2012) also reported that the higher degree of disruption was achieved through higher operating temperature and higher acid concentration added to the biomass (Halim, Harun, Danquah, & Webley, 2012). Lastly, acid treatment also can be applied simultaneously with other pre-treatment methods like autoclave. It is reported that the optimized condition for such process required low acid concentration, 0.5 M HCl to achieve around 85% of astaxanthin extractability (Xiao et al., 2009). This study shows the flexibility of acid treatment and the enhancement of cell disruption process with other pre-treatment method.

2.4.2.3 Alkali treatment

In alkali treatment, the common reagents used in treating most of the biomass are sodium hydroxide, ammonia (aqueous, liquid, gaseous), calcium hydroxide (lime) and sodium carbonate (J. S. Kim, Lee, & Kim, 2016). For the production of biofuel, the enzyme digestibility of biomass is vital, and this property can be enhanced through alkali treatment through the former three types of reagents mentioned. Alkaline treatment has emerged several advantages, for example, most of the reagents used in this method are non-polluting. The reagents are less corrosive than the chemicals applied for acid treatment and are favourable as it can be performed under milder condition, including soak in the alkaline reagent solutions. The operating temperature for alkali treatment is varied from low temperature (25 ^{o}C) to high temperature (210 ^{o}C), at which the time taken for the process varied depending on the biomass loading as well (J. S. Kim et al., 2016; T. H. Kim, Kim, Sunwoo, & Lee, 2003; Wyman et al., 2005; L. Yang et al., 2012). The sugars and other soluble compounds are breakable if the reaction conditions (time, temperature and reagent concentration) are severe to rupture the lignin and hemicelluloses of biomass. Several studies have showed that alkaline treatment reduces the average particle size of samples effectively with small cell fragments, and less visible cell wall structure is observed (Halim et al., 2012; Miranda et al., 2012). The modification of fibre's surface is achieved through alkaline treatment by removing lignin, hemicelluloses, wax and oils on the surface of natural fibres (Ouarhim, Zari, Bouhfid, & Qaiss, 2019). It often applied in anaerobic digestion to pretreatment sludge and bioconversion of lignocellulosic biomass for bioproducts (chemicals, food and feed) and bioenergy production. By applying alkaline treatment solely on the anaerobic

digestion, it is stated that there is no effect in treating the microalgae biomass for methane production. The combination of thermal with alkali treatment in treating microalgae biomass is proved to increase methane yield by 30 to 40% for biogas production at high temperature, around 120 ^{o}C (Bohutskyi, Betenbaugh, & Bouwer, 2014). Despite the effectiveness of alkaline treatment, it is not favourable for mild microalgae biorefinery as the high operating temperature to disrupt the cell denatures the protein (Günerken et al., 2015). In fact, alkaline treatment can be utilized for microalgae pretreatment to improve the lipid extraction efficiency. Several studies have proven the isolation of free fatty acid from microalgae via direct saponification using a mixture of potassium hydroxide and ethanol (Cartens, Grima, Medina, Giménez, & González, 1996; Grima, Medina, Giménez, & González, 1996). The combination of other techniques with chemical treatments to achieve mild operating conditions maybe more suitable for the microalgae biorefinery to obtain variety of end products (Günerken et al., 2015).

2.4.2.4 Ionic liquid

Besides acid and alkaline solution, ionic liquids known as green solvent is investigated in extracting and pre-treating biomass too. Ionic liquids are molten salts that remain as liquid form below 100 ^{o}C or at room temperature due to their low melting points. They often formed by organic cations and inorganic or organic anions (Pacheco-Fernández & Pino, 2020). The unique properties of ionic liquids such as high chemical and thermal stability, low vapour pressure at room temperature and nonflammability have made them a preferable substitution for conventional organic solvents. In addition, the desired physical

and chemical properties of ionic liquids (polarity, solvent miscibility, hydrophobicity and viscosity) can be tuned through the cations, anions and substituents of the ionic liquids (Z. Yang & Pan, 2005). However, the cost of ionic liquids is comparatively higher than conventional organic solvents, which required developing the process to be cost-effective by considering its potential to be applied in a larger-scale process. Hence, the recyclability of ionic liquids is often studied to reduce their high cost in a process in practical especially for industrial applications.

The usage of ionic liquids is commonly found to pre-treat the lignocellulosic or cellulosic biomass and extract organic compounds with or without other mechanical pre-treatment techniques. Several studies have presented the biomass pre-treatment using the ionic liquid, 1-ethyl-3-methylimidazolium acetate, [Emim][Ac] with recycle study (Shill et al., 2011; Weerachanchai & Lee, 2014). It is reported that the enzymatic digestibility is reduced along with the increasing number of pre-treatment recycles (Qiu & Aita, 2013). For the extraction of compounds from microalgae, various approaches have been investigated to improve the product yield. The extraction of phycobiliproteins was performed using a system consists of ionic liquid and potassium salts, where an extraction efficiency of 99% was achieved (Chang, Show, Lan, Tsai, & Huang, 2018). The ionic liquid and water mixture also has been studied by To et al. (2018) to extract the carbohydrate and lipid from microalgae (To, Procter, Simmons, Subashchandrabose, & Atkin, 2018). It was found that the lipids and sugars were successfully recovered from the biomass (30.6% of lipids and 71% of sugars) using cheaper material cost (mixture of ionic

liquid and water) rather than pure ionic liquids. Although To et al. (2018) did not study about the recyclability of ionic liquids in the mentioned study, this method has the potential to be commercialized with more analyses of technoeconomics, sustainability, exergy, emergy and more studies on pilot and large scale process are required to validate the method. Pan et al. (2016) has presented a microwave-assisted extraction using ionic liquids to extract lipids from microalgae. The study showed that the ionic liquids not only capable to cope with the thermal heat from microwave, the microalgae biomass was dissolved by the ionic liquid and enabled the lipids to be extracted easily (J. Pan et al., 2016).

2.4.3 Biological Pre-treatment

2.4.3.1 Fungi pre-treatment

Biological pre-treatment can be categorized into microbial and enzymatic pre-treatments, where microbial pre-treatment includes fungal, bacterial, microbial consortia and ensiling. In fungal treatment, the microalgae biomass exposed to the certain type of fungal species via submerged or solid-state culture technique. It is stated that the solid loading of submerged cultivation has to be limited whereas more solid loading can be utilized for solid-state culture technique (Zabed et al., 2019). Similarly, the fungal treatment has the capability to pre-treat lignocellulosic biomass like other treatments do, in modifying the biomass structure for ease of enzyme digestion. Different type of fungi reacts differently on biomass, for example lignin can be degraded using white-rot fungi while major effects are observed on the cellulose using brown- and soft-rot fungi with minor effects on lignin (Singh, Goyal, & Moholkar,

2018). Hence, the usage of white-rot fungi is mainly applied for biofuel processing due to its high efficiency and endproducts yield. The processing period for fungal treatment is comparatively longer compared to other microbial pre-treatments, which required weeks to months to process the complete treatment.

In the study of Hom-Diaz et al. (2016), 74% higher methane was produced compared to the non pre-treated biomass using the fungal broth of white-rot fungus, *Trametes versicolor*, in pre-treating the mix culture of green microalgae (Hom-Diaz, Passos, Ferrer, Vicent, & Blánquez, 2016). Lower yield of methane (20% increased compared to non pre-treated biomass) was obtained using enzymatic (laccase) treatment compared to fungal treatment in the same study, so they have concluded that the existence of other enzymes and radicals produced by the fungal have contributed to better solubilisation of the cells. However, higher methane yield was reported by Ehimen et al. (2013) using fungal broth to pre-treat the biomass at different contact time, proving the process period is one of the significant parameters to be investigated (Ehimen, Holm-Nielsen, Poulsen, & Boelsmand, 2013). Another study has reported that the green algal biomass able to produce higher biochemical methane content using fungal solid-state fermentation as pre-treatment compared to both acid and alkaline pre-treatments (Ben Yahmed, Carrere, Marzouki, & Smaali, 2017). This pre-treatment method shows effective production of biogas from microalgae and required less energy as well as reagents consumption through these studies, with the drawbacks of long pre-treatment time which is not favourable in industries.

2.4.3.2 Bacterial pre-treatment

On the other hand, the bacterial pre-treatment can be performed through exposing the biomass to lignin degrading bacteria or hydrolytic bacteria. Bacterial pre-treatment is commonly utilised for the biogas production from microalgae and required several days for the pre-treatment. In comparison to fungi pre-treatment, bacterial is more cost effective and has higher adaptability. Besides, the growth rate of bacteria is higher than fungi; hence, the culturing time is relatively shorter (Zabed et al., 2019). However, the drawback of bacteria pre-treatment is its lignin degradability is lower than fungi pre-treatment. It is reported that the methane production rate of microalgae biomass is increased through the bacterial pre-treatment on anaerobic digestion or liquefaction (S. He et al., 2016; Kavitha, Subbulakshmi, Rajesh Banu, Gobi, & Tae Yeom, 2017). The amount of methane obtained is found to be directly proportional to the dosage of bacteria applied in the pre-treatment, indicating that more dosage of bacteria to pre-treat microalgae biomass favours the methane production. Besides, thermophilic condition is discovered to have positive effect towards bacterial pre-treatment of microalgae in producing methane production. The methane production has increased by 13% compared to control group whereas a comparable methane yield is gained using the combination of thermal and bacterial pre-treatment under thermophilic condition (Vidmar, Fanedl, Marinšek Logar, & Panjičko, 2017). However, there is no effect found under mesophilic condition. Higher temperature has assisted in rupturing the cell wall along with the hydrolytic bacteria used in the process. In lipid extraction, Chen et al. (2013) have discovered the pre-treatment of microalgae with concentrated co-culture supernatant of bacteria can improve the extraction efficiency of lipid to nearly

100% increment, showing an environmental friendly and effective route for biofuel production (C.-Y. Chen, Bai, & Chang, 2013).

2.4.3.3 Microbial consortia pre-treatment

Microbial consortia pre-treatment is pre-treating the biomass through the co-culture systems that consist of bacterial-bacterial, fungal-bacterial or fungal-fungal. The development of such technique is to overcome the problems faced with pure culture systems such as unable to be performed in an open system and long pre-treatment time is required. The time of microbial consortia pre-treatment required is similar to bacterial pre-treatment and shorter than the fungal pre-treatment. In addition, the microbial consortia culture has improved the productivity of target product via the synergistic effect through mixtures of microorganisms and has higher adaptability than the pure culture (Dashtban, Schraft, & Qin, 2009). Bacterial consortium with protease, amylase and cellulose secreting bacterial strains (*Bacillus* species) are reported to have higher degradability in microalgae biomass and resulted in more production of volatile fatty acids during anaerobic fermentation (Kavitha, Yukesh Kannah, Rajesh Banu, Kaliappan, & Johnson, 2017). The mixed bacterial culture managed to disintegrate microalgae biomass for about 26%, showing better microalgae lyses rate than microbial consortium of protease and amylase secreting bacterial, and only cellulose secreting bacteria alone. Other than microalgae biomass, microbial consortium is applied to treat various type of feedstocks. It is reported that the pre-treated microalgae using bacterial consortium able to achieve simultaneous antibiotic and organic removal in treating the saline antibiotic wastewater contained amoxicillin (X. Shi, Yeap, Huang, Chen, & Ng, 2018). An

enhancement in pre-treating sludge like pulp and paper mill sludge with microbial consortium OEM1 (originated from spent mushroom substrate) is also discovered in successfully degrading lignin and adsorble organic halides (formed during the reaction between chlorine compounds and the lignin residuals from wood fibres in bleaching process) (Lin, Liang, Zeng, Wang, & Lin, 2017). The performance of anaerobic digestion for methane production depends on the pH of the system and the volume of pre-treated feedstocks with microbial consortium OEM1. The microbial consortia pre-treatment has showed viable applications through these studies with less energy demand process yet effective to achieve targeted product yield.

2.4.3.4 Enzymatic pre-treatment

Degradation of biomass through application of enzymes for pre-treatment is known to have minimal toxic product formed and perform under mild conditions. This technique is often performed prior to ethanol fermentation to release the fermentable sugars from feedstocks available. Compared to chemical pre-treatments, enzymatic pre-treatment is more favourable as no corrosion and caustic chemicals used in starch processing industries (S. P. Choi et al., 2010). Type of enzymes applied in this method includes protease, amylase, amyloglucosidase and cellulase (S. P. Choi et al., 2010; O. K. Lee et al., 2013). One of the enzymes, amyloglucosidase (AMG 300L) is genetically modified from the strain of fungus (*Aspergillus*) for their improved activity to utilise in liquefaction and saccharification. It is even proved that AMG 300L-catalyzed saccharification able to produce 42% yield of reducing sugar without any pre-treatment from the microalgae biomass residual (after lipid extraction), and a

total 80.9% (w/w) saccharification yield is obtained based on the total carbohydrates of the residual (O. K. Lee et al., 2013). Besides, a study shows that the methane yield from microalgae can be enhanced for 5 to 6.3-fold through the protease pre-treated microalgae compared to raw biomass (Mahdy, Ballesteros, & González-Fernández, 2016). The pre-treatment using solely one type of enzyme seems to have lower methane yield compared to a combined enzyme mixture. Ehimen et al. (2013) have investigated the comparison of individual enzyme and combined enzyme mixture on the anaerobic digestion of algae, showing an improved methane yield by more than 20% due to the hydrolysis of different biomass macromolecules through the synergistic effect of enzymes (Ehimen et al., 2013). Despite the improved methane production, shorter digestion time is observed by using the combined enzyme mixture in the pre-treatment. The work of Passos et al. (2016) is in agreement with the previous study, where a combined enzyme culture composed by cellulose, glucohydrolase and xylanase has increased the methane yield by 15% (Passos, Hom-Diaz, Blanquez, Vicent, & Ferrer, 2016). Therefore, the enzymatic pre-treatment is preferred to be performed using mixture of enzymes for algae biomass.

2.5 Extraction Techniques

In downstream processing, the extraction techniques have always been a concern to extract biomolecules or bioproducts from natural sources. The extraction of biomolecules from microalgae has recently emerged as potential alternative source due to the rapid growth rate, short cultivation time and low land usage compared to other type of agricultural biomass. The extraction of biomolecules is performed using various extraction techniques to overcome the

resistant microalgae cell wall in releasing them from microalgae. Conventional techniques such as solvent extraction and Soxhlet extraction are studied more for lipid extraction from microalgae instead of proteins and phycobiliproteins (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013; J.-Y. Lee et al., 2010; Ryckebosch, Muylaert, & Foubert, 2012). Besides from the intracellular compounds in microalgae, these techniques also are investigated for extraction of acrylamide from potato (Pedersen & Olsson, 2003), vanillin from vanilla pods (Jadhav, B.N, Gogate, & Rathod, 2009), butanols from fermentation (Groot et al., 1990) and glycotic enzymes from bakers' yeast (Johansson & Andersson, 1984). Although these techniques have been studied since 1879's and early 1970's (Soxhlet and solvent extraction, respectively), the problem faced using these techniques for downstream processing is yet to be overcome (Jensen, 2007; Su, Xia, Wang, & Xiao, 2017). Difficulties such as long processing time, requirement of toxic solvents, insufficient efficiency for extraction and possibly denature biomolecules like proteins, have shown that the discovery of alternative extraction methods is in desperate need. Therefore, emerging extraction techniques such as liquid biphasic system and three phase partitioning are introduced as potential solutions for downstream processing of biomolecules. The conventional and emerging extraction techniques are both evaluated and discussed in the following sections.

2.5.1 Conventional Extraction

2.5.1.1 Solvent extraction

Solvent extraction often involved two immiscible solvents, usually one polar (water) and another is non-polar (organic) solvent. Organic solvents are

usually lighter than water, so the upper phase is organic solvents and water usually is the bottom phase, but it is possible to have the opposite situation. In solvent extraction, the compounds or solutes firstly dissolved in one of the liquids will eventually separated to the respective phase (either polar or non-polar phase) based on their solubilities to the respective liquids. Variations in solubilities towards the solvents are caused by the strength of interaction of compounds with the solvents, leads to the uneven distribution of the compounds between two types of solvent (Rydberg, 2004). The schematic diagram of solvent extraction is shown in **Figure 2.1**. This extraction technique is widely applied for the recovery and separation of metal ions from aqueous solutions (Tachimori, Suzuki, Sasaki, & Apichaibukol, 2003; Tavlarides, Bae, & Lee, 1987). Ion-exchange is applied for rare earth metals extraction as well, but solvent extraction is accepted as the most suitable commercial technology to deal with larger volumes of dilute solvents. Therefore, ion-exchange is only applied to produce high purity of rare earth metals product in small quantities nowadays (F. Xie, Zhang, Dreisinger, & Doyle, 2014).

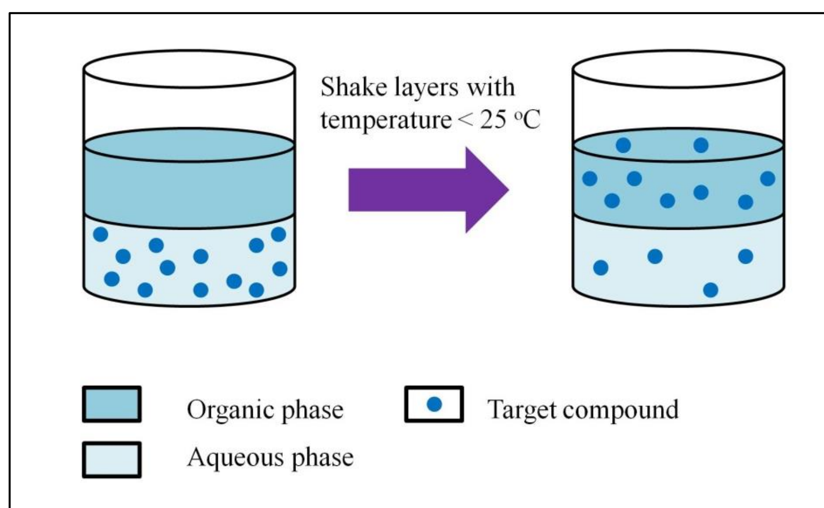


Figure 2.1: Schematic diagram of solvent extraction (C. Shi et al., 2017)

Several key parameters often influence the extraction efficiency of targeted compounds, such as type of solvents, ratio of solvent/sample, processing time and processing temperature. The selection of solvents used is extremely important and decided based on the selectivity and solubility of targeted compounds towards the solvents used. It is reported that the nearer values of polarity between the compounds and solvents can perform better extraction (Q.-W. Zhang, Lin, & Ye, 2018). Common type of solvents utilised in solvent extraction is alcohols especially methanol and ethanol. In the study of Oufnac et al. (2007), the extraction of antioxidants using three types of solvent were investigated. Methanol was found to be more effective than acetone and hexane in extracting total phenolic compound from wheat bran (Oufnac et al., 2007). Other studies also investigate the extractability of ethanol and water mixture for total phenolic compounds from leaves and fruit, reporting the high yield of phenolics obtained using alcoholic solvents (Dahmoune et al., 2014; Spigno, Tramelli, & De Faveri, 2007). Although ethanol can be used to extract phenolics, it is reported that ethanol does not show high selectivity for phenolics

and the mixture of water and ethanol shows better performance compared to solely ethanol as extractant (Spigno et al., 2007). The usage of single solvent is not sufficient to extract the desired compounds with relatively high activities possessed in them. The study of Jayaprakasha et al. (2001) has reported higher antioxidant activities (around 20 to 23% more) were gained using mixture of ethyl acetate and water compared to using single solvent such as methanol and acetone (Jayaprakasha, Singh, & Sakariah, 2001). It is further supported by Spigno and Faveri (2007) as higher purity of extracts can be obtained using mixture of ethyl acetate and water compared to ethanol (Spigno & De Faveri, 2007).

Besides, the optimization of solvents to water ratio is found to be essential as the selectivity for the extraction of targeted compound can be improved with appropriate ratio applied during the extraction. It is reported that the moderate ratio of ethyl acetate and water (17:3) has obtained higher antioxidants activity compared to higher (9:1) and lower (4:1) ratio of solvent to water (Jayaprakasha et al., 2001). The work of Pan et al. (2003) also showed that the extraction of phenolics compounds performed better using the ratio of ethanol to water lower than 50% (v/v) and decrement of extraction efficiency is observed using ratio more than 50% (v/v) (X. Pan, Niu, & Liu, 2003). Not only the ratio of solvents to water, the sample to solvent ratio needs to be considered as well. Higher amount of sample does not increase the yield of targeted compounds, in fact promotes the decrement of yield due to low surface contact of samples and solvents (Pinelo, Fabbro, Manzocco, Nuñez, & Nicoli, 2005). This statement is supported by the work of Galan et al. (2017), at which highest

recovery of phenolics is obtained using medium ratio of sample to solvent ratio (1:20) and started to decrease using ratio higher or lower (1:15 and 1:30, respectively) than the optimum ratio (Galan et al., 2017). The optimum ratio obtained in past works varies due to the difference in operation and the sample source like stalk or leaves from a plant. Hence, the comparison of sample source under similar experiment operation can be performed to examine the influence of sample to solvent ratio towards the extraction efficiency.

On the other hand, some studies claimed that processing time of the solvent extraction does influence the yield of targeted compounds. Lapornik et al. (2005) have stated that the yield of polyphenols increased with extraction time while Pekić et al. (1998) reported the yield of proanthocyanidins increasing slowly after 8 h as shown in the second part of the kinetics curves (Lapornik, Prošek, & Golc Wondra, 2005; Pekić, Kovač, Alonso, & Revilla, 1998). However, the findings are not in agreement with the Spigno and Faveri (2007) that they have concluded time does not influence the yield of extracts in solvent extraction (Spigno & De Faveri, 2007). More studies have to be performed to validate the findings of past work. In the search of other works, the solvent extraction is commonly performed in the time of less than 1 h to 48 h. The extraction temperature is crucial for the extraction of heat sensible compounds such as polyphenols and proteins. Higher temperature enhances the mass transfer of compounds to the solvents and leads to higher yield, but low purity possibly be obtained as the diffusion coefficient and solubility of other compounds are increased as well (Spigno & De Faveri, 2007). It is reported that the yield of oil from olive cake increased with higher temperature and the viscosity of oil is

reduced by the high temperature set (Amarni & Kadi, 2010). The extraction of phenolic compounds also showed better antioxidant activities using water at boiling temperature compared to room temperature (A. Sousa, Ferreira, Barros, Bento, & Pereira, 2008). This method consumes low amount of energy and easy to be operated in continuous mode despite the high amount of toxic solvents required. The current technology also combines solvent extraction with other technologies such as reverse micelles and supercritical fluid for the development of genetic engineering techniques (H. Chen & Wang, 2017).

2.5.1.2 Soxhlet extraction

Soxhlet extraction is a type of solid-liquid extraction that performed using the Soxhlet extractor. Initially, the conventional Soxhlet extraction is created to determine the fats in milk (Luque de Castro & García-Ayuso, 1998). The condensed fresh solvent is placed in the distillation flask and the thimble-holder is used to hold the sample. During the extraction, the sample is gradually filled with the condensed solvent until the liquid overflows. The siphon is then aspirates the solutes of thimble-holder and unloads into the distillation flask, which carries the extracted analytes into the bulk liquid. This process is repeated until the extraction is completed (Luque de Castro & García-Ayuso, 1998). The repeatedly contact between sample and fresh condensed fresh solvent during the extraction has helped in the displacement of the transfer equilibrium while the continuous heat supplied to the system maintains the temperature of system. As the system does not contain agitation device to accelerate the process, large amount of solvents is required for the evaporation or concentration process. The long processing time also cause in the thermal disintegration of targeted

compounds due to the exposure of samples at boiling point for a long period (Zaidel et al., 2019).

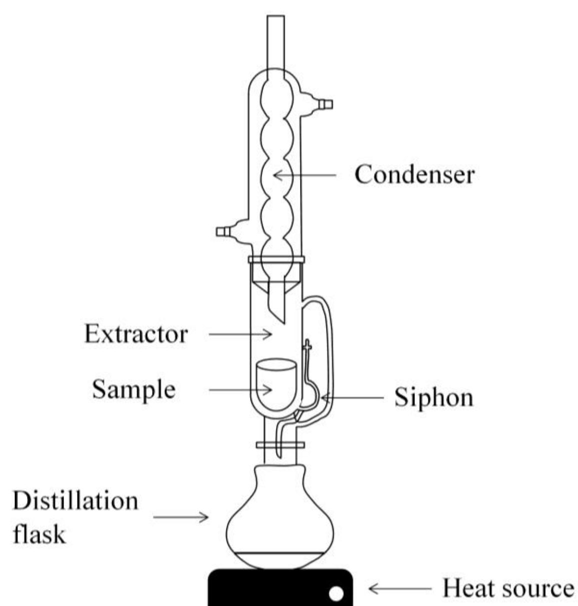


Figure 2.2: Conventional Soxhlet extractor (Luque de Castro & Priego-Capote, 2010)

In Soxhlet extraction, the selection of extracting solvent is extremely important as the extracts and extract's composition is affected by the type of solvents. Alcoholic solvents such as ethanol and methanol often utilised as extractants for biomolecules due to low boiling point and easy to be evaporated. Furthermore, ethanol is soluble with water and other organic solvents making it as multiuse liquid in all type of extractions while methanol can be used to extract both polar and non-polar compounds (Zaidel et al., 2019). The study of Silva et al. (2017) showed that the extracts obtained from Soxhlet extraction using ethanol as extractant consisted of various lipid fractions, for example myristic, palmitic, stearic and oleic that have wide industrial applications (de Sousa e Silva et al., 2017). The methanol extracts of the plant, *Arnebia benthamii*, obtained

using Soxhlet extraction (60–80 ºC) also proved to contain excellent antioxidant and antibacterial activities (Ganie et al., 2012). Other type of solvents such as acetone and hexane also studied as the extractant of Soxhlet extraction, especially hexane is reported as the most common solvent to extract edible oil from plant (Dicko et al., 2016; Zaidel et al., 2019). If the targeted compound is polar compound such as mangiferin, it can even be extracted using water as the solvent (Vrushali M Kulkarni & Rathod, 2014). Single type of solvent applied to extract compounds are commonly studied in past literatures, and the study of extractive solvents involved more than one type of solvent is not commonly found. Chloroform and hexane have known to be effective solvents for lipid extractions, and Ramluckan et al. (2014) also reported that these solvents able to extract >10% lipids extract from algal biomass. They also concluded that the binary mixtures of chloroform and ethanol as extractive solvents able to improve the extractive efficiency at the ratio of 1:1 (Ramluckan, Moodley, & Bux, 2014). However, it is reported that methanol is the most suitable solvent for anthocyanins and phenolic compounds extraction compared to the binary or ternary solvents mixtures of ethanol, methanol and acetone (Vega et al., 2017). Properties of solvent alters as they are mixed as extractive solvents which influence the solubility and mass transfer of targeted compounds to the solvents. Therefore, choosing the suitable solvent type as extractive solvent is very important as the affinity between targeted compounds and the solvent polarity plays a role during the extraction as well.

Alternatively, the ratio of sample to solvent can pose effect in recovering the compounds from main source to the solvent. The mass transfer from the

sample to solvent generally depends on the gradient of solutes concentration between samples and solvent, where the compounds will be extracted to the medium containing less solute. It is reported that the optimum yield of phenolic compounds is extracted using 1:20 g/ mL of solid to solvent ratio (Alara et al., 2018). Some studies have fixed higher ratio of sample to solvent, such as 1:50 or 1:75 in g/ mL to compare with other types of extraction on similar targeted compounds (Bimakr et al., 2011; Hawthorne, Grabanski, Martin, & Miller, 2000). Extraction time is an important parameter in Soxhlet extraction especially in the extraction of heat sensitive compounds. Prolonged extraction time representing possibility of degradation occurrence which is not desirable. It is reported that the recovery of phenolic compounds from bitter leaves decreases for extraction time more than 2 h although increasing the extraction period does enhance the recovery of targeted compounds (Alara et al., 2018). Zhang et al. (2018) have claimed that longer processing time does not allow higher recovery after the equilibrium state of solute was achieve in both solvent and sample (Q.-W. Zhang et al., 2018). The extraction temperature has to be controlled well to avoid degradation of compounds for effective extraction, which is recommended to control the temperature lower than 70 ^{o}C (Chin, Chong, Markus, & Wong, 2013). Except the heat sensitive compounds, substances like lipid which is not sensitive to high temperature has found to increase along with higher temperature. Yet, similar observation was obtained (decreasing yield) after a certain temperature point is reached (optimum extraction temperature). Efthymiopoulos et al. (2018) has concluded that the optimum extraction temperature is related to the type of solvent, as similar optimum extraction temperature was observed in the oil extraction ratio using n-hexane and iso-

hexane as the extractive solvents (Efthymiopoulos et al., 2018). The identical solvent properties of these solvents are the reason to obtained similar oil extraction ratio, while the octane requires higher extraction temperature for maximum extraction ratio (Efthymiopoulos et al., 2018).

2.5.2 Emerging Extraction Techniques

2.5.2.1 Liquid Biphasic System

Liquid biphasic system (LBS) also known as aqueous two phase system consist two types of solvents as phase forming components in a system. Different combination of phase forming components can be applied for separation and recovery of biological components (Chia et al., 2019; S. Y. Lee, Khoiroh, Ooi, Ling, & Show, 2017). Basically, the solvents used for LBS are miscible to each other, but they become immiscible above a particular concentration and temperature which forms two phases in the system. The properties to perform LBS can be obtained through phase diagram containing binodal curve for every specific combination (Raja, Murty, Thivaharan, Rajasekar, & Ramesh, 2011). For example, in the combination of alcohol/salt, the amount of salt (in w/w%) and alcohol (in w/w%) above the binodal curve will lead to formation of LBS; while the amount of salt and alcohol in the system lower than the binodal curve will form a single phase. The targeted compounds will be extracted to one of the phases in the system depending on the properties of both phase forming components and targeted compounds. Besides the combination of alcohol/salt which helps in extracting targeted compounds through salting-out effect, the combination of solvent/sugar assists in biological compounds extraction through sugaring-out effect. Several types of solvents used with sugar solution are

acetonitrile, ethanol and propylene to form two phases (Dai, Liu, & Xiu, 2015; Tubtimdee & Shotipruk, 2011). Top phase often consists of solvents while the bottom phase is the sugar-rich phase. The phase separation occurs due to the sugar solution added into the system which formed strong hydrogen bonding with water and subsequently pushed the other phase components (solvents) to the top phase; while the sugars dissolve in water and remain as bottom phase (Tubtimdee & Shotipruk, 2011). Sugaring-out can be performed under room temperature which is similar with salting-out. Various studies are performed, and different components are extracted through sugaring-out such as succinic acid (Yaqin Sun, Zhang, Zheng, Yan, & Xiu, 2019), syringic acid, ferulic acid (Bin Wang, Ezejias, Feng, & Blaschek, 2008), lactic acid (Yan, Sun, & Xiu, 2016) and phenolics compound (Tubtimdee & Shotipruk, 2011). This approach is said to be versatile as salting-out with the capability to overcome some issues faced in separation performed using salting-out (Dhamole, Mahajan, & Feng, 2010b). The advantages are the usage of sugars (monosaccharides or disaccharides) does not alter the pH value of the environment, prevent the corrosion of equipment and do not react with components of the system; hence, no unwanted reactions occur (Dhamole, Mahajan, & Feng, 2010a; Dhamole et al., 2010b).

Unlike conventional extraction techniques, LBS provide a suitable environment for biological products not to degrade or denature during the extraction and purification process. Biological products such as proteins, lipids and carotenoids can be successfully extracted using LBS (Chia et al., 2019; Leong, Ooi, et al., 2019). The high water content and low interfacial tension of the systems are the factors to protect the biological products and ensure their

stability. Parameters such as selection of top and bottom phase, volume ratio, tie-line length and temperature require optimization to obtain maximize recovery and purity of the targeted products (Babu, Rastogi, & Raghavarao, 2008; Pei, Wang, Wu, Xuan, & Lu, 2009). In general, most of the biological products are extracted to the top phase of system, like polymer phase, alcohol phase and ionic liquid (top phase) rather than salt phase (bottom phase). **Table 2.7** has showed the properties of different top phase forming components utilized in the system with salt solution as bottom phase. It is reported that bromelain (a type of enzyme) from pineapple and proteins (bovine albumin serum and trypsin) favour top phase such as polymer and ionic liquid while using salt as the bottom phase in system (Babu et al., 2008; Pei et al., 2009). Study of Chen et al. (2014) has utilized thermoseparating polymer, poly(ethylene glycol-ran-propylene glycol) (EOPO) in separating ciprofloxacin at which the phase component has recycled twice and still obtaining recovery around 103% in third recycling (B. Chen et al., 2014). The recycling of EOPO requires heating and stirring to recover from the solution as EOPO can be separated from solution if it is heated above cloud point (50 °C) (H. S. Ng et al., 2012). Hence, the operating temperature to recover EOPO usually more than 50 °C, is not encouraging for heat-sensitive compounds to avoid degradation.

Table 2.7: Properties of top phase forming components.

Component	Properties	Reference
Polymer (PEG)	• Separable through heating • Temperature required for separation depends on PEG molecular weight (95 ~ 180 ^{o}C) • Not economical compared to EOPO	(Pereira, Wu, Venâncio, & Teixeira, 2003)
Thermoseparating polymer (EOPO)	• Separable from solution via heating • Enabling reuse of both phases in next cycle (itself and salt) • Lower temperature required to separate from solution compared to PEG	(P. L. Show, Tan, Shamsul Anuar, et al., 2012)
Alcohol	• Inexpensive compared to polymers and copolymers • Low viscosity and settling time • Can be recovered through rotary evaporator	(Iqbal et al., 2016)
Ionic liquids	• Difficult to prepare and expensive • Green solvent with stable thermal and chemical properties • Fine-tuning properties available • Strong solubility power	(Pei et al., 2009)

Besides the type of phase components, the concentration of phase components which is related to the tie-line length of the system is one of the factors in the final recovery and purity of targeted compounds obtained. This parameter usually studied for the phase components such as polymer, alcohol, salt and sugar, while less attention was given for ionic liquid as phase component. Increment of salt concentration (bottom phase) leads to increasing partition coefficient of targeted compounds due to salting-out effect, but lower purity is obtained due to more proteins are partitioned to the top phase (Babu et al., 2008). Similar phenomenon was observed by Li et al. (2016), higher salt concentration increases extraction efficiency but immediately decreases as the salt concentration used is higher than optimum point (N. Li et al., 2016). Although the concentration of ionic liquid is not study, some studies have reported the volume of ionic liquid does affect on the performance of the system. The recovery is higher as volume of ionic liquid increases, but at certain point the recovery of targeted compounds slightly decreases or remains unchanged (M. Shao et al., 2014). Other than the parameters related to the properties of system, the crude load of sample influences the final results too. It is stated that the composition of the system might change due to the amount of sample loaded to the system which is supported by the study of Ng et al. (2012) (H. S. Ng et al., 2012). The results showed decrement of enzymes yield and selectivity, from 87 to 81% and 3.19 to 1.4, respectively, showing the partitioning behaviour was altered as the crude load is increased.

2.5.2.2 Liquid Biphasic Flotation

Liquid biphasic flotation (LBF) also known as aqueous two phase flotation, is an enhanced version of LBS technique which consists of the mechanisms of LBS and solvent sublation (Phong et al., 2017). This technique utilised two type of solvent with the addition of gas bubbles from bottom of the system to extract biomolecules from their main source. As the biomolecules are extracted to the top phase through salting-out effect via the mechanisms of LBS, more targeted biomolecules can be extracted through LBF with the assistance of gas bubbles. Targeted compounds are attached on the surface of gas bubbles and released after the gas bubbles collapse at the upper phase (Mathiazakan et al., 2016). Therefore, more targeted compounds will be accumulated in the upper phase of the system apart from those extracted through salting-out effect.

With the mechanisms of both LBS and solvent sublation, the LBF has inherent both the advantages of these techniques. The recovery of biological components can be obtained with higher concentration using this technique while multiple unit operations are performed in single step including concentration, separation and extractions (Phong et al., 2017). Other advantages such as low environmental impact, simple operation, high separation efficiency and recyclable phase components in convenient way are offered in this technique (P. L. Show et al., 2013). As LBF consists of the advantages of both LBS and solvent sublation, it has to be more efficient than these techniques theoretically. Comparison of these techniques on separating and concentrating penicillin G has been performed and LBF showed better separation efficiency (95%) and concentration coefficient compared to other techniques (Bi, Li, & Dong, 2009).

Another advantage is lower dosage of PEG was utilized in the study, only 7.5 mL of PEG 1000 in 15 mL of PEG solution are required to achieve such result.

The phase components utilized in LBF can be formed by those are mentioned in **Section 5.2.1**. Apart from salt and polymer as bottom phase, sugar such as glucose can be utilized to form biphasic with acetonitrile. It is reported that high concentration of sugars and low temperature allows better sugaring-out effect (Dhamole et al., 2010a). Key parameters affecting LBF are more likely similar with parameters mentioned in **Section 5.2.1**, with the addition of two more parameters such as flotation rate and flotation time. Flotation rate has to be studied as higher rate of gas bubbles are supplied, more compounds are possibly to be carried towards the upper phase; while low recovery of targeted compounds can be occurred due to low flotation rate supplies to the system. The optimum flotation rate has to be investigated as extremely high flotation rate will result in turbulent mixing at the interface of top and bottom phase (Bi, Dong, & Yuan, 2010). The performance of LBF will be affected in the way that accumulation of gas bubbles in the top phase and leads to re-dissolution of targeted compounds in bottom phase (Watcharasing, Kongkowit, & Chavadej, 2009). Optimum flotation rate of 30 mL/min was chosen for nitrogen flow in study of Show et al. (2011) while 10 mL/min was chosen for air flow in study of Ng et al. (2020). The previous study utilized EOPO/salt for lipase while the later applied combinations of alcohol/salt for extraction of membrane protein (H.-S. Ng et al., 2020; P. L. Show et al., 2013). The weight of polymer is denser than alcohol; therefore, it affects the optimum flow rate in obtaining best recovery of protein.

In addition, the flotation time desired can be adjusted according to the flotation rate, which shorter flotation time is required for higher flotation rate to obtain optimum recovery of targeted compounds. Longer flotation time represents more gas bubbles are supplies into the system. The flotation time for system using polymer as top phase are investigated for more than 60 min and the optimum time varies in each study. It is reported that the selectivity, purification fold and separation efficiency were in equilibrium after 60 min of flotation time and they increased along with the increasing of flotation time before 60 min (P. L. Show et al., 2013). In the study of Bi et al. (2009), the separation efficiency of penicillin G was found to be in equilibrium at 40 min of flotation time (Bi et al., 2009). For alcohol as top phase, the optimum flotation time is much shorter than polymer, where 15 min is required to obtain maximal betacyanins concentration, separation efficiency and partition coefficient (Leong et al., 2018). In term of reducing the usage of chemicals, recycling studies are performed to study the recyclability of the system for various combinations. Show et al. (2013) has reported that no significant difference for the end results (yield, separation efficiency, selectivity and purification fold of lipase) by using fresh and recycled chemicals in the system (P. L. Show et al., 2013). The upper phase component, EOPO copolymer in the system for the mentioned study is recovered up to 75% through thermoseparation, giving benefits in cost reduction as well as protecting environment through reducing the dosage of fresh organic solvents for extraction. Combination of alcohol and salt phase can be recycled as well. Most of the alcohol is separated from the targeted product and salt phase through rotary evaporator and fresh alcohol is added to the recycled alcohol for next cycle of extraction as the volume of alcohol decreases for each cycle of

extraction. It is found that the recovery yield and proportion of protein has increased by around 11% and 32% compared to the first extraction (Phong et al., 2017). This indicates that recycling phase components helps to reduce the dosage of fresh chemicals, while improving the extraction efficiency too.

2.5.2.3 Three Phase Partitioning

Three phase partitioning is an emerging bioseparation process performed for extraction and purification of proteins. It utilizes tert-butanol (T-butanol) and the addition of crude extraction to ammonium sulphate to form three phases after centrifugation, which consists of different components in all phases. The precipitation of proteins are often observed at the intermediate phase, top phase (T-butanol phase) will be rich with lipids, pigments and enzyme inhibitors while the bottom phase (salt phase) consists of polar compounds like saccharides (Wati, Theppakorn, Benjakul, & Rawdkuen, 2009). This technique is believed to be scalable easily and crude extract can be inserted directly in the system (Özer, Akardere, Çelem, & Önal, 2010). It is simple to operate, require short processing time (around 1 h) and more convenient compared to solvent extraction for lipid extraction (Shah, Sharma, & Gupta, 2004). All types of metabolites can be extracted using three phase partitioning as listed in **Table 2.8**. Key parameters of this technique are discussed as followed. Saturation of ammonium sulphate affects the partitioning of targeted compounds from the crude extract in the system. Generally, the saturation of ammonium sulphate is investigated starting with the lowest range of 20% to 50% or 60% (Akardere, Özer, Çelem, & Önal, 2010; Chaiwut, Pintathong, & Rawdkuen, 2010; Özer et al., 2010). Higher salt saturation can result in more recovery in the intermediate

phase. However, it is reported that the purification of targeted compound may decreases along with the increasing salt saturation (Narayan, Madhusudhan, & Raghavarao, 2008). One of the studies in **Table 2.8** has added more salt to the bottom phase of first step of three phase partitioning as the proteases obtained in the bottom phase (salt phase) is in the form of aqueous phase. The increment of salt has increased the yield and purification fold of the proteases (Chaiwut et al., 2010). Akardere et al. (2010) have reported that the recovery of invertase from Baker's yeast favours aqueous phase (bottom phase) and has the highest activity recovery in the mentioned phase (Akardere et al., 2010). However, this statement is not in agreement with Özer et al. (2010), claiming the 190% activity recovery of invertase in a single step (Özer et al., 2010). These two studies have obtained similar optimum salt saturation, 50% w/v with different sources of invertase and optimum pH values. 50% salt saturation is suitable to apply in extracting peroxidase from orange peels as well as stated by Vetal and Rathod (2015) (Vetal & Rathod, 2015).

Table 2.8: Metabolites extracted using Three Phase Partitioning.

Source	Metabolites	Saturation of $(NH_4)_2SO_4$	Ratio (T-butanol to crude extract)	Temperature	pH	Yield/ recovery	Reference
Fungus, *Aspergillus niger*	Inulinase	30% w/v	1:0.5 (v/v)	25 °C	4	88%	(Vinoth Kumar et al., 2011)
Papaya peels	Proteases	20% (1st cycle), increase to 55% (2nd cycle)	1:0.5	-	-	89.4%	(Chaiwut et al., 2010)
Leaves of *Ipomoea palmata*	Peroxidase	30%	1:1	37 °C	-	81%	(Narayan et al., 2008)
Baker's yeast (*Saccharomyces cerevisiae*)	Invertase	50% w/v	1:2	25 °C	4	363%	(Akardere et al., 2010)
Tomato	Invertase	50% w/v	1:1	25 °C	4.5	190% activity recovery	(Özer et al., 2010)
Pepino (*Solanum muricatum*)	α- galactosidase	50% w/v	1:1.5 (crude:t-butanol)	25 °C	5.25	127% activity recovery	(Şen, Eryılmaz, Bayraktar, & Önal, 2011)
Viscera of giant catfish	Proteases	50%	1:2	-	-	163% protease recovery	(Rawdkuen, Vanabun, & Benjakul, 2012)

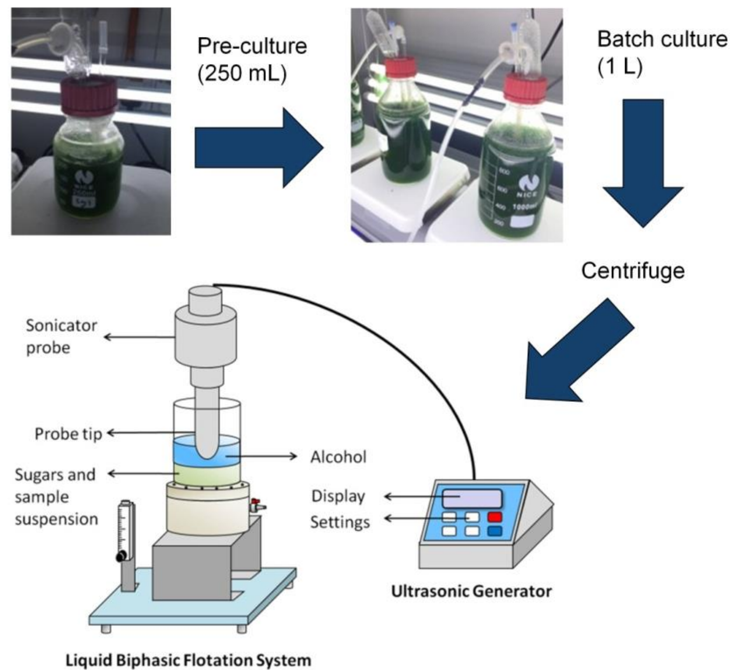
Table 2.8 (Continue)

Source	**Metabolites**	**Saturation of $(NH_4)_2SO_4$**	**Ratio (T-butanol to crude extract)**	**Temperature**	**pH**	**Yield/ recovery**	**Reference**
Wheat germ	Protein inhibitor	30% w/v	1:1	20 ºC	-	85% activity recovery	(A. Sharma & Gupta, 2001)
Orange peels (*Citrus sinenses*)	Peroxidase	50% w/v	1:1.5 (v/v) (broth:t-butanol)	30 ºC	6	93.96%	(Vetal & Rathod, 2015)
Potato crude extract	Catalase	40% w/v	1:1 (v/v)	-	-	262%	(Duman & Kaya, 2013)

On the other hand, the ratio of t-butanol to crude extract (or crude extract to t-butanol) is significant in managing the partitioning of compounds to the selective phase as well. This is because less t-butanol does not synergise with the salt solution and higher amount of t-butanol than optimum ratio can possibly lead to protein denaturation (A. Sharma & Gupta, 2001). Some studies have showed that the increment of t-butanol volume has resulted in the decrement of purification fold of targeted compounds (Akardere et al., 2010; Chaiwut et al., 2010), while a study has showed increasing trend until the optimum ratio is achieved and decreasing trend with higher ratio (Özer et al., 2010). Besides, the pH of system strongly affected the partitioning behaviour of protein as the salting-out of protein is depending on the net charge of proteins as well. The activity recovery of invertase from tomato is the highest at pH 5 and purification fold at pH 4.5, indicating most of the contaminant proteins in aqueous phase while at pH 3, 6 and 7, the comparatively low activity recovery and purification fold has showed the precipitation of invertase with other contaminants (Özer et al., 2010). Akardere et al. (2010) have presented different optimum pH value for purification fold and similar ones for activity recovery compared to Özer et al. (2010), stating that maximal purification fold was obtained at pH 4 while highest activity recovery was gained at pH 5 (Akardere et al., 2010). For peroxidase, the optimum pH seems to be pH 6 as both the maximal activity recovery and purification fold are obtained at this particular value (Vetal & Rathod, 2015). Since enzymes are heat sensitive and possibly degrades under high temperature, the effect of temperature is investigated in some studies towards the recovery and purity of targeted products. A temperature range from 4 ºC to 37 ºC in partitioning protein inhibitor showed that 4 ºC and 37 ºC are not suitable to be

performed and the system works best at 20 ºC (A. Sharma & Gupta, 2001). Most of the studies in **Table 2.8** reported that 25 ºC was the optimum temperature for the metabolites despite some studies reported other operating temperature may works best for their systems. Although the speed of centrifugation was not investigated as one of the parameters in most of the studies, each study has applied different centrifugation speed and time for their samples. The agitation speed for three phase formation is believed to enhance the mass transfer rate between the crude extract to one of the phases. Higher agitation rate has increased the activity recovery and purity of targeted compounds. Significant improvement can be observed from 100 rpm to 300 rpm but little increment is found after 300 rpm for a total agitation time of 80 min (Vetal & Rathod, 2015). Compare to study of Vetal and Rathod (2015), some studies have applied much higher agitation speed, such as 4000 rpm and 12000 *x*g for much shorter agitation period for the phase separation (Akardere et al., 2010; A. Sharma & Gupta, 2001). Therefore, the shorter agitation time requires higher agitation speed for the formation of three phases.

CHAPTER 3 ISOLATION OF PROTEIN FROM *CHLORELLA SOROKINIANA* CY1 USING LIQUID BIPHASIC FLOTATION ASSISTED WITH SONICATION THROUGH SUGARING-OUT EFFECT



This chapter covers application of ultrasound probe in liquid biphasic flotation to extract and isolate protein from microalgae biomass. Isolation of protein using wet microalgae biomass through sugaring-out effect has been studied. Effects of sonication and liquid biphasic system on the protein yield and separation efficiency were investigated and optimum condition was identified. This chapter consists of thesis-version of work published in the Journal of Oceanology and Limnology (Chia S.R. et al., 2019, *Journal of Oceanology and Limnology*, 37: 898-908).

3.1 Abstract

Microalgae, a sustainable source of multi beneficial components has been discovered and could be utilised in pharmaceutical, bioenergy and food applications. This study aims to investigate the sugaring-out effect on the recovery of protein from wet green microalga, *Chlorella sorokiniana CY-1* which was assisted with sonication. A comparison of monosaccharides and disaccharides as one of the phase-forming constituents shows that the monosaccharides, glucose was the most suitable sugar in forming the phases with acetonitrile to enhance the production of protein (52% of protein). The protein productivity of microalgae was found to be significantly influenced by the volume ratio of both phases, as the yield of protein increased to 77%. The interval time between the sonication as well as the sonication modes were influencing the protein productivity as well. The optimum protein productivity was obtained with 10 s of resting time in between sonication. Pulse mode of sonication was suitable to break down the cell wall of microalgae compared to continuous mode as a lower protein yield was obtained with the application of continuous mode. The optimum condition for protein extraction were found as followed: 200 g/L glucose as bottom phase with volume ratio of 1:1.25, 10 s of resting time for ultrasonication, 5 s of ultrasonication in pulse mode and 0.25 g of biomass weight. The high yield of protein about 81% could be obtained from microalgae which demonstrates the potential of this source and expected to play an important role in the future.

Keywords

Sugaring-out; *Chlorella sorokiniana* CY1; Liquid Biphasic Flotation; Sonication; Microalgae; Extraction

3.2 Introduction

Microalgae are being studied intensively as a potential replacement of feedstock for the next generation in the bioenergy production (Chia et al., 2018a). The carbohydrates and lipid content within most of the microalgae species are the major constituents to produce biofuels through transesterification, biochemical conversion and thermochemical conversion (Chia et al., 2018; Ho et al., 2017; Xia, Wan, Li, Sang, & Zhang, 2013), while other constituents such as pigments, vitamins, polyphenols and polyunsaturated fatty acids are the high valuable compounds that are beneficial to mankind and animal health (Chew et al., 2017; Ma et al., 2011; H. Yang, Zeng, Dong, Liu, & Li, 2010) with biological activities such as antitumor, antimicrobial, anti-diabetes etc. (Guo, Li, Li, Shi, & Han, 2011; J. Liu, Zhang, Sun, & Lin, 2010). Besides these constituents, the protein content in microalgae was identified to be a potential substituent to conventional protein source. Microalgal protein is often discussed and investigated as the consumers are paying more attention to the health benefits and notably it offers an alternative source of protein to vegan or allergenic consumers (Hariskos & Posten, 2014). The protein content of microalgae could be as high as 40 to 70% (Becker, 2007), and has a comparable composition with the lipid content within microalgae. This high composition of protein could possibly substitute the main protein supply for human nutrition due to the rapid growth rate of microalgae and their high photosynthetic efficiencies in specific environments (Markou & Nerantzis, 2013).

Conventional techniques for the extraction of bioactive compounds require longer processing time, cost-consuming with complex scale-up (Asenjo

& Andrews, 2012). In order to overcome these significant problems in the extraction of bioactive compounds, an efficient liquid biphasic flotation (LBF) method was proposed. LBF is the formation of two types of immiscible solutions, with the induction of air bubbles to the system. LBF is a combination of the aqueous two-phase system (ATPS) with solvent sublation (SS) (Phong et al., 2017). LBF is proposed mainly due to the wide application of ATPS in the separation of biomolecules and could be performed better with an enhancement of SS (Platis & Labrou, 2009). Among all the downstream processing methods, ATPS is scalable, cost-effective, and a good recovery of biomolecules could be obtained from biomass in various studies (Frampton, Tsuei, White, Abraham, & Takayama, 2015; Zimmermann et al., 2018). ATPS acts as an effective and simple platform for the purification of biomolecules with good performance, which paves a way towards industrial applications (Soares, Azevedo, Van Alstine, & Aires-Barros, 2015). On the other hand, SS is able to concentrate the targeted biomolecules from the aqueous solution with a suitable gas flow rate and type of solvents applied in the system (Sobianowska, Walkowiak, & Kozłowski, 2009). ATPS is a liquid-liquid fractionation technique with water as the main medium, which prevents the denaturation of biomolecules. The principle of SS is based on nonfoaming adsorptive bubbles separation, by allowing the biomolecules absorbed to the surface of bubbles and the bubbles subsequently float up from the bottom liquid phase to the upper liquid phase (Sobianowska et al., 2009). Therefore, LBF is claimed to have the advantages of both ATPS and SS, as mentioned above.

Apart from the extraction methods of biomolecules, the cell disruption method is equally essential in order to break down the cell wall of microalgae to release the targeted biomolecules. There are several types of cell disruption methods to release protein from the biomass, for example, manual grinding, ultrasonication, bead-beating, high pressure cell disruption and alkaline treatment (Carl Safi, Ursu, et al., 2014). In this study, sonication was exploited to assist in the extraction of protein. The mechanism induced by sonication such as erosion, sonoporation, fragmentation and more (Khadhraoui et al., 2018), often enhance the extraction of the targeted component. These mechanisms allowed the inner medium of natural sources to be released by damaging the membrane or cell wall of the plant. Sonication is used as "green and innovative" approach in green processing, pasteurization, and extraction as it can be used to overcome the current issues faced in industries, such as low production efficiency, time-consuming procedures and a large quantity of wastewater (Chemat, Rombaut, Meullemiestre, et al., 2017). The green impacts of sonication for downstream processing are a reduction of wastewater, time and energy-saving (use of the low volume of water, shorten or simplified procedures for heating and stirring, etc.) and elimination of hazardous substances which preserve the environment. Sonication can be performed using a bath sonicator or probe horn. The bath sonicator is often known as indirect sonication while the probe type is considered to be direct sonication. The sonicator probe generally provides more ultrasound energy compared to sonicator bath (J. Wu, Lin, & Chau, 2001). The cell wall structure of microalgae was taken into consideration as its cell wall consists of polysaccharides and glycoprotein matrix, which made the cell wall intrinsic and provides formidable defence towards the environment

(Gerken, Donohoe, & Knoshaug, 2013). Hence, sonication was performed using the sonicator probe.

In this study, the extraction of protein from microalgae was carried out by using LBF assisted with probe type sonication. The protein extracted through sugaring-out effect has been investigated with different types and concentrations of sugar as well as the working volume of both phases in the system. Besides, this study was performed with *Chlorella sorokiniana*, a microalgal that consists of higher protein content. The studies related to sonication such as pulse mode and continuous mode for assisting the extraction of protein were determined to evaluate the optimum parameters during the extraction process.

3.3 Materials and Methods

3.3.1 Materials

Glucose, sucrose, maltose, fructose, acetonitrile and Bradford reagent were obtained from R&M Chemicals (Malaysia). The solutions used for protein recovery were prepared from distilled water and the medium used for microalgae cultivation was prepared from deionized water. The working standard solutions of BSA were prepared by appropriately diluting the stock solution of 2 mg/ml using distilled water. All the purchased chemicals and solvents were analytical grade.

3.3.2 Apparatus

A liquid biphasic flotation (LBF) equipment with a total volume of 500 ml was connected to the oilless air compressor (Model: PAC750-240F) with a

capacity of 24 L tank, motor speed of 1450 rpm, maximum pressure of 8 bar and an air displacement of 107 L/min for the generation of air bubbles. The LBF equipment is a 15 cm height of glass column with an inner diameter of 7 cm, equipped with the sintered disk of G4 porosity at the bottom of the glass column. The thickness of the glass column is 0.4 cm. The air flowrate of the system was regulated using the rotameter (Dwyer, USA) in a range from 25 to 250 ml/min. The ultrasound treatment was introduced using Bandelin Sonopuls (UW 2200, Germany) with a titanium horn sonotrode (TT 13/FZ). The schematic of LBF equipment is as shown below (**Figure 3.1**).

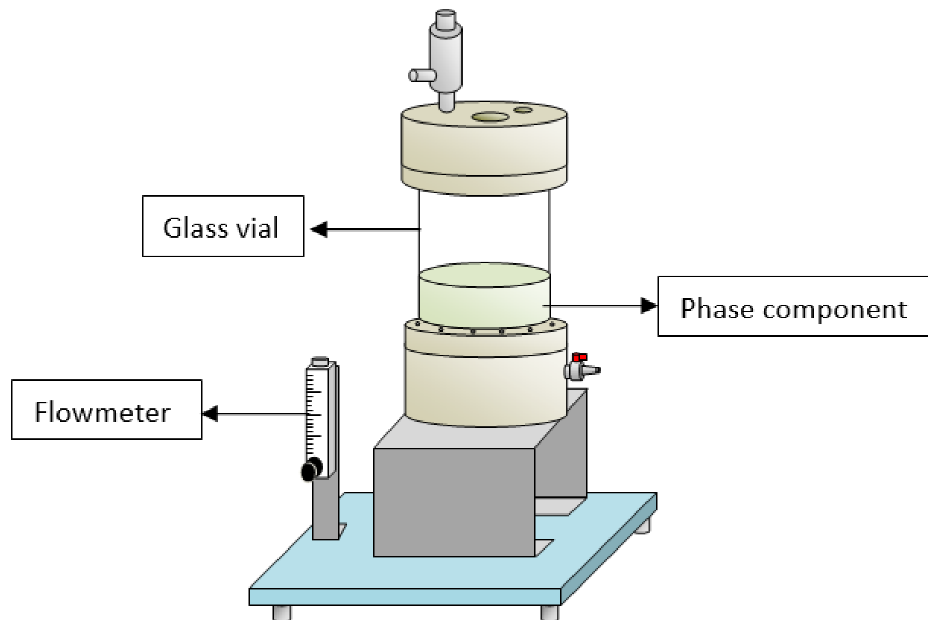


Figure 3.1: Schematic of the equipment used in liquid biphasic flotation (LBF).

3.3.3 Medium Composition and Microalgae Cultivation

A green microalgae strain, *Chlorella sorokiniana CY-1*, was selected in this study. The medium used in the pre-cultivation and batch cultivation for the microalgae is BG-11 medium with a continuous supply of 2.5% CO_2. The composition of BG-11 medium is as follows: 1.5 g/L of $NaNO_3$, 0.03 g/L of

K_2HPO_4, 0.075 g/L of $MgSO_4.7H_2O$, 0.006 g/L of citric acid, 2 g/L of Na_2CO_3, 3.6 g/L of $CaCl_2.2H_2O$, 0.6 g/L of ferric ammonium nitrate, 0.1 g/L of EDTA, 2.86 g/L of H_3BO_3, 1.81 g/L of $MnCl_2.4H_2O$, 0.222 g/L of $ZnSO_4.7H_2O$, 0.39 g/L $Na_2MoO_4.2H_2O$, 0.079 g/L of $CuSo_4.5H_2O$ and 0.049 g/L of $Co(NO_3)_2.6H_2O$.

The initial culturing of microalgae and the transferring of inoculums into photobioreactor were carried out within the laminar flow chamber to reduce the possibility of biological contamination. The pre-cultivation of microalgae was performed for one week, whereas the batch cultivation was performed within 10 to 14 days. The culture was illuminated continuously with a light intensity of 200 µmol/m^2 s^{-1} throughout the cultivation. The nitrate content was determined on daily basis and the wet microalgae were harvested when the nitrate content of microalgae was about 10% of the original nitrate content at Day 1 of batch cultivation (nitrogen starvation). The nitrate content of microalgae biomass was determined by centrifuging the harvested biomass at 6000 rpm for 5 min (Eppendorf, 5430). The supernatant was obtained, and 10 times dilution was performed prior to analysis. The sample was tested at a wavelength of 220 nm by using UV-Vis spectrophotometer (Chew, Chia, Show, Ling, et al., 2018).

3.3.4 Determination of Protein Content

The protein content of microalgae biomass was examined using a modified Bradford method (Bradford, 1976). 0.25 ml of sample was mixed with 2.5 ml of Bradford reagent to measure the extracted protein content from microalgae. A wavelength of 595 nm was used to determine the extracted protein

by using UV-Vis spectrophotometer. The concentration of protein extracted was determined by converting the obtained absorbance values via calibration between OD595 and BSA protein concentrations. The protein calibration curve was obtained using BSA as standard which is showed in **Figure A 1**.

3.3.5 Determination of Separation Efficiency (E), Total Protein Content and Yield (Y)

The separation efficiency (E) for the extraction of protein was calculated using Eq. (1):

$$E = \frac{V_T \times C_T}{V_B \times C_B} \quad (1)$$

where V_T is the volume obtained for the top phase (mL), V_B is the volume obtained for bottom phase (mL), C_T is the concentration of the top phase (mg/mL) and C_B is the concentration of bottom phase (mg/mL). The total protein content in the microalgae (P_T) was calculated using Eq. (2):

Total protein content in microalgae (%) = Nea × NTP (2)

where N_{ea} is the total nitrogen (%) of *Chlorella sorokiniana* and a value of 7.05% was obtained using an elemental analyzer (LECO CHN628S, UK). The elemental analysis was performed at temperature of 960 ^{o}C according to Phong et al. (2018). The combustion gas is pure oxygen and carrier gas is pure helium for a complete combustion of 2 mg sample (Phong et al., 2018). NTP is a constant value of the nitrogen-to-protein conversion factor, which is 4.78.

The yield of protein (Y) from microalgae was calculated using Eq. (3):

$$Y = \frac{Total\ protein\ recovered\ in\ top\ phase}{Initial\ total\ protein\ in\ microalgae} \times 100\% \quad (3)$$

3.3.6 Cell Disruption with Sonication

Sonication-assisted LBF composed of sugar solution and solvent was implemented for the extraction of protein from microalgae. Firstly, the sugar solution was prepared and mixed with the weighed microalgae biomass. The mixture was poured into LBF equipment, followed by a measured volume of solvent was added. The flotation time initially was set to 5 min with an air flowrate of 10 ml/min. The compressor was supplying at 0.5 bar and the air was filtered through a sterile air filter. The filtered air will be connected to the bottom joint of equipment, where the generated air bubbles passed through the solution (consist of sugar solution, acetonitrile, and wet microalgae biomass) from the bottom of the system. The sonicator probe was inserted into the system and was placed between the interface of the top and bottom phase. The targeted product will be brought to the upper phase of the system and took for further analysis.

The initial condition for sonication-assisted LBF was stated as followed: 150 mL of 200 g/L sugar solution (glucose), 150 mL of pure acetonitrile, 0.5 g of wet microalgae biomass, 20% amplitude of a 200 W maximal power, pulse mode (5s ON/ 10s OFF), sonication time of 5 min and flotation time of 5 min. The initial volume ratio used is 1:1 as reported in the previous work which used a small working volume (Dhamole et al., 2010b). The air flowrate was maintained at 100 cc/min throughout the experiment to avoid any disturbance caused to the equilibrium of the two-phase system.

The operating parameters of sonication-assisted LBF were performed as followed: selection and concentration of sugar, the volume ratio of the system,

resting time for sonication, pulse and continuous mode and concentration of microalgae, were shown in **Table 3.1**. These parameters were studied and optimised using one-factor-at-a-time (OFAT) approach.

Table 3.1: Operating parameters for sonication-assisted LBF through the sugaring-out effect.

No.	Operating parameters	Variables	Unit
1.	Selection of sugar	Sucrose, fructose, maltose, and glucose	N/A
2.	Concentration of glucose	150, 175, 200, 225, 250, 275	g/L
3.	Volume ratio	1 to 0.75, 1 to 1, 1 to 1.1, 1 to 1.25, 1 to 1.33	N/A
4.	Resting time	5, 10, 15, 20, 30	s
5.	Pulse and continuous mode	Pulse: 3, 5, 10, 15 Continuous: 2, 5, 7.5, 10	Pulse: s Continuous: min
6.	Concentration of microalgae	0.25, 0.5, 0.75, 1, 1.25, 1.5	g

3.4 Results and Discussion

3.4.1 Selection of Sugar

In liquid biphasic flotation (LBF), the common chemicals used as phase construction materials are polymers, salts, alcohols and solvents. Other than salt solutions, sugar solutions could be used to form the two phases with solvents as well. In this case, the targeted product that had to be isolated from the source would be separated from the biomass through sugaring-out effect. The sugaring-out effect is similar to the salting-out effect, where the types of sugars are the crucial phase constructing components in the system. The effect of sugaring-out

agents could result in variations in the ability of phase separation as reported earlier (Tu et al., 2018; C. Zhang, Huang, Yu, & Liu, 2012).

The protein partitioning was determined using different types of sugars, for example, sucrose, fructose, glucose, and maltose. These sugars were chosen as they are common sugars that are either fall under the category of monosaccharide or disaccharide. Glucose and fructose are monosaccharides while sucrose and maltose are disaccharides. Acetonitrile has been chosen to be the top phase of the system due to the suitability and stability of forming phases with the selected sugar solution (Dhamole et al., 2010a). In addition, the phase separation of acetonitrile and water can be induced at very low temperatures such as 1 °C using sugars (Bin Wang et al., 2008), which can also be achieved at low and at room temperatures in the range of 6 to 24 °C (Dhamole et al., 2010b). For this experiment, pure acetonitrile with a similar working volume of sugar solution was used and the experiment was performed at room temperature. **Figure 3.2** shows the protein yield (Y) and separation efficiency (E) of protein from the biomass.

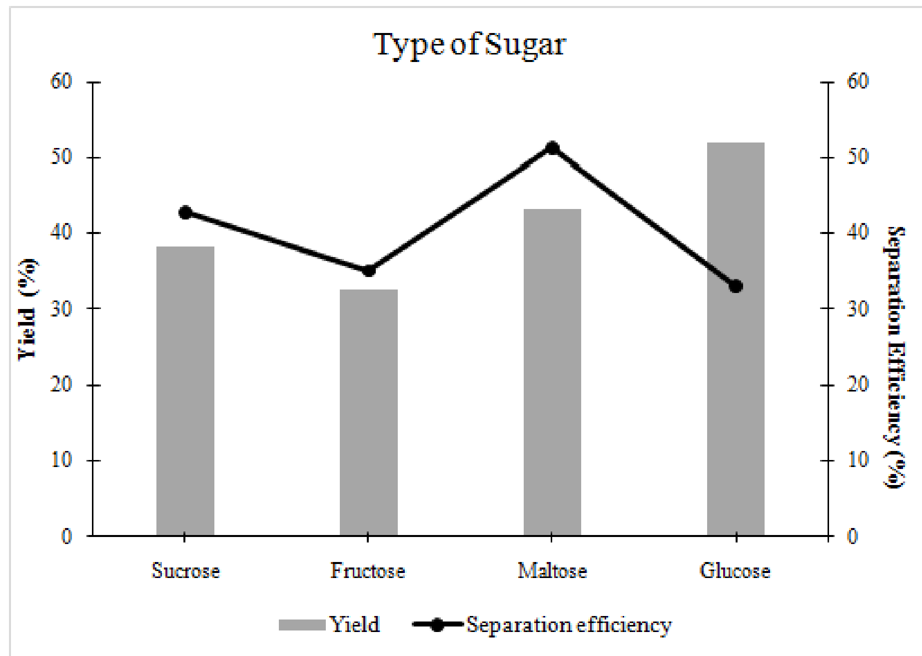


Figure 3.2: Effect of type of sugar on the protein yield and separation efficiency.

From **Figure 3.2**, the highest Y recovered from the biomass was around 52% and the lowest Y was around 33%, for glucose and fructose respectively. Glucose as the bottom phase gave the highest Y but comparable E of protein with the system consisting of fructose. The performance of both monosaccharides i.e. glucose and fructose in protein partitioning, were completely different, as glucose had the best performance on Y while fructose was not. The study of Cardoso et al. (2013) suggested that the hydration ability of sugars is proportional to the tendency of phase separation (de Brito Cardoso et al., 2013). According to their study, glucose (an aldose with a 6-sided ring of carbon atoms) is more effective in inducing the formation of two aqueous phases compared to fructose, due to the presence of ketose. On the other hand, the system using maltose as the bottom phase had the highest E compared to other systems. The disaccharides have comparable Y and E, and they had slightly

better performance than fructose either in Y or E. The disaccharides showed similar capabilities in isolating protein from biomass due to their structure. Sucrose consists of glucose and fructose while maltose consists of two units of glucose. As disaccharides and glucose have lower compatibility with acetonitrile molecules, more of these acetonitrile molecules were separated out from aqueous solutions, resulting in the higher amount of protein being transferred to the acetonitrile-rich phase (C. Zhang et al., 2012). Glucose has been chosen as the bottom phase of LBF as highest Y is obtained.

3.4.2 Concentration of Glucose

From the study related to types of sugars, glucose has been chosen among the carbohydrates due to the high recovery of protein and comparable separation efficiency (E) obtained. The induction of two phase depends on the concentration of glucose as well. In this study, the concentration of glucose as the bottom phase was altered as, 150, 175, 200, 225, 250, and 275 g/L. The initial glucose concentration was 200 g/L as followed in the study of Shishov et al. (2017). In the mixture of acetonitrile and water, Shishov et al. (2017) reported that the addition of glucose above a critical concentration was able to induce two phases. The sugar concentration of 200 g/L and above extracted amylmetacresol effectively in both phases (Shishov, Nechaeva, Moskvin, Andruch, & Bulatov, 2017). Hence, lower sugar concentrations (150 and 175 g/L) were examined along with the high concentration of sugar for larger working volume to investigate the potential of utilizing lower glucose concentration to extract the targeted product. However, a faint line was observed in both the systems containing 150 and 175 g/L of glucose in the bottom phases. As the glucose

concentration increased, phase separation in the system was clearly observed. This finding corresponds to the observations of Timofeeva et al. (2017), where a complete phase separation was only observed at the concentration of glucose in an aqueous solution which was higher than 200 g/L (Timofeeva, Shishov, Kanashina, Dzema, & Bulatov, 2017).

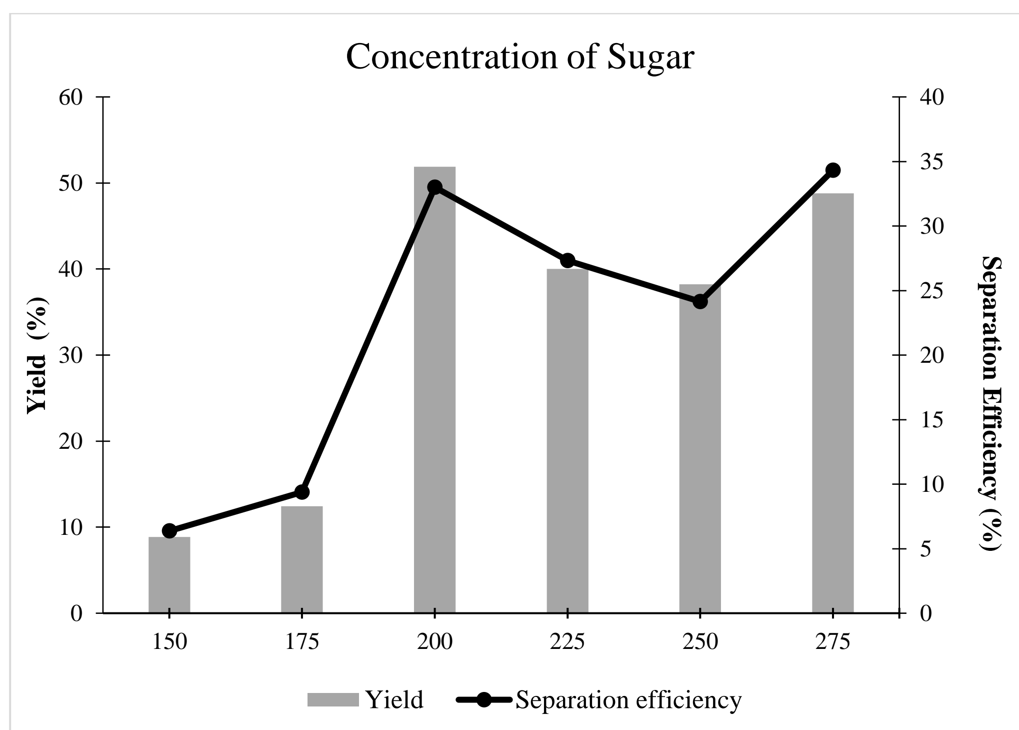


Figure 3.3: Effect of sugar concentration on the protein yield and separation efficiency.

From **Figure 3.3**, the protein recovered using 150 and 175 g/L of glucose were comparatively low, which were only able to recover 8.86 to 12.45% of protein yield (Y) compared to using a higher concentration of glucose. The results obtained had shown that Y was increasing from 150 g/L to 175 g/L with an increase in the volume of organic extract due to an increase in the concentration of glucose. The increasing trend of Y and E was observed from

150 g/L to 200 g/L and from 250 g/L to 275 g/L of glucose. It is believed that the sonication along the process breaks down the cell wall and the bubbling effect (flotation) could bring the soluble proteins from the glucose solution (bottom phase) to the acetonitrile-rich phase. In addition, the higher glucose concentration added into the bottom phase has resulted in lesser hydrogen available for proteins to bond. Therefore, more proteins are moved to the acetonitrile-rich phase, showing a significant difference of E and Y between low and high glucose concentration (Sankaran, Manickam, et al., 2018). However, the highest glucose concentration did not recover most of the protein from the biomass, instead 200 g/L of glucose was able to recover the highest percentage of protein among all the parameters. The systems consisted of glucose concentration higher than 200 g/L have a lesser water content in the glucose mixture as the percentage of glucose is higher. Thus, it is reasonable to speculate that the maximum capacity of protein soluble in the water was reduced due to the limited water content (Tu et al., 2018). This would reduce the amount of soluble protein in the acetonitrile-rich phase under sonication and flotation. Indeed, the separation efficiency of protein was at a peak in 275 g/L of glucose and was the lowest in 150 g/L of glucose. The volume ratio of acetonitrile-rich phase and glucose were varied from the initial volume ratio, where most of the higher glucose concentration formed a higher volume of acetonitrile-rich phase. This is similar to the salting-out effect, where a higher concentration of glucose resulted in an increased partition behaviour of protein to acetonitrile-rich phase (Goja, Yang, Cui, & Li, 2013). In this study, the glucose concentration of 200 g/L was chosen as the optimum concentration for protein extraction.

3.4.3 Volume Ratio

From previous studies, glucose with 200 g/L was chosen to study the volume ratio in the system. This is an important study for determining the optimum volume ratio required to isolate the protein without denaturing and to obtain an optimum yield (Y) and separation efficiency (E) from the biomass. By using acetonitrile as top phase, the volume ratio was altered in the ratio of 1:0.75/ 1/ 1.1/ 1.25 and 1.33 (volume of bottom phase: volume of top phase). The volume of top phase was altered due to more protein which was separated to the top phase compared to the bottom phase (data not shown) and the increasing working volume of top phase would possibly improve Y and E of protein. The total working volume of the system remained the same (300 mL) similar to studies shown in **Section 3.4.1 and 3.4.2**. The volume ratio of acetonitrile to water mixture with the addition of sugars is 1:1 for conducting the sugaring-out effect (Nugbienyo et al., 2017; B. Wang, Feng, Ezeji, & Blaschek, 2008). Therefore, the initial volume ratio of 1:1 was used as the standard and the range was set as shown in **Figure 3.4**.

The results obtained in this study are not similar to both the previous studies. The Y and E have been shown to be in an increasing manner with an increase in the working volume of acetonitrile. The higher Y in acetonitrile-rich phase was obtained due to more soluble protein released from the biomass through sonication and flotation was able to solubilise in the acetonitrile-rich phase. However, a slight decrement of both Y and E was observed in the highest volume ratio (1:1.33) of the system (**Figure 3.4**). As the initial volume of acetonitrile-rich phase greater, the lower the working volume of glucose-rich

phase. The lower working volume of glucose-rich phase was not consisting of a similar amount of water content as with another volume ratio. Thus, the lower water content in glucose-rich phase was not capable to solubilise a higher amount of protein extracted from microalgae biomass through flotation even sonication was performed to disrupt the cell wall (Naveena, Armshaw, & Tony Pembroke, 2015).

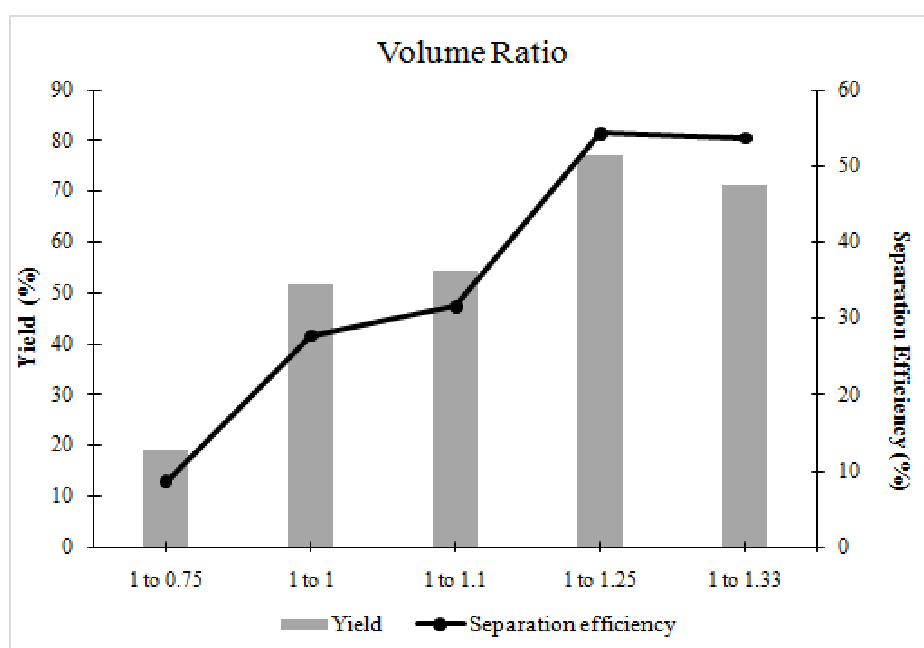


Figure 3.4: Effect of volume ratio on the protein yield and separation efficiency.

Furthermore, the working volume of acetonitrile should be at least the same working volume with the bottom phase to obtain more than 50% of recovered protein. The low working volume of acetonitrile was not able to extract more protein from the biomass even in the presence of sonication. The protein released through sonication may not be able to be extracted into the acetonitrile-rich phase as only a certain amount of protein can be solubilised into the limited capacity of acetonitrile-rich phase. With the discussion mentioned

above, the volume ratio of 1:1.25 has been chosen as the best condition to perform the next study, the resting time of ultrasonication.

3.4.4 Resting Time

The studies related to liquid biphasic flotation (LBF) were discussed in detail in **Section 3.4.1** to **3.4.3**. Hence, the studies focusing on sonication were investigated for the integrated extraction. Resting time is the resting period in between the sonication process during the pulse mode. The resting time of sonication would influence the extraction as the sonicator probe would have a higher temperature (which sound energy is transformed into heat energy) if longer sonication time is applied for the extraction (Ravanfar, Tamadon, & Niakousari, 2015). Generally, the regularity of sonication for longer resting time is not as much as compared to the shorter resting time between the sonication period (Sankaran, Manickam, et al., 2018). Hence, the effect of resting time on the protein extraction was investigated as the cavitation effect generated reduces during the ultrasonication. In this study, only the resting time was altered while the sonication time was fixed at 10 s in a total extraction time of 5 min. The resting time in between the sonication time was ranged from 5 s to 30 s in order to sustain sufficient cavitation effect for breaking the cell wall of microalgae. **Figure 3.5** shows that 10 to 30 s had similar protein yield (Y) recovered from the biomass except for a resting time of 15 s.

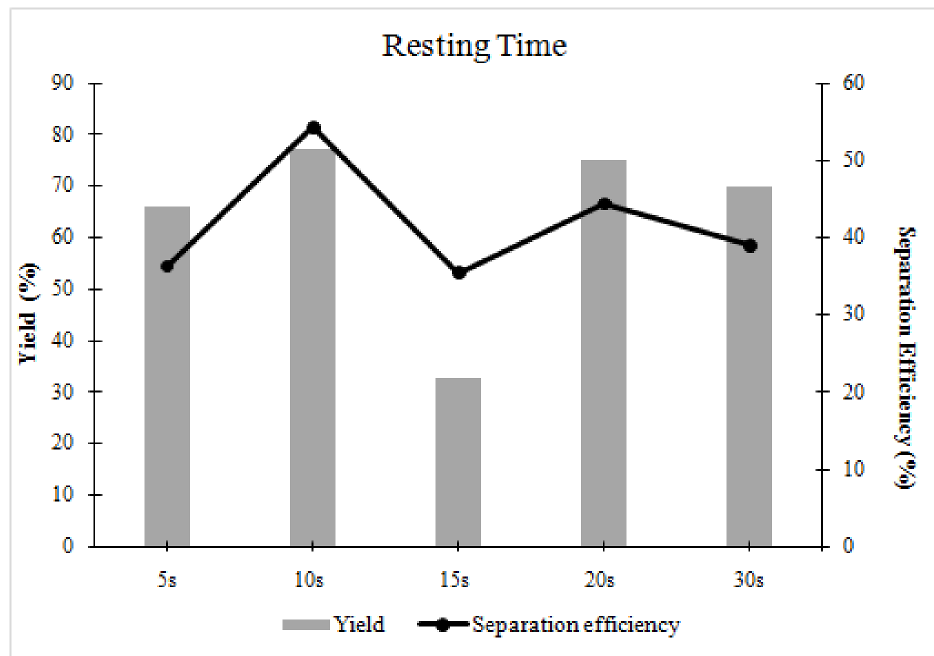


Figure 3.5: Effect of resting time of ultrasonication on the protein yield and separation efficiency.

From **Figure 3.5**, the Y was found to be decreasing drastically at 15 s of resting time, which was around 30%, even 5 s of resting time had higher protein yield than 15 s. This is mainly due to the times of sonication generated in 5 min as more times of sonication generated in 5 s of resting time than in 15 s of resting time. The continuous oscillatory motion of liquid medium generated by the radial movement of cavitation bubbles, namely micro-convection, which subsequently result in the growth of nuclei. When the cavitation bubbles grow to the maximum size (4-300 mm diameter) and acoustic energy reached sufficient intensity, these microbubbles become unstable and collapse. The times of sonication generated was critical to the breakage of cell wall as the collapse of these microbubbles create microjets with a velocity of more than 100 m/s (Naveena et al., 2015). As the resting time in between sonication increases, the times of sonication decreases. However, 20 to 30 s of resting time has better Y

and separation efficiency (E) of protein than 15 s of resting time. The mixing process induced by flotation and sonication for 15 s of resting time was more vigorous than 20 and 30 s of resting time, resulting into turbulence in the medium which pushes the extracted protein away from the surface of microbial cells. Furthermore, the vigorous mixing process causes the extracted protein dispersed in between the two phases, without remaining in the acetonitrile-rich phase. Besides, the E of each resting time was different. The higher resting time had similar E, but the lowest resting time had a similar result with the moderate resting time (15 s). This is due to the volume ratio of both phases which affected through the turbulence generated by flotation and sonication, as indicated in the previous paragraph. The 10 s of resting time was the most suitable for pulse mode and was chosen to be studied for the next parameter as the highest E and Y was obtained.

3.4.5 Pulse and Continuous Mode

The pulse and continuous mode of sonication were studied to examine the suitable mode for the experiments after investigating the resting time in between sonication. The pulse mode of sonication was examined from 3 to 15 s with a fixed resting time of 10 s and the continuous mode was studied from 2 to 10 min of non-stop sonication without any resting time in between. As reported by Chemat et al. (2017) and Sivakumar et al. (2017), the extraction of components through different modes of sonication could affect the yield of components extracted (Chemat, Rombaut, Sicaire, et al., 2017; V. Sivakumar, Rani, & Kumari, 2017). The range selected for continuous mode was lower than the ranges studied in the previous reports (Pradal, Vauchel, Decossin, Dhulster,

& Dimitrov, 2016; Shirsath et al., 2017), due to the mechanisms as both sonication and flotation lead to mixing in the system.

The obtained results for pulsed and continuous mode of sonication-assisted with flotation have been shown in **Figure 3.6**. Among all the sonication time set for pulse mode, 5 s of sonication time could achieve the highest protein yield (Y) and separation efficiency (E). The sonication time of 3 s was not sufficient for the complete breakage of the cell wall, causing low Y and E of protein obtained. However, similar results were obtained by 10 and 15 s of sonication time with 3 s of sonication time, only 5 s of sonication time was the best.

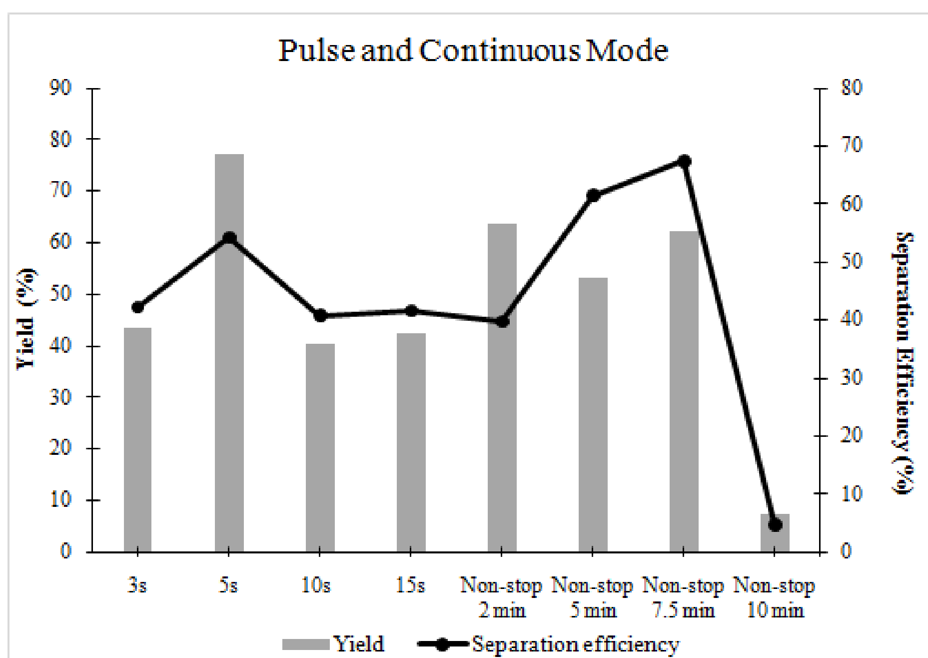


Figure 3.6: Effect of pulse and continuous modes of ultrasonication on the protein yield and separation efficiency.

5 s of sonication time has higher Y than 10 s and 15 s of sonication time as the occurrence of sonication regularity at a given time had been increased in shorter pulse mode, leads to a better Y and E (Sankaran, Manickam, et al., 2018). Overall, the average yield obtained by using continuous mode was better than pulse mode, yet the highest yield and separation efficiency was obtained using pulse mode. The pulse mode of the sonicator probe could perform better than the continuous mode in extracting the protein due to the cavitational events. The gas content of the liquid increased as pulse mode was applied to the system, which creates a higher number of cavities resulting in higher cavitational events, thereby enhancing the Y (M. Sivakumar, Senthilkumar, Majumdar, & Pandit, 2002). As for the continuous mode, the decrement of Y was caused by the application of continuous ultrasound, where the nuclei were destroyed rapidly and resulted in degassing of the cavitation bubbles (P. S. Kumar, Kumar, & Pandit, 2000). Besides, the continuous mode with 10 min of sonication obtained the lowest Y as well as E, which is mainly due to the vigorous mixing process, as mentioned in **Section 3.4.4**.

3.4.6 Concentration of Microalgae

The study related to the sample weight was carried out and the examined weight of biomass was ranged from 0.25 to 1.5 g of wet microalgae. This study was performed due to the previous studies which stated that the concentration or weight of crude biomass does affect the targeted product recovered from the crude biomass (Phong et al., 2017; P. L. Show et al., 2011). The partition behaviour of protein altered accordingly when biomass was loaded into the system. In the study of Show et al. (2011), the separation efficiency was

decreased as the higher amount of feedstock was loaded to the system, which influenced the composition of the system (P. L. Show et al., 2011). Furthermore, the protein recovered from the biomass should be increasing as the crude load of biomass was increased. In fact, the obtained result was not corresponding to the hypothesis. **Figure 3.7** illustrates that the yield (Y) and separation efficiency (E) were correlated to each other as the lower yield has lower separation efficiency for 0.75 to 1.5 g of biomass used. However, the highest separation efficiency did not correspond to the highest yield obtained in this study. The precipitation of cell debris or other components (beside proteins) may occur at the interface of the system, resulting in the protein not to be sugaring-out from the bottom phase (glucose-rich phase) (Selvakumar, Ling, Walker, & Lyddiatt, 2010).

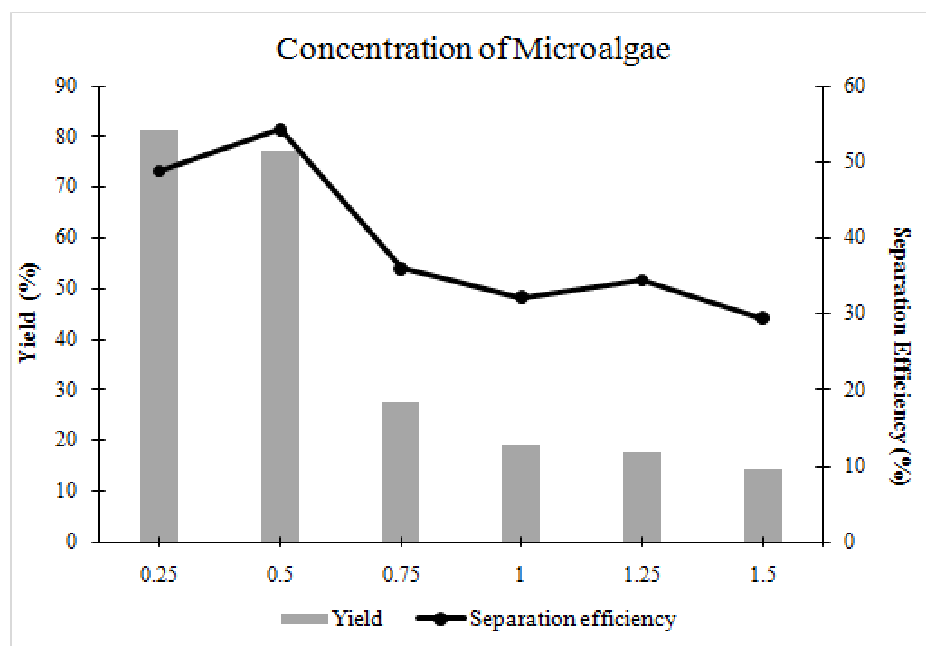


Figure 3.7: Effect of concentration of microalgae on the protein yield and separation efficiency.

The best performance of the system was observed using the lightest biomass weight (0.25 g) to obtain the highest Y of protein, around 80% with a comparable E of protein with 0.5 g of biomass. This may be due to the complete sonicated biomass by using the sonicator, which causes a higher amount of protein extracted from the biomass. Nonetheless, the biomass weight with 0.75 g to 1.5 g was only capable to extract 10 to 30% of protein from the biomass which was much lesser than using 0.25 and 0.5 g of biomass in the system. The Y for biomass weight from 0.75 to 1.5 g decreased drastically while having similar E of protein in these systems. These phenomena would probably occur as the volume ratio of systems was similar after the extraction process but obtaining lesser protein in the top phase (acetonitrile-rich phase).

3.4.7 Potential Industrial Application and Up-scaling

Sonication process often shows green impact such as lower energy consumption, reduction of eco-footprint of process and act as an economical model. Therefore, the parameters of sonication along with the parameters of LBF were included in this work. In order to utilize the energy input effectively, the resting time of sonication and mode of sonication have been studied and optimized. The optimum condition to extract protein is resting time of 10 s and pulse mode in sonication-assisted LBF. The resting time of sonication is significant as it implies the energy required for the sonication process, whereby more energy input is needed for the shorter resting time of sonication and vice versa. Besides, sonication in pulse mode requires lower energy input from the generator compared to the sonication in a continuous mode as it requires a continuous supply of energy to perform the sonication.

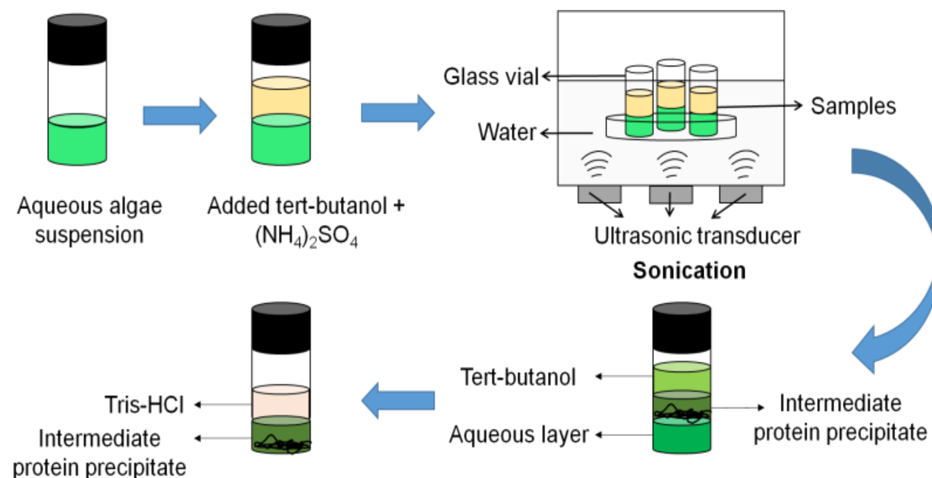
A preliminary assessment of the energy consumption for both conventional and sonication-treated extraction of seeds have been performed in the study of Sicaire et al. (Sicaire et al., 2016). The study takes into account preparation of seeds, desolventization step, distillation step and heat recovered from the gas from total distillation. Reduction of heat and steam consumption was observed which subsequently leads to lower global environmental impact by using sonication-treated extraction.

This work has indicated potential industrial application as the sonication-assisted process have speed-up the processing time, where the extraction only required 5 min of sonication with 5 s of sonication and 10 s of resting time. A significant time reduction in extraction process could lead to a reduction of processing cost implied in energy production. Hence, the sonication-assisted LBF is highly recommended as the extraction method, LBF is ease to scale up to industrial scale. However, further investigation is required as the lab scale extraction may vary with the industrial scale extraction. The impact in industrial has to be investigated as it might clearly to be beneficial to the industry.

3.5 Conclusion

In conclusion, this study demonstrates an effective technique to extract the protein content from green microalga, *Chlorella sorokiniana* CY1, assisted with sonication that aids in breaking down the cell wall during extraction. The results presented in this work show that the protein could be isolated from microalgae through sugaring-out effect. In liquid biphasic flotation (LBF), the type of sugar solution, the concentration of sugar, the weight of wet microalgae were investigated as the factors influencing the performance of extraction. Besides, the protein extraction was influenced by the working volume of both phases significantly, obtaining around 77% of protein yield and 54% of separation efficiency. It has also been found that the protein extraction is higher for pulse mode compared to the protein extracted by the continuous mode. The highest protein yield of 80% and the corresponding separation efficiency of 49%, were achieved after optimisation through various parameters, suggesting that the high content of protein within microalgae could be extracted on an industrial scale.

CHAPTER 4 MICROALGAL PROTEIN EXTRACTION FROM *CHLORELLA VULGARIS* FSP-E USING TRIPHASIC PARTITIONING TECHNIQUE WITH SONICATION



This chapter covers the application of sonication-assisted three phase partitioning technique for the extraction and purification of proteins from microalgae. Comparison of three phase partitioning (TPP) and sonicated-assisted TPP was investigated for feasibility of the enhanced technique in proteins extraction. Various parameters were optimized after acknowledged the suitable method, which is sonicated-assisted three phase partitioning, in achieving optimum conditions for maximal yield and separation efficiency. A scaled-up study and analysis on surface of microalgae biomass after treatment was performed. This chapter consists of a thesis-version of the manuscript accepted in the Frontiers in Bioengineering and Biotechnology (Chia S.R. et al., 2019, *Frontiers in Bioengineering and Biotechnology*, 7, 396).

4.1 Abstract

Green microalgae containing various bioactive compounds and macronutrients such as lipids, carbohydrates and proteins, have attracted much attention from the global community. Microalgae have the potential to be applied in food industries due to its high protein content, rapid growth rate and ability to survive in harsh conditions. This study presents a simple yet efficient technique of sonication-assisted triphasic partitioning process, also known as ultrasonic-assisted three phase partitioning (UATPP), for the extraction of proteins from *Chlorella vulgaris* FSP-E. Comparison studies between three phase partitioning (TPP) and UATPP was conducted to investigate the feasibility of the enhanced technique on proteins extraction. Types of salt, ratio of slurry to t-butanol, salt saturation, sonication frequency, power, irradiation time, and duty cycle as well as biomass loading were studied. UATPP was found to be an improved technique compared to TPP. An optimum separation efficiency and yield of 74.59 ± 0.45% and 56.57 ± 3.70% was obtained, respectively, with the optimized conditions: salt saturation (50%), slurry to t-butanol ratio (1:2), sonication power (100%), irradiation time (10 min), frequency (35 kHz), duty cycle (80%) and biomass loading (0.75 wt%). A scaled-up study was performed to validate the reliability of UATPP for protein extraction. The outcome of the study revealed that UATPP is an attractive approach for downstream processing of microalgae.

Keywords

Protein Extraction; Ultrasonic; Triphasic Partitioning Technique; Microalgae; Bio-separation

4.2 Introduction

Nowadays, the growth of global human population is increasing at an incredible speed each year. An estimated 70% increase in food production will be needed for the growing human population (by around 2.3 billion) by the year 2050 (Tester & Langridge, 2010). The remarkable supply growth of food productions have reduced the proportion of global hunger, despite the world population doubling over the last half-century (Bleakley & Hayes, 2017). Nevertheless, the world is facing a great challenge in sustaining adequate food production to meet the rising demands. Methods and techniques used conventionally to produce food will soon no longer be a solution due to the emission of greenhouse gases by these processes, nutrient run-off causing environmental pollutions, degradation of soil and disruption of ecosystem caused by over-harvesting of aquatic foods (Tester & Langridge, 2010). Specifically, protein is one of the macronutrients that will be in shortage at the near future. A substitution or alternative protein source and more efficient production techniques needs to be discovered and developed in order to meet the global demand.

Microalgae is well known as a viable source of biological components such as carbohydrates, lipids, pigments, vitamins and polyphenols, especially proteins (Chia et al., 2018; Hsieh & Wu, 2009). The protein quality in microalgae is known to be similar with some of the traditional protein sources like milk, meat and egg (Bleakley & Hayes, 2017). In general, the protein content of microalgae constitutes a major portion compared to lipid and carbohydrate (Lavens & Sorgeloos, 1996). The total protein content from microalgae,

especially *Chlorella* sp. is about 43% to 50% (Phukan, Chutia, Konwar, & Kataki, 2011; Ramazanov & Ramazanov, 2006). In the studies of Richmond (2017) and Thompson et al. (1996), the nutritive quality of *Chlorella sp.* was proved to be influenced by light environment during the microalgae cultivation stage (Richmond, 2017; Thompson, Guo, & Harrison, 1996). Higher protein production was obtained with the increment of light irradiance and longer photoperiod (Seyfabadi, Ramezanpour, & Amini Khoeyi, 2011), since the efficiency of protein solubilisation are influenced by the chemical composition, structural characteristics and the morphology of microalgae (Ursu et al., 2014). *Chlorella* protein are also proven to be safe for consumption through various clinical and animal studies, and positive health effects such as lower high blood pressure, glucose and cholesterol levels are seen with the dietary of *Chlorella* protein (Waghmare, Salve, LeBlanc, & Arya, 2016).

Apart from that, there are several other advantages of using microalgae as main source of proteins, for example, rapid growth rate, high productivity, can withstand harsh conditions, ability to be cultivated using exhaust industrial gases and indirectly reducing greenhouse gas emissions (Chew, Chia, Show, Yap, et al., 2018; Jeon, Cho, & Yun, 2005). The cultivation of microalgae does not require freshwater and it can be cultivated using non-arable land that does not affect the need for land to grow food crops. These advantages have attracted huge interests to further investigate the potential of microalgae in pharmaceutical, food, cosmeceutical and bioenergy applications. Nevertheless, the production cost of biomolecules from microalgae in industrial-scale is relatively high and the commercialization of microalgae technology still remains

a challenge to the current industry (Chia et al., 2018). Thus, the development of an efficient technique for scalable production is deemed vital in order to maximize the recovery of biomolecules as well as the industrial profitability.

Conventional separation techniques like membrane separation, column chromatography, precipitation and crystallization often consist of multiple unit operations and high consumption of toxic organic solvents. The multiple processing stages consequently contributes to more time consumption and product loss throughout the entire process, and this leads to lower concentrations of the end products. Currently, the industry is searching for alternative techniques to overcome the mentioned drawbacks using greener and efficient techniques. Hence, three phase partitioning (TPP) was introduced as an efficient approach in extracting and purifying enzymes and biomolecules (Akardere et al., 2010; Gagaoua et al., 2014). TPP is easy to operate, efficient and scalable, where the salt is added to the aqueous solution containing the targeted product, followed by the addition of t-butanol to form three phases. The top phase of TPP is the t-butanol layer, middle phase is the protein precipitate layer and bottom phase is the aqueous layer where the solubility of t-butanol with water will change with the addition of salt. Since it is a three phase system, the targeted product may partition to either of the phases due to the operational conditions and physicochemical properties of targeted product (Avhad, Niphadkar, & Rathod, 2014).

The partitioning behavior of targeted product depends on the mass transfer phenomenon, where the purification fold and partitioning of end-product

can be improved through the increment of mass transfer (Niphadkar & Rathod, 2015). The utilization of ultrasound has been well applied in various processes such as extraction, absorption, bioremediation and fermentation to improve the mass transfer of targeted products (Gogate & Kabadi, 2009; Sulaiman, Ajit, Yunus, & Chisti, 2011; Tay, Lau, & Shariff, 2016). The mass transfer of targeted products is intensified when the ultrasonic waves generate cavitational bubbles in the medium. The shock waves and mechanical shear will be imparted to the surrounding environment due to the collapsing of these bubbles (Avhad et al., 2014). The cavitational phenomenon caused by the difference in mechanical shear and local energy densities will speed up the mass transfer across the phases in the system. Therefore, the integrated approach for protein extraction was conducted to improve the purification and mass transfer of protein.

The aim of the present study was to achieve a maximum recovery of proteins from *Chlorella vulgaris* FSP-E through triphasic partitioning techniques, three phase partitioning (TPP) and ultrasound-assisted three phase partitioning (UATPP). The first attempt was performed using TPP and UATPP with similar operating conditions to extract and purify proteins from microalgae in a single unit operation. Several operating parameters like types of salt, salt saturation, slurry to t-butanol ratio, ultrasonic power, ultrasonic frequency and time, duty cycle and biomass loading were then studied and optimized.

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

T-butanol, ammonium sulphate ($(NH_4)_2SO_4$), sodium sulphate (Na_2SO_4), magnesium sulphate ($MgSO_4$), magnesium acetate ($Mg(CH_3COO)_2$), dipotassium hydrogen phosphate (K_2HPO_4), Bradford reagent, and tris-HCl buffer were purchased from R&M chemicals (Malaysia). Bovine serum albumin (BSA) standards were purchased from Merck (Malaysia). All chemicals used were of analytical grade.

4.3.2 Microalga

The microalga selected is the green microalgae, *Chlorella vulgaris* (strain FSP-E). BG-11 medium was used for pre-culturing the selected microalgae for around 7 days and then the microalgae was cultivated in batch mode by supplying 5% of CO_2 continuously. The BG-11 medium used was prepared with: 1.5 g/L of $NaNO_3$, 0.03 g/L of K_2HPO_4, 0.075 g/L of $MgSO_4.7H_2O$, 0.006 g/L of citric acid, 2 g/L of Na_2CO_3, 3.6 g/L of $CaCl_2.2H_2O$, 0.6 g/L of $H_8N_8CeO_{18}$, 0.1 g/L of EDTA, 2.86 g/L of H_3BO_3, 1.81 g/L of $MnCl_2.4H_2O$, 0.222 g/L of $ZnSO_4.7H_2O$, 0.39 g/L $Na_2MoO_4.2H_2O$, 0.079 g/L of $CuSO_4.5H_2O$ and 0.049 g/L of $Co(NO_3)_2.6H_2O$.

The microalgae were cultivated in batch mode using a 1 L indoor glass vessel photo-bioreactor (PBR) by inoculation from the pre-culture cultivated in a PBR (250 mL). Light source was provided continuously to illuminate the culture by mounting external LED lights on the both sides of the PBR. The light intensity of external LED lights is 200 $\mu mol/m^2s^{-1}$. The initial culturing of

microalgae and transfer of inoculum from the agar plate to the PBR were performed under a UV laminar flow chamber to prevent biological contamination. The biomass productivity, nitrate content and pH values of the microalgae culture were monitored during the batch cultivation. Biomass productivity of the culture was measured at wavelength of 680.8 nm and the nitrate content was measured at wavelength of 220 nm using supernatant of microalgae culture (10 times dilution) via UV-Vis spectrophotometer. The pH value of culture was determined using pH meter. The biomass was harvested when the growth of microalgae achieved a stationary phase. The microalgae culture took 14 days to achieve stationary phase. The harvested biomass was centrifuged at 6000 rpm using a centrifuge (Eppendorf, 5430) for 5 min to remove liquid content. The remaining biomass paste was frozen at -20 ^{o}C for 24 hours before freeze-dried prior to the extraction experiments.

4.3.3 Determination of Protein Content

The proteins extracted were examined and quantified through modified Bradford method (Bradford, 1976). A total amount of sample containing proteins, 0.25 mL, was mixed with 2.5 mL of Bradford reagent and measured at the wavelength of 595 nm using a UV-Vis spectrophotometer. The readings obtained in absorbance unit were converted into protein concentration using the calibration curve performed (**Figure A 1**) while using BSA as standard.

4.3.4 Determination of Separation Efficiency (*E*), Total Protein Content and Yield (Y)

Separation efficiency of the extracted protein was calculated using Equation (1):

$$E = \frac{V_M C_M}{V_T C_T + V_M C_M + V_B C_B} \quad (1)$$

where V_T, V_M and V_B are volume of top, middle and bottom phase, respectively; C_T, C_M and C_B are concentration of top, middle and bottom phase, respectively. The total protein content in microalgae biomass (P_T) was calculated using Equation (2) (Carl Safi et al., 2013):

$$P_T = N_{ea} \times NTP \quad (2)$$

where N_{ea} is the total nitrogen (%) in *Chlorella vulgaris* FSP-E and a value of 4.18% was obtained through elemental analysis (Perkin Elmer CHNS/O 2400 Series II). A complete combustion of 1 mg dried sample was performed at 950 ^oC in a pure oxygen environment and the carrier gas is helium gas. NTP is a constant value of nitrogen-to-protein conversion factor, which is 4.78 (Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004; Templeton & Laurens, 2015). Therefore, the final value of P_T used in this work is 19.98%. The elemental composition of microalga is carbon %: 49.32, hydrogen %: 8.24, nitrogen %: 4.18 and sulfur %: 0.59. Yield of proteins (Y) from microalgae biomass was calculated using Equation (3):

$$Y = \frac{V_M C_M}{\frac{P_T}{100} \times W_i} \times 100\% \quad (3)$$

where W_i is the initial weight of biomass microalgae used in the study.

4.3.5 Three Phase Partitioning (TPP)

The study of TPP was conducted with a total working volume of 10 mL using the predetermined initial conditions. The system was prepared using 5 mL of 30% saturation $(NH_4)_2SO_4$, 5 mL of pure t-butanol and 0.5 wt% of freeze-dried microalgae biomass. The amount of freeze-dried microalgae biomass used is measured according to the total weight of bottom phase. Firstly, the microalgae biomass was dissolved in deionized water before mixing with salt solution. 5mL of t-butanol was then added to the mixture of microalgae biomass and salt solution in a small beaker. The mixture was stirred using a magnetic stirrer at 200 rpm for 1 hr and was allowed to separate for 30 min at room temperature. Three phases were observed and separated carefully by pipetting them out from the beaker. The intermediate protein precipitate was dissolved in appropriate amount of tris-HCl buffer and analyzed for protein content. The separation efficiency and yield of proteins in all three phases were analyzed and compared with the ultrasound-assisted three phase partitioning process. All the experiments were repeated three times and the mean of the values were reported.

4.3.6 Ultrasound-assisted Three Phase Partitioning (UATPP)

Ultrasound-assisted three phase partitioning of proteins was conducted using an ultrasonic water bath. The initial conditions of UATPP such as the working volume, saturation of salt solution and weight of biomass were similar with TPP for the comparison study. The preparation and mixing procedure of UATPP was similar with TPP, but a glass vial was used for ultrasonic treatment. The initial volume ratio used is 1:1. The glass vial containing the mixture was placed into the ultrasonic water bath, operating with 35 kHz and 100% sonication

power for 5 min. The glass vial was then taken out and allowed to separate for 30 min at room temperature. The intermediate protein precipitate was dissolved with tris-HCl and proteins in all three phases were analyzed through the modified Bradford method. All the experiments were done in triplicate and the mean values were reported.

4.3.7 Optimization of Operating Parameters

The optimization of UATPP was applied for proteins extraction, where the system composed of t-butanol and salt solution with sonication effect. Operating parameters of UATPP which includes the type and saturation of salt, ratio of slurry to t-butanol, sonication power, frequency, irradiation time, duty cycle and biomass loading, were studied in the optimization step. Initial conditions and subsequent variables for each parameter were shown in **Table 4.1**. Type of salt was set as the first parameter to determine the interaction of different salts with t-butanol in order to precipitate out the protein from the biomass. Optimum salt saturation was investigated to reduce the usage of salt in the whole system, where the highest salt saturation studied was up to 60%. The third parameter, slurry to t-butanol ratio was studied before the optimization of sonication properties. The relationship between the volume of slurry and t-butanol used in experiment was studied as it is critical in optimizing the TPP process (A. Sharma, Sharma, & Gupta, 2000). The sonication parameters were optimized to use energy more efficiently and save operating cost where possible while achieving optimum proteins extraction. The last parameter, biomass loading was examined due to possible change in equilibrium of three phases in

the system. The effects of operating parameters were studied using one-factor-at-a-time (OFAT) approach.

Table 4.1: Operating parameters for UATPP.

No.	Operating parameters	Initial settings	Variables	Unit	Justification
1.	Types of salt	$(NH_4)_2SO_4$	Na_2SO_4, $MgSO_4$, $Mg(CH_3COO)_2$, K_2HPO_4	N/A	First parameter to optimize the salt saturation
2.	Salt saturation	30	20, 40, 50, 60	%	-
3.	Slurry to t-butanol ratio	1:1	1:0.5, 1:1.5, 1:2, 1:2.5	N/A	The ratio of slurry is set as 1 (slurry:t-butanol)
4.	Sonication power	100	20, 40, 60, 80	%	-
5.	Sonication frequency and irradiation time	35 kHz and 10 min	Set A (35 kHz): 2.5, 5, 7.5, 12.5 min Set B (130 kHz): 2.5, 5, 7.5, 10, 12.5 min	kHz, min	-
6.	Duty cycle	100	20, 40, 60, 80	%	100% is continuous sonication
7.	Biomass loading	0.5	0.25, 0.75, 1.00, 1.25	wt%	-

4.3.8 Field Emission Scanning Electron Microscope (FESEM)

Field Emission Scanning Electron Microscope (FESEM, Quanta 400F) (FEI, USA) analysis was used to analyze the morphological structure of microalgae biomass before and after extraction (with and without UATPP). The intermediate phase consisting of the protein precipitation was dissolved and

washed with Tris-HCl buffer. The obtained samples were centrifuged and lyophilized for a day prior to the analysis. Three samples are subjected to the analysis, which are the fresh microalgae biomass, the samples after sonication only using water as medium, and the samples after UATPP.

4.3.9 Statistical Analysis

The data was subjected to one-way ANOVA (with significance $p \leq 0.05$) data analysis and the mean differences were compared using Tukey HSD post-hoc test. All studies were conducted in triplicate and the values were expressed as mean ± standard error. The statistical analysis was performed using IBM SPSS statistics software (SPSS version 26.0 for window; IBM Corporation; United States).

4.4 Results and Discussion

4.4.1 Comparison Study

The study of TPP and UATPP showed that the addition of sonication resulted in better extraction of proteins from microalgae. The results obtained using both methods were tabulated in **Table 4.2**.

Table 4.2: Comparison study between TPP and UATPP.

Methods	Yield (Y, %)	Separation efficiency (E, %)
TPP	25.15 ± 1.04	49.78 ± 0.44
UATPP	40.01 ± 4.51	52.26 ± 2.47

In both cases, the proteins favorably partitioned to the intermediate phase and less proteins were partitioned to the top and bottom phase. These precipitated

proteins formed in between the top and bottom phases created the three-phase formation (Chew, Ling, & Show, 2018). Better results were obtained using UATPP compared to TPP due to the mechanism of ultrasonication which has helped to break the cell wall of microalgae. The cavitation bubbles induced by ultrasonic waves disrupt the microalgae cell wall, leading to the release of proteins from microalgae cells. In addition, TPP requires 1 hr to partition proteins from microalgae biomass while UATPP only needs 10 min to achieve higher yield of proteins. This significant finding showed that higher yield and better separation efficiency of proteins could be achieved within shorter processing time by UATPP as supported by Pakhale and Bhagwat (2016) (Pakhale & Bhagwat, 2016). The findings are in agreement with Zhang et al.'s work, where the recovery of targeted product increased using UATPP compared to conventional TPP (X. F. Zhang, Wang, & Luo, 2017). The reduced processing time subsequently leads to lower operating cost via UATPP.

4.4.2 Optimization of UATPP Process Parameters

4.4.2.1 Types of salt

The first parameter that was optimized in this study is the compatibility of t-butanol with various types of salt for protein extraction. Salts type plays an important role in UATPP as the partitioning behavior of proteins is influenced through the interaction of salts and t-butanol depending on the ionic strength of solution. All the systems showed a slight difference (around 9%) in separation efficiency, ranging from 45.48 ± 1.90 to $54.48 \pm 2.58\%$, excluding the system consisting of $Mg(CH_3COO)_2$ from **Figure 4.1**. The protein yield for Na_2SO_4, $MgSO_4$, and K_2HPO_4 was comparable, falling in the range of 31.94 ± 0.24 to

32.63 ± 4.04%, while $(NH_4)_2SO_4$ obtained the highest yield of proteins. Based on the statistical analysis, the yield of protein was significantly affected by the types of salt ($p<0.05$).

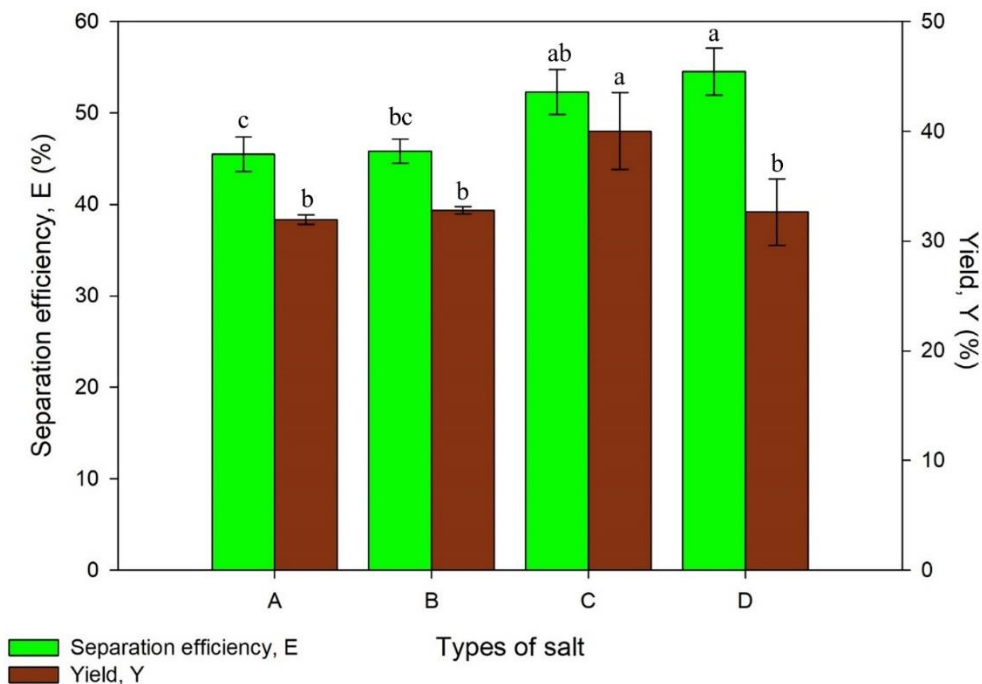


Figure 4.1: Effect of types of salt on separation efficiency (E) and yield of protein (Y) where A is Na_2SO_4, B is $MgSO_4$, C is $(NH_4)_2SO_4$ and D is K_2HPO_4. Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

The combination of selected salts with t-butanol have formed a distinct three phase except for $Mg(CH_3COO)_2$, where only two phases were observed with a blur separation interface. This phenomenon may be due to the salting-in effect by $Mg(CH_3COO)_2$ at a particular molality. However, the salting-out effect of $Mg(CH_3COO)_2$ with IL was stronger than K_2HPO_4 as stated by Neves et al. (2015), proving that every combination of salt and solvent have to be investigated for their suitability to extract specific products (Neves et al., 2015).

$(NH_4)_2SO_4$ obtained the highest yield of proteins as it has higher hydration capacity and affinity of water compared to other types of salt. The hydration energy of sulphate ions has increased its effective radius by involving large proportions of water molecules and promoting the stability of proteins through hydrophobic interactions (Chew, Chia, Lee, Zhu, & Show, 2019). Large ions gather together and isolate the proteins through precipitating the proteins out of the water phase. Besides that, the sulphate ions are positioned in the front part of the Hofmeister series. They interact well with water molecules as they act as dehydrating agent, forming H-bonds as well as dehydrate proteins (Pakhale & Bhagwat, 2016). T-butanol with the presence of high $(NH_4)_2SO_4$ concentration acts in more lipophilic manner, increasing the hydrophobicity and exclusion from water due to the higher effective dielectric constant of water (Waghmare et al., 2016). Various studies have utilized $(NH_4)_2SO_4$ in both TPP and UATPP for its salting out ability, and have successfully extracted the desired products (Gagaoua et al., 2017; Pakhale & Bhagwat, 2016). Therefore, the system of $(NH_4)_2SO_4$ and t-butanol was chosen for further studies.

4.4.2.2 Effect of $(NH_4)_2SO_4$ saturation

Saturation of $(NH_4)_2SO_4$ is a significant parameter on UATPP due to the salting-out phenomenon. The effect of $(NH_4)_2SO_4$ saturation was investigated from 20% to 60% (w/v). **Figure 4.2** showed that increasing $(NH_4)_2SO_4$ saturation enhanced the separation efficiency and yield of proteins due to the salting out effect. The protein yield showed an increasing trend along with the increased saturation of salt up to 50% saturation, where it starts to decrease, this is in agreement with results reported by previous research (Niphadkar & Rathod,

2015). The yield of proteins decreased at higher saturation of $(NH_4)_2SO_4$ (>50%) due to the irreversible denaturation of proteins with too much salt concentration. Insignificant changes of yield from 20% to 40% salt saturation were observed due to weaker salting-out effect. The maximum separation efficiency was obtained using 60% saturation of $(NH_4)_2SO_4$ while maximum yield was obtained using 50% saturation of salt. The protein was more likely to be partitioned at higher $(NH_4)_2SO_4$ saturation which leads to higher separation efficiency due to the stronger salting-out effect. Higher saturation of $(NH_4)_2SO_4$ will cause water from the solvation layer around proteins to be dissipated more easily. This will lead to the exposure of hydrophobic patches of protein surfaces, causing the interaction with hydrophobic patches of other protein surfaces to occur. As a result, aggregation and precipitation of proteins would be observed in the intermediate phase (Niphadkar & Rathod, 2015). The results obtained by Niphadkar and Rathod (2015) was slightly lower than the result from this experiment, which is 40% $(NH_4)_2SO_4$. The experiment was conducted under 1:0.5 of crude extract to t-butanol ratio, pH 6, 60% duty cycle with 25 kHz frequency and 3 min irradiation time (Niphadkar & Rathod, 2015). However, it was found that the result obtained was in agreement with the study of Zhang et al. (2017), 50% of $(NH_4)_2SO_4$ was the optimized $(NH_4)_2SO_4$ concentration to recover phycocyanin from *Spirulina* sp. (X. F. Zhang et al., 2017). The conditions performed for the phycocyanin extraction was 1:1 crude extract to t-butanol ratio, pH7 at 25 kHz frequency with 50% duty cycle. The statistical analysis showed that the yield of protein was significantly affected ($p<0.05$) by the salt saturation parameter. Lower protein yield was observed in lower $(NH_4)_2SO_4$ saturation (20%) as it is insufficient to modify the hydrophobic

patches surfaces of proteins. Similar results have been observed in the work of Avhad et al. (2014) where lower recovery and purification fold was found at lower $(NH_4)_2SO_4$ saturation (20% w/v) (Avhad et al., 2014). The highest yield was obtained using 50% saturation of $(NH_4)_2SO_4$, therefore it was selected for the next study.

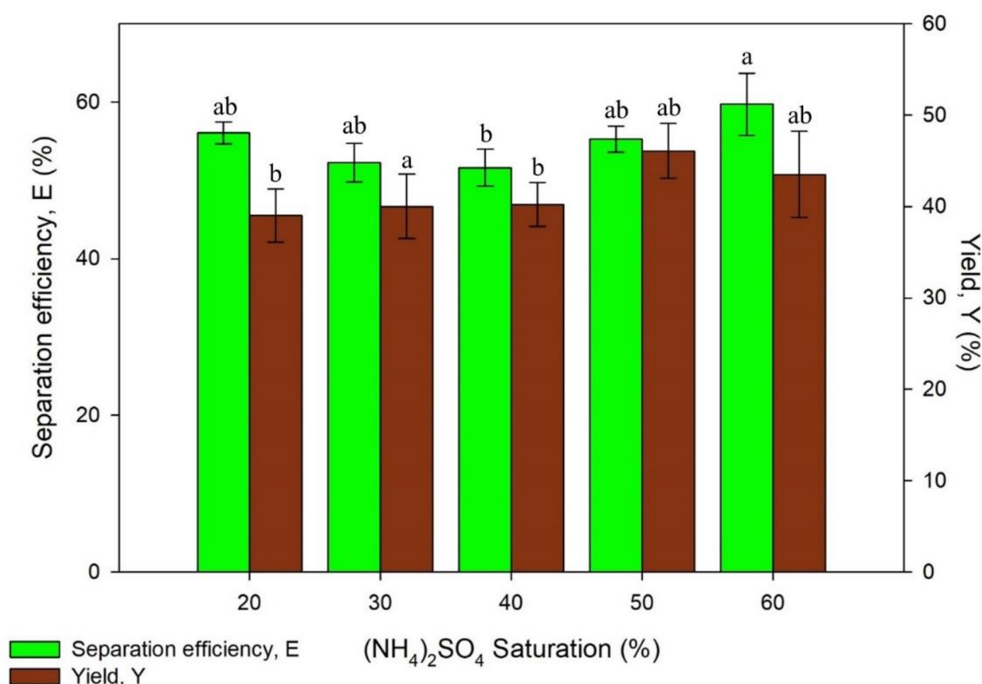


Figure 4.2: Effect of $(NH_4)_2SO_4$ saturation on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

4.4.2.3 Effect of slurry to t-butanol ratio

The alcohol used for UATPP was set as t-butanol as it is commonly used in past studies (P. D. Patil & Yadav, 2018; Yadav, Gupta, & Khare, 2017). Dhananjay and Mulimani (2009) have stated that at room temperature, t-butanol tends to be kosmotropic, contributing to enzyme-t-butanol co-precipitates that

floats above the bottom phase (Dhananjay & Mulimani, 2009). Another unique characteristic of t-butanol is that it could not permeate into folded proteins due to its structure and size, which can avoid protein denaturation (R. Dennison, 2000). The effect of slurry to t-butanol ratio on UATPP of protein was examined by altering the working volume of t-butanol while the slurry remained constant throughout the experiment. The ratio of slurry to t-butanol such as 1:0.5, 1:1, 1:1.5, 1:2 and 1:2.5, was studied with the optimized $(NH_4)_2SO_4$ saturation. The working volume of t-butanol was chosen to be adjusted rather than $(NH_4)_2SO_4$ because previous studies stated that the inherent properties of t-butanol have offered great advantages in partitioned enzyme (Avhad et al., 2014), as it aids in enhancing the protein yield through UATPP. Inherent properties such as higher molecular size and kosmotropic have prevented the denaturation of protein as it is unable to permeate into folded three-dimensional structure of protein and the phase partitioning is enhanced due to kosmotropic effect of t-butnaol (Avhad et al., 2014; C. Dennison & Lovrien, 1997). The effect of slurry to t-butanol ratio on both separation efficiency and yield of protein was illustrated in **Figure 4.3** as followed.

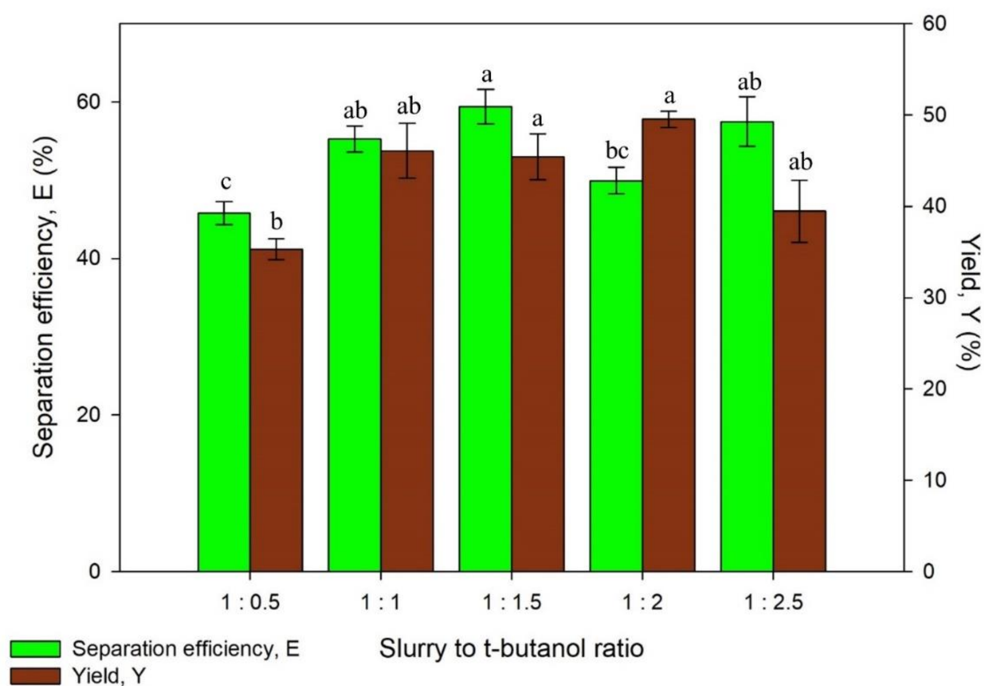


Figure 4.3: Effect of slurry to t-butanol ratio on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

It was observed that the separation efficiency and yield of proteins were rising with increasing slurry to t-butanol ratio from **Figure 4.3**. However, the yield has decreased at the highest ratio (1:2.5). The denaturation of protein was more likely to occur if the volume of t-butanol is high (>1:2), where this was also observed in extraction of enzymes (Narayan et al., 2008). The increment of surface tension caused by high concentration of t-butanol also leads to lower yield. This is due to the high surface tension that has decreased cavitation (Santos, Lodeiro, & Capelo‐Martínez, 2008). Kulkarni and Rathod (2014) have reported similar trends where the yield of mangiferin decreased with the increasing ratio of slurry to t-butanol (Vrushali M Kulkarni & Rathod, 2014).

The separation efficiency of protein using all systems was more than 50% except from the ratio of 1:0.5, in which the synergistic effect of $(NH_4)_2SO_4$ to recover proteins could not be achieved since the volume of t-butanol is much less. On the other hand, the yield obtained was varied through different ratios. The protein yield obtained falls in the range of 35.28 ± 1.16% to 49.50 ± 0.89%, with the highest yield obtained being 1:2 of slurry to t-butanol ratio. Chew et al. (2019) have reported a 1:1 ratio of slurry to t-butanol as the optimum ratio for protein extraction from *Chlorella* sp. which was not in agreement with the obtained result due to different TPP conditions, for example, the optimized salt concentration was 30% in their work (Chew, Chia, Lee, et al., 2019). Thus, the appropriate ratio of slurry to t-butanol should be determined for specific optimized extraction. The ratio of slurry to t-butanol significantly affected the protein yield ($p<0.05$). The optimum value of separation efficiency and yield were obtained using the ratio of 1:2 and this was selected for next study.

4.4.2.4 Effect of ultrasonic power

The ultrasonic power was studied as part of the optimization of sonication in the system. This study was conducted with the optimum values from previous experiments and initial settings of other parameters by varying the power of the ultrasonic bath. The ultrasonic power is an important factor that affects the cost of the process when operated at industrial scale (Niphadkar & Rathod, 2015; X. F. Zhang et al., 2017). On top of that, the ultrasonic power has to be optimized to avoid denaturation of proteins. Generation of large amplitude ultrasonic waves may produce more cavitation bubbles through higher ultrasonic power. The higher cavitation effect may result in denaturation of proteins

although higher mass transfer can be achieved. **Figure 4.4** showed the effect of sonication power on both separation efficiency and yield of protein.

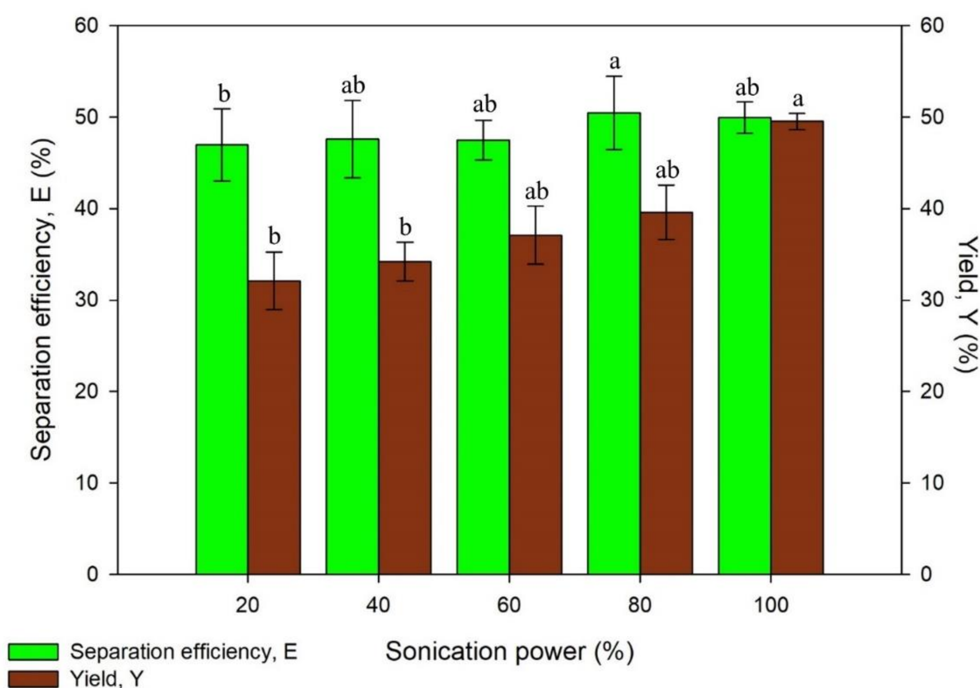


Figure 4.4: Effect of sonication power on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

As illustrated in **Figure 4.4**, the results obtained have shown an increasing trend in the separation efficiency and yield of proteins along with higher ultrasonic power. This is because the collapsing of cavitation bubbles has imparted vicious mechanical shear to the system. The mechanical shear induced resulted in the better disruption of microalgae cell wall and subsequently enhanced the mass transfer and protein yield (X. F. Zhang et al., 2017). The statistical analysis also showed that the recovery of protein was significantly affected ($p<0.05$) by the ultrasonic power. The difference of separation efficiency obtained using 20 to 100% of ultrasonic power did not vary beyond 4%, which was a considerably small difference in values. The separation

efficiency obtained indicates that the partitioning behavior of proteins was not influenced much by the ultrasonic power. However, the yield of proteins considerably increased around 10% using 100% of ultrasonic power compared to the lowest yield obtained, which is more favorable in the process. Thus, 100% of ultrasonic power was chosen for further studies due to the highest yield obtained.

4.4.2.5 Effect of ultrasonic frequency and irradiation time

The study on ultrasonic frequency and time was categorized into two sets, Set A and Set B as listed in **Table 4.1**. Set A consists of irradiation time ranging from 2.5 to 12.5 min with the constant frequency of 35 kHz while Set B consisted of same time range with the frequency of 35 and 130 kHz, both the results obtained are illustrated in **Figure 4.5** and **Figure 4.6**, respectively. Ultrasonic frequency and duration of ultrasonic irradiation was altered as the former regulates power dissipated to the system and the dose of ultrasound was quantified by the latter. The results for yield obtained in 35 kHz shown an increasing trend from 5 to 10 min of irradiation and reduced significantly at 12.5 min.

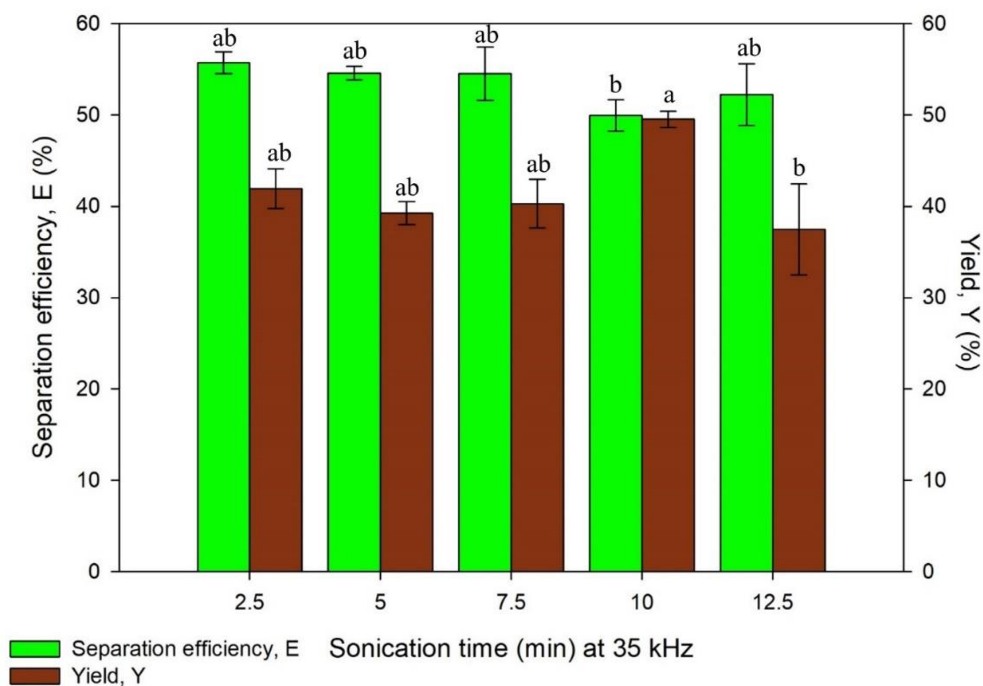


Figure 4.5: Effect of sonication time at frequency, 35 kHz on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). Analysis tests were performed with data in **Figure 4.6** (130 kHz frequency).

As for 130 kHz, the yield was in an increasing trend as irradiation time increased and later decreased after 10 min of irradiation time. The reduction of yield at longer irradiation time may be resulted from the excessive ultrasonic irradiation time that altered the protein conformation and led to the degradation of proteins. Furthermore, the increment of temperature from the long irradiation time would contribute to the degradation and activity loss of proteins. The findings were in agreement with previous studies where UATPP of fibrinolytic enzyme and polyphenol oxide were performed (Avhad et al., 2014; Niphadkar & Rathod, 2015). It was observed that the shorter the irradiation period, lower yield was obtained. This is possibly due to insufficient ultrasonic irradiation to

disrupt the rigid microalgae cell wall. If the ultrasonic irradiation is not sufficient, the agitation could not lead to the formation of large turbulence in the solvent phase, thus the mass transfer could not be enhanced (Niphadkar & Rathod, 2015). In all sets of experiment, the highest yield was obtained at 10 min of irradiation time for lower frequency, 35 kHz. Higher frequency of ultrasonic has decreased the cavitation yield due to formation of smaller and less energetic cavitation bubbles compared to low frequency, leading to lower yield of products according to the work of Kirpalani & McQuinn (Kirpalani & McQuinn, 2006). However, the opposite phenomenon was observed in this study in which higher yield of protein was attained using 130 kHz compared to 35 kHz. As compared to the study performed by Zhang et al. (2017), the higher frequency (40 kHz) obtained lower purity and recovery of phycocyanin compared to the lower frequency (25 kHz) (X. F. Zhang et al., 2017), supporting as well the statement of Kirpalani and McQuinn (2016). The frequency of sonication and irradiation time significantly affected the protein recovery through the statistical analysis ($p<0.05$). There have been reports that concluded low frequency was favorable for biomolecules extraction (Capote & de Castro, 2007; Vrushali M Kulkarni & Rathod, 2014). Highest yield of protein was gained using 10 min of irradiation time with 35 kHz. Therefore, 10 min of irradiation time with 35 kHz of ultrasonic frequency was selected for the next study.

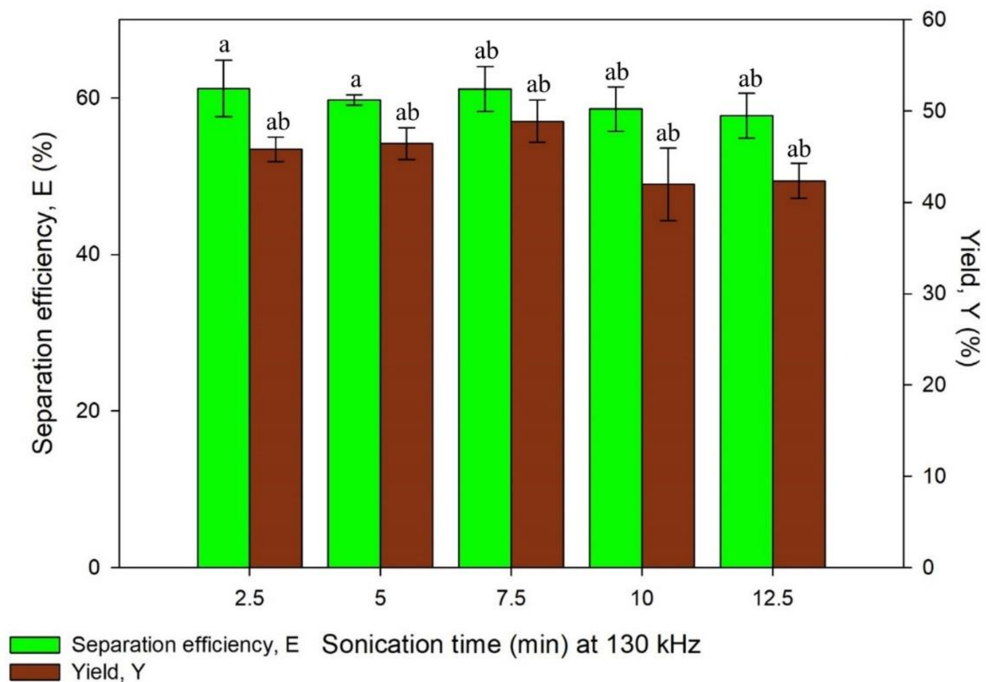


Figure 4.6: Effect of sonication time at frequency, 130 kHz on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). Analysis tests were performed with data in **Figure 4.5** (35 kHz frequency).

4.4.2.6 Effect of duty cycle

Duty cycle was varied by switching the ON and OFF time of ultrasonic process at 35 kHz. Higher percentage of duty cycle has higher fraction of ultrasonic irradiation in one period. It was found that the separation efficiency and yield has increased up to 80% of duty cycle (48 s ON and 12 s OFF) and thereafter it decreases as shown in **Figure 4.7**. This is probably a result of the excessive ultrasonic dose of UATPP. The degradation of protein structure could occur at higher duty cycle as the temperature rose along with the mechanical shear caused by the implosion of cavitation bubbles. The results obtained were in agreement with previous studies, where the phycocyanin recovery decreased

after 90% of duty cycle (X. F. Zhang et al., 2017). However, the purification of serratiopeptidase from *Serratia marcescens* NRRL B 23112 using UATPP only required 40% duty cycle for 5 min to obtain highest purity and recovery (Pakhale & Bhagwat, 2016). The decrement was caused by excessive sonication dose towards the system. Based on the statistical analysis, the duty cycle of sonication significantly affected the protein yield ($p<0.05$). Sonication can be operated in continuous mode or pulse mode. The pulse mode of sonication to intensify bioprocesses shows great potential due to various advantages. It is preferable as the energy utilized in pulse mode is lower than in continuous mode and more energy efficient as unnecessary heat and energy loss can be avoided. It is stated that the pulse mode of sonication was able to intensify the recovery as well as reduce the occurrence of heat sensitive compounds degradation (Vrushali M Kulkarni & Rathod, 2014). On top of that, the lifespan of transducers can be prolonged compared to continuous mode (Dey & Rathod, 2013). Thus, the duty cycle of 80% was chosen as the optimized condition in this study.

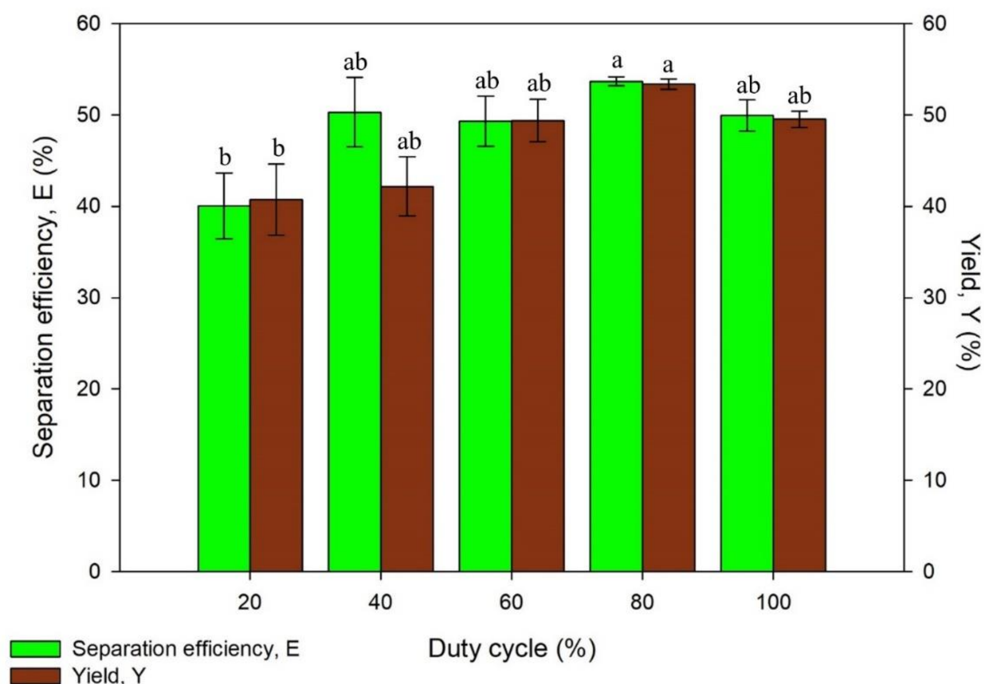


Figure 4.7: Effect of duty cycle on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

4.4.2.7 Effect of biomass loading

Apart from the optimization on sonication, the biomass loading was altered to study its effect on protein extraction via UATPP. The biomass loading in a range of 0.25 to 1.25 wt% were examined. It is known that the yield of proteins could be increased by increasing the biomass loading. The studies related to aqueous two phase system have proven that protein separation is saturated once the biomass loading has achieved the critical quantity (Selvakumar et al., 2010). However, there is no research reporting the effect of biomass loading on UATPP whereby the equilibrium between the three phases could possibly be affected by the weight of biomass loaded into the system.

Figure 4.8 showed the effect of biomass loading on both separation efficiency and yield of protein as followed.

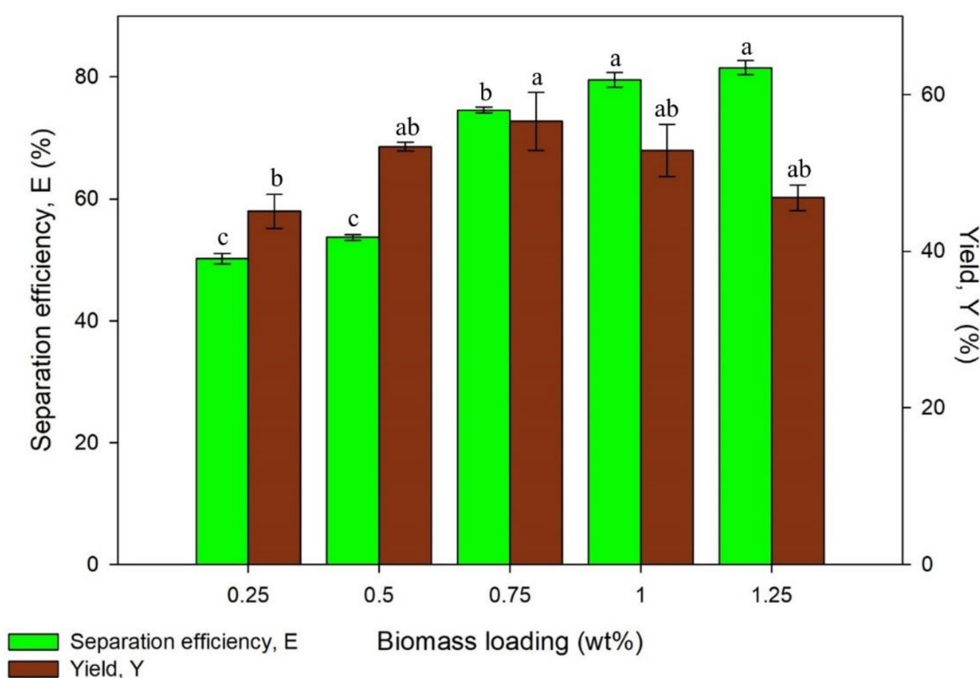


Figure 4.8: Effect of biomass loading on separation efficiency (E) and yield of protein (Y). Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test).

From **Figure 4.8**, an increasing trend was observed from 0.25 wt% to 0.75 wt% of biomass loading and the protein yield was decreased after 0.75 wt% of biomass. The highest yield was discovered at 0.75 wt% of biomass with moderate separation efficiency. It is the optimum biomass loading because the sonication irradiation was fully utilized to disrupt the microalgae cells, and subsequently improved the protein recovery. Besides, it was observed that the lower protein yield has been obtained by lower biomass loading. This is due to the initial quantity of proteins that already exists in the biomass loading is low,

such that the microalgae cell wall was fully disrupted by sonication, and the total amount of proteins present remain the same. It was observed that the proteins recovered at higher biomass loading (> 0.75 wt%) was also decreased. The obtained results were found to be in agreement with work of Chew et al. (2019) where the recovery of protein decreased at higher microalgae percentage, 0.75 wt% (Chew, Chia, Lee, et al., 2019). The biomass loaded to the system has influenced the separation efficiency and yield of protein such that higher loading of biomass into the system leads to uneven distribution of ultrasonic irradiation and may cause the incomplete breakage of cell wall for protein extraction. Therefore, a biomass loading of 0.75 wt% was taken for further studies with the consideration of separation efficiency and yield obtained. The separation efficiency of protein was increasing for higher biomass loading because higher ratio of proteins was separated to the top phase as lesser proteins were released through the breakage of the cell wall. The statistical analysis showed that biomass loading significantly affected ($p<0.05$) the yield of proteins in the system.

4.4.3 Scale Up

The integrated system of sonication and TPP on protein extraction in a larger scale was examined and explored for its feasibility, reliability, as well as the possibility of this technique to be scaled-up. The system was conducted with all the optimized parameters such as 50% of $(NH_4)_2SO_4$ saturation, 1:2 of slurry to t-butanol ratio, 100% of power, 35 kHz of ultrasonic frequency, 10 min of irradiation time, 80% of duty cycle and 0.75 wt% of biomass loading. The large-scale study was performed with the total working volume of 10 times higher than

the lab scale study. A total working volume of 150 mL with 50 mL of slurry and 100 mL of t-butanol were used. The yield obtained in larger scale system (56.57 ± 3.70%) was comparable with the yield obtained with the lab scale system (57.03 ± 1.32%). Relatively high separation efficiency of protein was obtained in large scale as well. Based on the results shown in **Table 4.3**, the reliability of this technique in scale-up has been verified and could be further up-scaled towards an industrial scale.

Table 4.3: Comparative evaluation of other studies using TPP and ultrasound-assisted TPP.

Studies	**Target compound**	**Findings**	**Total working Volume**	**References**
Extraction of protein from *Chlorella* sp. in lab-scale	Protein	A total yield of 56.57% ± 3.70 and 74.59% ± 0.45 separation efficiency were obtained.	10 mL	This study
Extraction of protein from *Chlorella* sp. in large-scale	Protein	A total yield of 57.03% ± 1.32 and 70.88% ± 1.02 separation efficiency were obtained.	150 mL	This study
Recovery of astaxanthin from *Paracoccus* NBRC 101723 using ultrasound-assisted three phase partitioning (UATPP)	Astaxanthin	Recovery of 428 µg/g of wet biomass was achieved using 40% $(NH_4)_2SO_4$, 1:0.75 wet biomass to solvent ratio assisted with sonication as pretreatment using 100% amplitude for 20s.	50, 100 and 250 mL	(Chougle, Singhal, & Baik, 2014)

Table 4.3 (Continue)

Studies	Target compound	Findings	Total working volume	References
Ultrasound-assisted three-phase partitioning of polyphenol oxidase from potato peel (*Solanum tuberosum*)	Polyphenol oxidase	70% recovery of polyphenol oxidase and 6.3 purification factor were obtained using 40% $(NH_4)_2SO_4$, 1:1 extract to t-butanol ratio, pH 7, 40% duty cycle with 25 kHz frequency.	250 mL	(Niphadkar & Rathod, 2015)
Concentration and characterization of microalgae proteins from *Chlorella pyrenoidosa* (three phase partitioning)	Protein	78.1% w/w protein concentration was obtained using 40% $(NH_4)_2SO_4$, pH 6, 1:1.5 of slurry to t-butanol ratio with enzymatic treatment (combination of Stargen and Carezyme).	40 mL	(Waghmare et al., 2016)
Ultrasound-assisted three phase partitioning of phycocyanin from *Spirulina platensis*	Phycocyanin	94.3% recovery of phycocyanin with 6.69 purification factor were obtained using 50% $(NH_4)_2SO_4$, 1:1 crude extract to t-butanol ratio, pH 7, 50% duty cycle with 25 kHz frequency.	20 mL	(X. F. Zhang et al., 2017)

A comparative study was performed using the results obtained in current study and literature from previous studies. There are three studies that found that 40% $(NH_4)_2SO_4$ was the suitable salt saturation % for recovering their targeted compounds (Chougle et al., 2014; Niphadkar & Rathod, 2015; Waghmare et al., 2016), which was not in agreement with our findings. It was found that most of the condition utilized in the mentioned studies was not similar with the current

study and the recovery of compounds obtained was generally higher. The recovery of protein in Waghmare et al. (2016) was performed with enzymatic treatment which requires higher slurry to t-butanol ratio compared to this study in order to achieve optimum results (Waghmare et al., 2016). The results obtained in the study of Zhang et al. (2017) was similar with results obtained in current study, which requires 50% $(NH_4)_2SO_4$ and low irradiation frequency (25 kHz) to recover phycocyanin from microalgae, a different type of protein (X. F. Zhang et al., 2017).

4.4.4 Field Emission Scanning Electron Microscopy (FESEM) Characteristics of Microalgae Surfaces

Figure 4.9 shows the FESEM images of the surface of microalgae before sonication treatment, after sonication treatment (with water as medium) and after UATPP treatment (with solvents of UATPP). In **Figure 4.9(A)**, the surfaces of microalgae before any sonication treatment were observed to be smooth and round-shape cells that are clumped on the surface. After treatment with sonication, the microalgae cells shrunk and became rougher compared to the microalgae cells without ultrasonic treatment (**Figure 4.9(B)**). However, the structure of microalgae was more severely altered, and became flat and more shrunken after the treatment with UATPP (**Figure 4.9(C)**). This indicates that UATPP can deal more disruptive damage to the microalgae cell wall compared to treatment with sonication only, where the use of TPP solvents can further help to recover higher portions of the compounds in microalgae. The obtained results have proven that UATPP is very effective in disrupting the microalgae cells and leads to the enhancement of protein extraction.

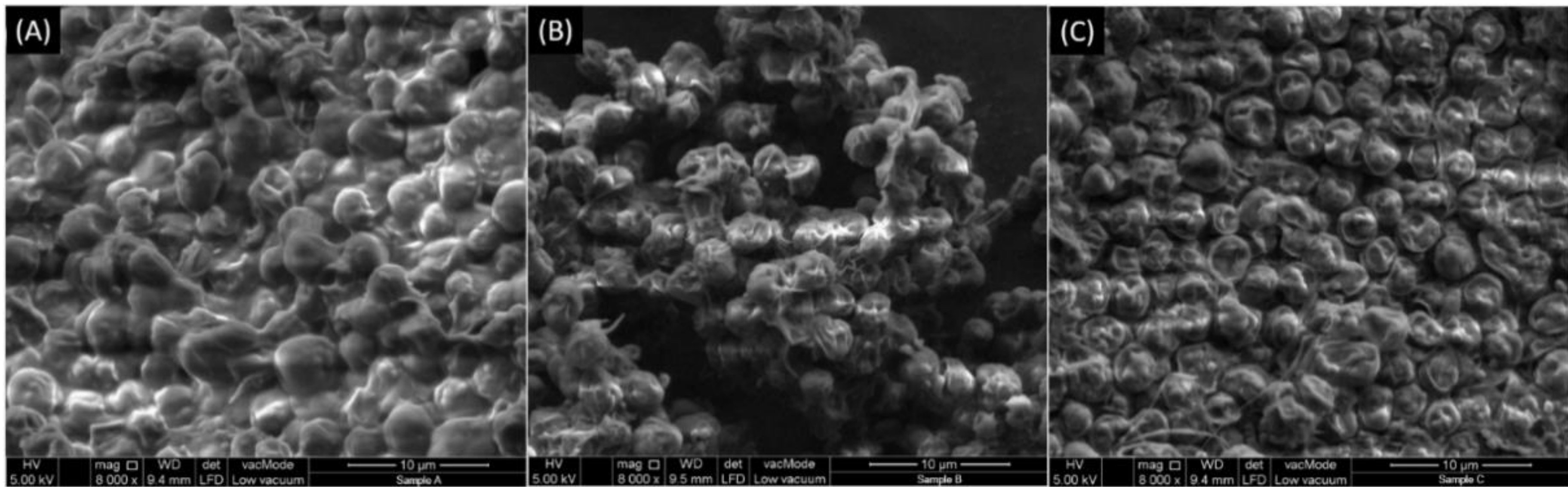
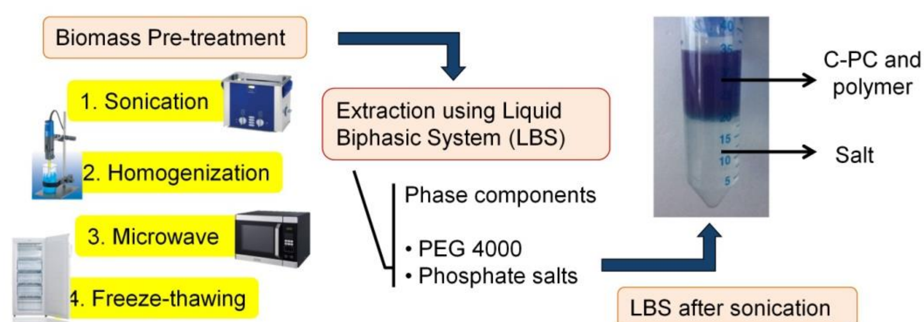


Figure 4.9: FESEM images of microalgae cells (A) before sonication treatment; (B) after ultrasound with water as medium; (C) after ultrasound with UATPP medium.

4.5 Conclusion

In this work, proteins from *Chlorella vulgaris* FSP-E was successfully extracted using sustainable bioprocessing technique, ultrasound-assisted three phase partitioning. Several parameters such as types of salt, ratio of slurry to t-butanol, salt saturation, sonication frequency, power, irradiation time, duty cycle and biomass loading were studied. UATPP was found to be an improved technique compared to TPP, where UATPP has obtained around 15% higher yield and 2.5% higher separation efficiency of protein compared to TPP. The optimized conditions are 50% of salt saturation, 1:2 of slurry to t-butanol ratio, 100% of sonication power, 10 min of irradiation time, 35 kHz of sonication frequency, 80% of duty cycle and 0.75 wt% of biomass loading in gaining the maximized 74.59 ± 0.45% of separation efficiency and 56.57 ± 3.70% of protein yield. The enhanced yield and separation efficiency with reduced processing time and capability to be scaled up, indicates that UATPP is an effective approach for future integrated bio-separation technique for biomolecules extraction from microalgae. The optimized conditions for protein recovery was identified and discussed to move towards the realization to produce low cost nutritional food for the society as well as to develop and improve the current downstream bioprocessing techniques.

CHAPTER 5 *SPIRULINA PLATENSIS* BASED BIOREFINERY FOR THE PRODUCTION OF VALUE-ADDED PRODUCTS FOR FOOD AND PHARMACEUTICAL APPLICATIONS



This chapter covers the application of liquid biphasic system as the extraction and purification method for the targeted compound from microalgae, c-phycocyanin. Four types of cell disruption methods were studied as the pre-treatment of microalgae biomass prior to extraction and compared for the maximal recovery of c-phycocyanin and purification factor. Parameters for each cell disruption method were optimized while the parameters of liquid biphasic system were fixed. The molecular weight characterization of c-phycocyanin obtained after the process of optimum pre-treatment method and liquid biphasic system was performed. This chapter consists of a thesis-version of the manuscript submitted to the Bioresource Technology journal (Chia S. R. et al., 2019, *Bioresource Technology*).

5.1 Abstract

In this present study, microalgal phycobiliproteins were isolated and purified via potential biphasic processing technique for pharmaceutical as well as food applications. The algal pre-treatment techniques were studied to enhance the yield of microalgal phycobiliproteins from the biomass. The proposed methods were optimised to obtain the best recovery yield of phycobiliproteins that can be isolated from the biomass. The phycobiliproteins were further purify using liquid biphasic system. The results showed that microalgal phycobiliproteins of high purity and yield was achieved using sonication treatment (20% power, 50% duty cycle and 7 min of irradiation time) with the biphasic system, where the purification fold was 6.17 and recovery yield was 94.89%. This work will provide insights towards the effective downstream processing of biomolecules for microalgae.

Keywords

Microalgae; Phycobiliproteins; Downstream Processing; Purification; Extraction

5.2 Introduction

In past decades, researchers have focused on the discovery of high valuable compounds from natural resources in replacing the chemicals used in daily life. The researches were initiated due to the biohazard and toxicity of chemicals used which required a cheaper, safer and accessible natural bioactive compounds. Natural bioactive compounds are often existed in natural sources such as plant, animal, fungi, even cyanobacteria. In the search of natural bioactive compounds, microalgae as a third generation of renewable fuel feedstock has attracted attention from the industries as well as researchers, mainly due to the high areal oil productivity, rapid biomass accumulation and non-competitive with other food crops (Vanthoor-Koopmans, Wijffels, Barbosa, & Eppink, 2013). These features have encouraged the researchers to explore more about microalgae as the potentiality seen in the future. Clearly, the studies showed that the content of valuable compounds within microalgae is remarkable. It is reported that the microalgae contain constituents like protein, lipid, carbohydrate, vitamins and phycobiliproteins which can be applied in pharmaceutical, cosmeceutical, and bioenergy applications (Chew et al., 2017; Y. Xie et al., 2015).

Among the valuable compounds aforesaid, phycobiliproteins is the highly demand compound in immunological analysis and fluorescent applications in clinical (Moraes & Kalil, 2009). Phycobiliproteins were categorised into phycocyanin, allophycocyanin and phycoerythrin, acting as the major component of light-collecting complexes in particular microalgae (Kannaujiya, Kumar, Pathak, & Sinha, 2019). C-phycocyanin is the major

photosynthetic pigment from *Spirulina* sp. while allophycocyanin only exist in minor portion in *Spirulina* sp. (C.-Y. Chen, Kao, Tsai, Lee, & Chang, 2013). Both of the pigments are blue coloured which can be used as natural dyes and fluorescent marker in biomedical research (Oi, Glazer, & Stryer, 1982). Microalgal phycobiliproteins were seek as potential substitution of therapeutic agent in oxidative stress-induced disease and utilization as functional food. Furthermore, these pigments have biological activities such as hepatoprotective, neuroprotective, anti-inflammatory, antioxidants and antitumor which can be beneficial to human health (Chaiklahan, Chirasuwan, Loha, Tia, & Bunnag, 2011).

In order to isolate the C-phycocyanin from the microalgae, the extraction method has played a major role to maximise the recovery of pigments. Processes such as rupturing the microalgae cell and releasing the pigments from the cells are involved in the extraction of phycobiliproteins. The extraction methods are significant for this particular pigments as cyanobacteria is tremendously resistant for disruption of their cell wall (Sivasankari & Ravindran, 2014). The conventional methods in recovering C-phycocyanin have been studied such as Soxhlet extraction or maceration required large solvent quantity, longer processing time but low recovery obtained (Vernès, Granvillain, Chemat, & Vian, 2015). The high consumption of toxic solvent can be a burden to the environment and longer processing time meaning a loss of profit. Higher yield of the C-phycoycanin recovered from the biomass will lead to cheaper production cost. Therefore, a better alternative has to be introduced to overcome the mentioned drawbacks in producing C-phycocyanin in industrial scale.

Liquid biphasic system (LBS) is an economical downstream processing technique that is designed for the large scale of protein extraction and purification. This technique was studied intensively over decades as a separation technology without denaturing the biological products. It was reported that LBS capable to isolate various type of components such as alkaline phosphatase from *Bacillus licheniformis* MTCC 1483 (Pandey & Banik, 2011), lipase from *Burkholderia cenocepacia* strain ST8 (P. L. Show, Tan, Anuar, et al., 2012) even separate sugars and hydrolytic enzymes to avoid enzymatic inhibition (Bussamra et al., 2019). Several types of LBS are generally utilised for biomolecules separation, for example polyethylene glycol (PEG)/PEG, PEG/salt, alcohol/salt, acetonitrile/sugar and ionic liquid/salt (Raghava Rao & Nair, 2011). The efficiency of biomolecules extraction mostly depends on these parameters: the phase forming components, pH, volume ratio and load of biomass. The advantage of this technique includes the simple, scalable operation, environmental-friendly and most important, is highly efficient in concentrating and purifying biomolecules. Therefore, this technique is highly recommended to apply in extracting C-phycocyanin from *Spirulina* sp.

The objective of this research is to maximise recovery and purity of C-phycocyanin from *Spirulina* sp. through LBS with the assist of different pre-treatment methods. Pre-treatments such as sonication, microwave, homogenisation and freeze-thawing were performed to observe the compatibility of these cell disruption methods with LBS in enhancing the recovery of C-phycocyanin. The pre-treatments were evaluated through the studies of time, duty cycle, speed and power used. The combination of phase components for

LBS were obtained from literature and the parameters were fixed throughout the study to ensure the consistency of the experiments conducted (F. S. Antelo, A. Anschau, J. A. Costa, & S. J. Kalil, 2010). This study is to discover an effective method to extract and purify the C-phycocyanin with greater yield compared to conventional method.

5.3 Materials and Methods

5.3.1 Chemicals and Reagents

Phosphate buffer, potassium dihydrogen phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4) and polyethylene glycol 4000 (PEG 4000) were purchased from R&M chemicals (Malaysia). Dried *Spirulina* sp. powder was purchased from Charming & Beauty Co. Ltd. (Taipei, Taiwan). All the chemicals used were analytical grade. The algae were disrupted through different pre-treatments before subjected to Liquid Biphasic System (LBS). An UV-Vis spectrophotometer (Shimadzu Corporation, Japan) was used to measure the absorbance of the C-phycocyanin.

5.3.2 Cell Disruption Methods

Four types of cell disruption methods were studied and evaluated in this work, such as microwave, sonication, homogenisation as well as freeze-thawing. A small scaled system was performed for sonication, microwave and freeze-thawing, where a total weight of 100 mg *Spirulina* sp. powder was measured using analytical balance prior to pre-treatment. The weighed samples were mixed with phosphate buffer in a 15 mL centrifuge tube. For homogenisation, a total weight of 300 mg sample was measured and mixed with phosphate buffer.

Higher volume of sample was used in homogenisation due to the equipment used for disrupting *Spirulina* sp. The mixture of sample and phosphate buffer was vortexed using Vortex Genius 3 (Ika, Germany) for homogenisation. The mixture was placed into a 4 ^{o}C refrigerator overnight prior to analysis after pre-treatment. All the experiments were performed triplicate. The operating parameters and variables of the studies were listed in **Table 5.1**.

Table 5.1: Parameters studied in various pre-treatment methods.

No.	Type of pre-treatment	Operating parameters	Initial settings	Variables	Unit
1.	Sonication	Power	20	20, 40, 60, 80, 100	%
		Duty Cycle (ON/OFF)	100	25 (15/45), 50 (30/30), 75 (45/15), 100 (60/0)	% (s/s)
		Time	5	3, 5, 7, 9, 11	min
2.	Microwave	Duty cycle (ON/OFF)	100	25 (15/45), 50 (30/30), 75 (45/15), 100 (60/0)	% (s/s)
		Time	3	1, 2, 3, 4, 5	min
3.	Homogenisation	Time	5	1, 3, 5, 8, 10	min
		Speed	1	1, 2, 3, 4	N/A
4.	Freeze-thawing	Time	-	8, 16, 24, 48	h

5.3.2.1 Sonication

The sonication was performed using the ultrasonic water bath (Transsonic TI-H-15, Malaysia). Initially, the sample was treated with sonication continuously for 5 min with the power of 20%, 40%, 60%, 80% and 100%,

operating at 35 kHz. Parameters to be optimized for sonication were power, duty cycle and time.

5.3.2.2 Microwave

The microwave was performed using a microwave oven (Samsung, Malaysia). The microwave pre-treatment was conducted at 100 W for 2 min with different duty cycle, such as 25%, 50%, 75% and 100%. The parameters to be optimized were duty cycle and time.

5.3.2.3 Homogenisation

The sample was treated with Homogeniser (Ika Ultra Turrax T18, Germany). The prepared mixture was poured into 100 mL glass beaker and homogenised at a speed of 1 (3500 rpm). The other speeds of homogeniser studied: 2, 3 and 4 are equivalent to 7000, 11000 and 15500 rpm, respectively. The homogenisation time was tested in a range of 1, 3, 5, 8, 10 min. The parameters to be tested for homogenisation were time and speed.

5.3.2.4 Freeze-thawing

The mixture was placed in the -20 ^{o}C refrigerator for 8 hours. After 8 hours, the mixture was taken out from the refrigerator to thaw at room temperature. To fasten the thawing process, the centrifuge tube contained the mixture was placed inside a beaker filled with water for 20 min. The mixture was taken out every 24 hours for the set parameter, 48 hours. For this pre-treatment, time is the only parameter to be optimized.

5.3.3 Preparation of Liquid Biphasic System

Liquid Biphasic System (LBS) was performed after the pre-treatment of mixture. The system was constructed by polyethylene glycol (PEG) 4000 and potassium dihydrogen phosphate (KH_2PO_4) in order to obtain the recovery of C-phycocyanin (Francine S Antelo et al., 2010). The effect of pre-treatment could be observed if the extraction method remained similar for every sample, therefore the system used for each sample has fixed parameters such as phase constructing components, stirring time and volume ratio of the phases. The phase systems were prepared in centrifuged tubes by weighing 25 w/w% of PEG 4000 and 20 w/w% of KH_2PO_4. The weighed PEG 4000 was dissolved by deionized water using vortex. Since C-phycocyanin was sensitive to extreme pH values, the pH of salt phase was adjusted using dipotassium hydrogen phosphate (K_2HPO_4) to pH 7. The sample extracts were added into the systems prepared and stirred at 300 rpm for 1 h. The LBS was performed in darkness and room temperature (25 ^oC) since C-phycocyanin can be influenced by light, pH and temperature. LBS was left for another 1 h after stirring in order to stabilize both phases prior to analysis. The volume of phases was measured after the separation. The concentration of C-phycocyanin in top (polymer) and bottom (salt) phase were tested using UV-Vis spectrophotometer. All tests were performed in triplicate.

5.3.4 Determination of C-phycocyanin

The concentration of C-phycocyanin was defined according to Bennett and Borogad (1973) (Bennett & Bogorad, 1973), as stated as below:

$$PC = \frac{[OD_{615}\text{-}0.474 \times OD_{652}]}{5.34} \quad (1)$$

where PC is the C-phycocyanin concentration (mg mL^{-1}), OD_{615} is the optical density of the sample at 615 nm and OD_{652} is the optical density of the sample at 652 nm. The purity of C-phycocyanin was calculated as below:

$$\text{Purity} = \frac{\text{OD}_{620}}{\text{OD}_{280}} \quad (2)$$

where OD_{280} is the optical density of the sample at 280 nm and OD_{620} is the optical density of the sample at 620 nm. The absorbance of 620 nm represents the concentration of C-phycocyanin while the absorbance of 280 nm represents the total concentration of proteins in the extract. Equation (2) indicates the C-phycocyanin purity with respects to various forms of contaminating protein. The purification factor of C-phycocyanin was calculated as below:

$$P_F = \frac{\text{Purity}_P}{\text{Purity}_C} \quad (3)$$

where P_F is the purification factor of C-phycocyanin, $Purity_P$ is the purity of extract after LBS and $Purity_C$ is the purity of crude extract. Recovery of C-phycocyanin (%) was calculated as stated below:

$$R\ (\%) = \frac{[PC_{Phase} \times V_{Phase}]}{(PC_{TOP} \times V_{TOP}) + (PC_{BTM} \times V_{BTM})} \times 100\% \quad (4)$$

where PC_{Phase} is the concentration of C-phycocyanin in the top phase, PC_{TOP} is the concentration of C-phycocyanin in top phase, PC_{BTM} is the concentration of C-phycocyanin in bottom phase, V_{Phase} is the volume of top phase, V_{TOP} is the volume of top phase and V_{BTM} is the volume of bottom phase.

5.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Analysis (SDS-PAGE)

SDS-PAGE was performed by using 12% Mini-Protean TGX Precast Protein Gels system (Bio-Rad) with the purified sample extracts according to their respective analytical standards. A total amount of 15 µl sample solution which contained loading buffer and fraction from LBS was loaded into each well. The 50 mM of Tris-MOPS buffer which consisted of 50 mM Tris, 50 mM MOPs, 1 mM EDTA and 0.1% (m/v) SDS was utilised as electrode buffer. The slab gel was stained in solution containing 0.05 w/w% of Coomassie Brilliant Blue R250 after the electrophoresis and destained using deionized water. The purified sample extracts were visualized using Chemidoc Touch Imaging System.

5.4 Results and Discussion

5.4.1 Sonication

5.4.1.1 Power

Spiruline sp. was pre-treated by sonication using different sonication power, such as 20%, 40%, 60%, 80% and 100%, and followed by LBS. This cell disruption method has been proven by many studies in breaking down the cell wall of *Spirulina* sp. (W. Choi & Lee, 2018; Furuki et al., 2003; Caroline Costa Moraes, Luisa Sala, Guido Picaluga Cerveira, & Susana Juliano Kalil, 2011). The higher the degree of sonication power, the more cavitation bubbles were created and collapsed, resulting shock wave as well as mechanical shear in the medium. The cavitational phenomenon is able to destroy the resistant multi-layered cell walls of *Spirulina* sp. and aid in the release of C-phycocyanin

(Avhad et al., 2014). The power of sonication was altered as the first parameter as it is one of the main factors in influencing the cost of operation at larger scale.

The purification factor (P_F) and the recovery (R) % of C-phycocyanin using various sonication power were displayed in **Figure 5.1(a)**. C-phycocyanin was found to be recovered mostly in the top phase (PEG) compared to the bottom phase (salt) of system (data not shown). From **Figure 5.1(a)**, it was observed that the higher sonication power has led to lower P_F and R% of C-phycocyanin. The obtained data may result by the denaturation of C-phycocyanin at higher power than 20% of sonication. Highest P_F of C-phycocyanin was discovered as 4.90-fold with the highest R% obtained was 85.05% of C-phycocyanin at 20% of sonication power. The lowest P_F and R% was found at 80% of sonication power. The obtained results have proved that the cell wall of *Spirulina* sp. could be ruptured with only 20% of sonication power and no excess energy was required. Similar result was reported by Entezari et al. (2004) where low sonication power was the suitable power to extract the date syrup (Entezari, Hagh Nazary, & Haddad Khodaparast, 2004). A similar R% was obtained at 60% and 100%, but the P_F was varied in these parameters (where a difference of 7% was observed). In general, 20% of sonication power was chosen for the next parameter based on the P_F and R% of C-phycocyanin obtained.

5.4.1.2 Duty cycle

Duty cycle was examined in the pre-treatment of *Spirulina* sp. using sonication. The optimum sonication power, 20% was set in this study while the duty cycle has been altered to 25%, 50%, 75% and 100% accordingly. Duty

cycle was studied in this work for the reason that utilising energy efficiently as well as prolonged the lifespan of the ultrasonic transducers (Avhad et al., 2014). 100% duty cycle for sonication represented the continuous mode where sonication was performed non-stop within the proposed time while other duty cycles were pulse mode.

Figure 5.1(b) has illustrated the recovery (R) % and purification factor (P_F) of C-phycocyanin using the proposed duty cycles. It was noted that the R% of C-phycocyanin obtained at all duty cycles were above 80% while the P_F falls into 3.59 to 4.95-fold of C-phycocyanin. The sonication in pulse mode (25%, 50% and 75%) and continuous mode (100%) were able to disrupt the cell wall of *Spirulina* sp. effectively (Caroline Costa Moraes et al., 2011), and able to yield such a high R and P_F of C-phycocyanin with the aid of LBS in current study.

The highest R% of C-phycocyanin was obtained at 50% of duty cycle with a purity-fold of 4.96, whereas the lowest R% was obtained at 25% duty cycle. The obtained C-phycocyanin has better quality than other studies (Caroline Costa Moraes et al., 2011; Vali Aftari, Rezaei, Mortazavi, & Bandani, 2015a). The lowest R% of C-phycocyanin was obtained at 25% duty cycle due to the lesser number of times for the sonication performed, which might result into lower amount of C-phycocyanin released from the biomass sample. It was reported that duty cycle lower than 100% (which was pulse mode) could perform better in extraction as the continuous sonication has led to the degassing of cavitation bubbles, causing lower R% to be obtained (P. S. Kumar et al., 2000).

Chia et al. (2019) has concluded that the pulse mode (5s ON/ 10s OFF) in a duration of 5 min sonication has obtained around 20% higher yield of protein than 5 min of continuous sonication (Chia et al., 2019). In conclusion, the optimum duty cycle to extract C-phycocyanin was 50% duty cycle of sonication.

5.4.1.3 Time

This study has been performed by changing the sonication time (3 min, 5 min, 7 min, 9 min, 11 min) with 50% duty cycle, 20% of sonication power and 30 kHz of sonication frequency. The irradiation time quantifies the dose of sonication; therefore the optimum irradiation time has to be examined for C-phycocyanin extraction. The irradiation time would influence the recovery of C-phycocyanin as longer irradiation time can contribute to higher temperature. The high temperature would breakdown the hydrogen bonding interaction between amino acid residue and surface water of protein, led to the decrement of purity ratio (X. Zhang, Zhang, Luo, Yang, & Wang, 2015).

The obtained data of purification factor (P_F) and recovery (R) % of C-phycocyanin using different sonication irradiation time was presented in **Figure 5.1(c)**. Longer sonication irradiation time has improved the P_F and R% of C-phycocyanin, resulting to the average value of 5.56 of P_F and 91% of R% in the tested range of sonication time (Gerde et al., 2012). The study of Eldalatony et al. (2016) has illustrated that the total reducing sugars (mg/g) recovered from microalgal biomass at 30 min was around 2-fold of the total reducing sugars obtained at 15 min (Eldalatony et al., 2016). In additional, Gerde et al. (2012) has reported that the increased of energy input resulted by longer sonication time

treatment would improve the extraction of intracellular materials within microalgal biomass (Gerde et al., 2012).

From **Figure 5.1(c)**, the purity fold and R% of C-phycocyanin was discovered to be lowest at least irradiation time, 3 min while the highest P_F, 6.34-fold of C-phycocyanin was discovered using 9 min of irradiation time. The P_F obtained from 7 min and 11 min of sonication time only had 0.3% difference, and the R% is differed by 0.7%. After considering both the P_F and R%, the data with higher R% which was 7 min was chosen to be the optimum time for this study.

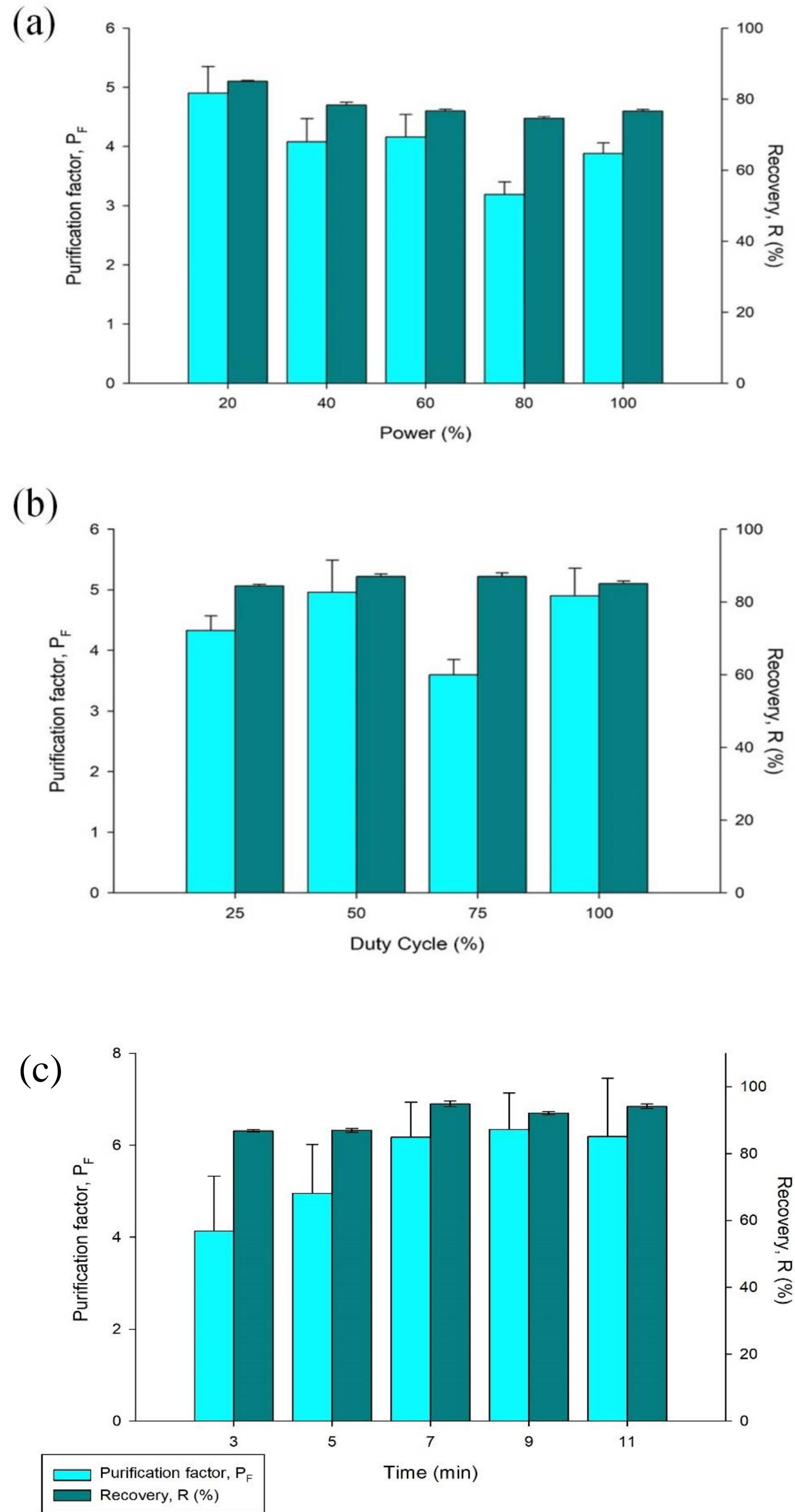


Figure 5.1: Recovery and purification factor of C-phycocyanin using sonication.

5.4.2 Microwave

5.4.2.1 Duty cycle

Microwave was the second proposed cell disruption method to extract C-phycocyanin as microwave is a powerful tool for sample preparation or pre-treatment compared to traditional extraction methods (Reyes et al., 2009). The first parameter to be manipulated was duty cycle. A similar range of duty cycle with **Section 5.4.1.2** was altered in this study, 25% to 100% duty cycle of microwave irradiation. In this study, other parameters such as power and time were fixed at 100 W and 3 min of irradiation period throughout the experiment to observe the effect of duty cycle towards C-phycocyanin extraction.

Figure 5.2(a) has presented the calculated results for purification factor (P_F) and recovery (R) % of C-phycocyanin at several duty cycles of microwave. It was discovered that the purity fold of C-phycocyanin increasing from 25% to 50% duty cycle but decreasing from 75% to 100% duty cycle of microwave irradiation. The study of Chew et al. (2019) was shown that the recovery yield of protein decreased caused by the prolonged dose of microwave irradiation (Chew, Chia, Lee, et al., 2019). Duty cycle less than 100% were pulse mode, which could lower the molecular friction that may rupture the protein content in sample extracts (Chia et al., 2019). The least P_F of C-phycocyanin was obtained at 100% duty cycle, whereby the highest P_F of C-phycocyanin was found at 50% duty cycle. The highest R% of C-phycocyanin was corresponded to the purity fold of C-phycocyanin extracted, which were 83% of recovered C-phycocyanin with 3.99-fold of crude extract. The R% obtained at 75% duty cycle has the lowest R% but considerably high purity fold was achieved. In general, the C-

phycocyanin extracted from the biomass pre-treated with microwave was having purity fold above 3 and R% above 70% which was considerably high compared to other studies (Juin et al., 2015; Vali Aftari et al., 2015a). Kulkarni and Rathod (2015) has reported that 50% duty cycle was the optimum duty cycle for extracting mangiferin with an irradiation time of 5 min, which was in agreement with the obtained data (Vrushali M. Kulkarni & Rathod, 2015). As a conclusion, the duty cycle of 50% was selected among the studied duty cycles for further optimisation study.

5.4.2.2 Time

The microwave irradiation time study was conducted after the optimisation of microwave duty cycle. The range of time selected for this study was 1 min to 5 min due to the microwave heating caused by electromagnetic radiation. The activity of C-phycocyanin was not able to be sustained under high temperature, therefore the period of samples exposed to the microwave needed to be examined and kept in short irradiation time (Juin et al., 2015).

In **Figure 5.2(b)**, the purification factor (P_F) and recovery (R) % of C-phycocyanin at different irradiation time were illustrated. C-phycocyanin was able to be purified into 4.15-fold of the crude extract by using 2 min of irradiation time. The irradiation time required for microwave for cell disruption often very short compared to other techniques (Sparr Eskilsson & Björklund, 2000). This also observed in the work of Pan et al. (2003) which the best recovery of tea caffeine was gained at 4 min of irradiation time and decreased with irradiation time more than 4 min (X. Pan, Liu, Jia, & Shu, 2000). In additional, the R% of

C-phycocyanin at 2 min of irradiation time was the highest amongst all tested variables, where 88.48% of C-phycocyanin was recovered. At 1 min of irradiation time, the cell wall of *Spirulina* sp. was not disrupted as much as other irradiation time, resulting to a lower R% of C-phycocyanin had been obtained. This result could relate to the past literature as 1 min of microwave irradiation was not sufficient to obtain highest recovery of targeted biomolecules (Tong, Gao, Xxiao, & Pan, 2012). Aftari et al. (2015) were verified that the enhanced C-phycocyanin concentration could be obtained with 25 min of microwave irradiation (Vali Aftari et al., 2015a). As observed from **Figure 5.2(b)**, the R% of C-phycocyanin at 5 min was similar with R% obtained at 2 min, but with a lower P_F of C-phycocyanin. It was stated by Kovacs et al. (1998) that longer irradiation time did not contribute to higher extraction recovery of amino acid (Kovács, Ganzler, & Simon-Sarkadi, 1998). Thus, the optimum irradiation time for this study was 2 min.

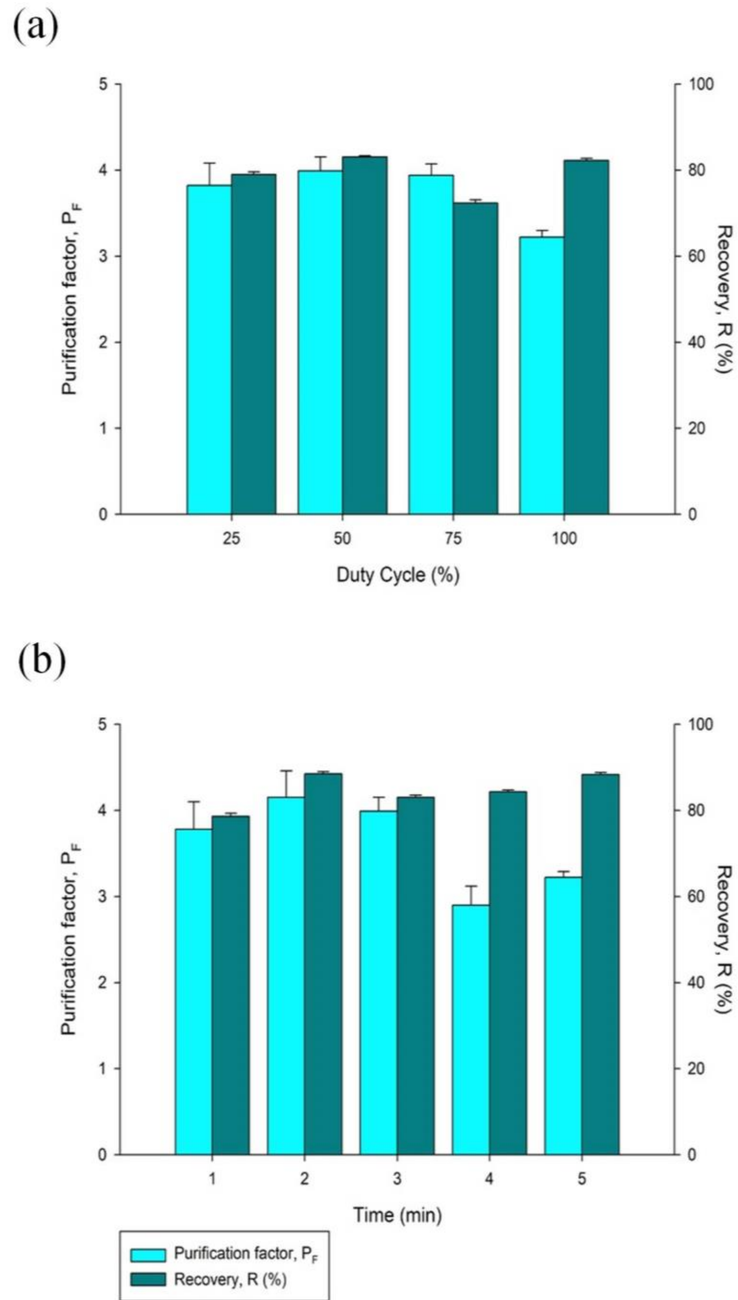


Figure 5.2: Recovery and purification factor of C-phycocyanin using microwave.

5.4.3 Homogenisation

5.4.3.1 Time

Homogenisation is a type of mechanical disruption method, and it is categorized as bead-based homogenisation and rotor-stator disruption. In this work, the homogenisation applied was rotor-stator disruption that housing a rapidly moving rotor by a stationary stator. This method could be used to disrupt animal and plant tissue effectively, even the resistant algae cell wall through the shearing force created by the equipment. Time was studied as one of the parameters due to the operating cost for the energy usage in the whole process. The range of time determined were 1 min, 3 min, 5 min, 8 min and 10 min while the speed of homogeniser was set as 1 for this study.

The purification factor (P_F) and recovery (R) % of C-phycocyanin obtained from different homogenisation period were shown in **Figure 5.3(a)**. The P_F of C-phycocyanin in this study was generally above 4.1-fold, representing that the cell wall of *Spirulina* sp. was successfully disintegrated through this method. It was observed that the R% of C-phycocyanin decreased as the time of homogenisation prolonged. Guedes et al. (2013) has stated that mechanical disruption method has to be gentle in rupturing the cell wall of biomass to avoid the denaturation of intracellular compounds (Guedes, Amaro, Gião, & Malcata, 2013). The P_F of C-phycocyanin was lowest with the least homogenisation period (1 min), which probably resulted by less C-phycocyanin was released from the cells compared to other homogenisation periods. The obtained result was in agreement with Horvath et al. (2013) whereby low extraction efficiency was obtained for homogenisation lower than 2 min (Horváth et al., 2013). The

C-phycocyanin was having the best P_F and R% at 3 min of homogenisation, around 4.3-fold and 89%. Thus, 3 min of homogenisation was decided to be applied in the next parameter.

5.4.3.2 Rotor's speed

In this study, the homogenisation was performed with 3 min of homogenisation and speed of rotor varied from 1 to 4. Other speeds of the rotor such as speed 5 and 6 were not determine in this study as heat will be generated through the high speed of rotor, which probably leads to denaturation of C-phycocyanin. **Figure 5.3(b)** has displayed the purification factor (P_F) and recovery (R) % of C-phycocyanin obtained using various rotors' speed at 3 min of homogenisation. The R% of C-phycocyanin was similar using rotor's speed of 1 to 3 but the R% of C-phycocyanin was gradually decreased using rotor's speed of 4. The decrement in R% may cause by the heat generated during pre-treatment of sample extract, and damaged parts of the C-phycocyanin in sample solution. In additional, lowest C-phycocyanin's purity fold was observed at the rotor's speed of 4 as well, which is 4.08. A 4.6 purity-fold was obtained at the highest R% of C-phycocyanin (90.33%), which was obtained through a rotor's speed of 2. However, the highest P_F of C-phycocyanin was around 5.6-fold, with a slightly lower R% of C-phycocyanin obtained, which was 89.51% by a rotor's speed of 3. Recovery and purity levels of proteins should be considered in optimizing the extraction and purification parameters (Berg, Tymoczko, & Stryer, 2002). Therefore, the optimum rotor's speed selected in recovering and purifying C-phycocyanin was 3.

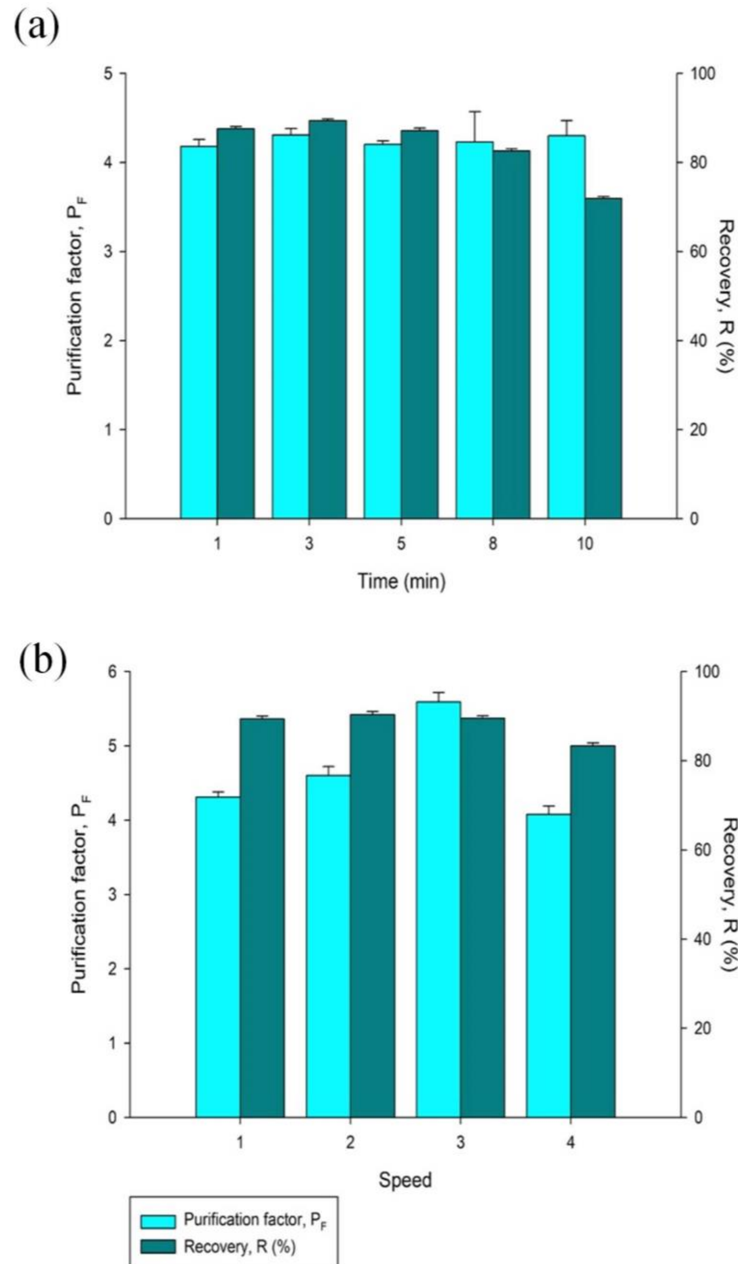


Figure 5.3: Recovery and purification factor of C-phycocyanin using homogenisation.

5.4.4 Freeze-thawing

The fourth cell disruption method was freeze-thawing where the freezing time of crude extract and sample extract were altered. The sample extracts were frozen for several freezing periods, which were 8 h, 16 h, 24 h and 48 h, then

followed by 20 min of thawing at room temperature (25 ^{o}C). Freeze-thawing was consider as "mild" homogenisation procedure (Gagné, 2014), and the cell could be swelled and broken as ice crystals form during the freezing process, followed by contracting during thawing, which led to cell lysis (Farrant, Walter, Armstrong, & Medawar, 1967).

The purification factor (P_F) and recovery (R) % of C-phycocyanin obtained through different freezing period were presented in **Figure 5.4**. The P_F of C-phycocyanin was decreasing with the increasing of freeze-thawing time in the current study. According to Mogany et al. (2018), the purity of C-phycocyanin was at the highest when the samples were frozen for 6 h and decreased thereafter (Mogany, Kumari, Swalaha, & Bux, 2018). The low purity of C-phycocyanin obtained indicated the release of contaminant proteins from the sample. The purity of C-phycocyanin could be as low as 2.25-fold of the crude extract with the obtained R% value of 79.83% at 48 h of freezing time. The freezing time of 48 h was a repeated cycle of 24 h freeze-thawing (2 cycles) which recovered higher yield compared to 8 h of freeze-thawing. It was reported that repeated cycles of freeze-thawing able to increase the yield of C-phycocyanin, where the first cycle of freeze-thawing able to recover <1000 µg/l and repeated cycles increased the yield nearly to 2250 µg/l (Horváth et al., 2013). However, the highest R% of C-phycocyanin, 80.31% was obtained with a slightly higher purity of C-phycocyanin, which was 3.25-fold at 24 h of freezing time. Since the C-phycocyanin recovered throughout all the proposed time falls into the range of 76 - 80%, the obtained P_F of C-phycocyanin would be the key to select the optimum time for this method. The sample frozen at 8 h was notable

as the P_F of C-phycocyanin was highest among all, such that the C-phycocyanin was purified to 4.15-fold of the crude extract, with a considerably lower R%, 77.10% in the experiment. Therefore, 8 h was chosen as the optimum freeze-thawing time for pre-treatment of C-phycocyanin.

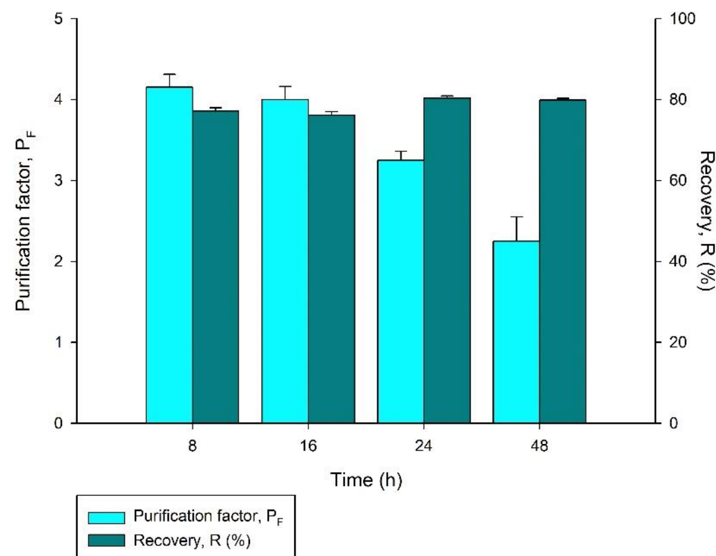


Figure 5.4: Recovery and purification factor of C-phycocyanin using freeze-thawing.

5.4.5 Comparison of Cell Disruption Methods

The suggested cell disruption methods were performed using same batch of *Spirulina* sp. powder. From the studied methods, purification factor (P_F) and recovery (R) % of C-phycocyanin obtained using the optimum parameters of the proposed methods varied after applied the extraction method, LBS. The effect of pre-treatment methods to the extraction of C-phycocyanin was studied in this work, by observing the P_F and R% of C-phycocyanin obtained after extraction. The extraction applied to C-phycocyanin with pre-treatment of biomass has resulted in better recovery and purity of C-phycoyanin compared to stand alone

extraction method (only sonication, freeze-thawing, homogenisation or microwave) in past literature (Tavanandi, Mittal, Chandrasekhar, & Raghavarao, 2018). C-phycocyanin was released more as the cell wall of biomass was ruptured through the pre-treatment methods, and the extraction of C-phycocyanin was enhanced and purified using LBS. The optimum parameters for each cell disruption methods were tabulated in **Table 5.2**.

Table 5.2: Optimum values of pre-treatment methods.

Cell Disruption Method	Parameters	Purification Factor (P_F)	Recovery (R), %
Sonication	Power: 20% Duty cycle: 50% Time: 7 min	6.17	94.89
Microwave	Duty cycle: 50% Time: 2 min	4.15	88.48
Homogenisation	Time: 3 min Speed: 3	5.59	89.51
Freeze-thawing	Time: 8 h	4.15	77.10

It was observed that the best P_F and R% of C-phycocyanin were obtained when the sample was treated with sonication from **Table 5.2**. Meanwhile, the lowest P_F and R% of C-phycocyanin, 4.15 and 77.10%, were gained by freeze-thawing the sample. Sample pre-treated by microwave was obtaining similar P_F of C-phycocyanin with the sample pre-treated by freeze-thawing, but different R% of C-phycocyanin was observed. Additional 11.38% of C-phycocyanin was recovered through microwave with similar quality of C-phycocyanin obtained through freeze-thawing. The obtained R% represented that microwave able to break down the cell wall of biomass more effective compared to freeze-thawing,

thus allowed higher amount of C-phycocyanin to be recovered. This could be supported by various studies where microwave only required comparatively short irradiation time compared to other methods (İlter et al., 2018; Juin et al., 2015).

The effectiveness of homogenisation and sonication were better than microwave and freeze-thawing. Nearly 90% of C-phycocyanin was recovered through homogenisation while more than 90% of C-phycocyanin was recovered through sonication. The P_F of C-phycocyanin through homogenisation and sonication was high as well, which were 5.59 and 6.17, respectively. In Horváth et al. (2013), homogenisation using Polytron Homogeniser was the least effective method in extracting C-phycocyanin which was in disagreement with current study. It was believed that the time range optimised for homogenisation should be prolonged and the suitable speed of homogeniser should be determined for better results as Horváth et al. (2013) only studied the homogenisation time from 15 s to 120 s (Horváth et al., 2013). Guedes et al. (2013) have claimed that homogenisation was better in rupturing the cell wall of microalgae and cyanobacteria compared to sonication (Guedes et al., 2013). However, the study of Wong and Lee (2018) demonstrated sonication has the highest extraction yield of C-phycocyanin (Caroline Costa Moraes et al., 2011) which was supported by other research works (Furuki et al., 2003; Sutanto & Suzery, 2016).

Although homogenisation only required 3 min to obtain such R% of C-phycocyanin, yet the sonication showed some advantages such that higher R%

and purity of C-phycocyanin compared to homogenisation were obtained. The disruption of cells through homogenisation also requires more cumbersome equipment management compared to sonication, where washing and sterilising the rotor-stator have to be performed prior to reuse. There is an alternative but disposable attachment will lead to higher operating cost. Meanwhile, sonication can be performed without dipping any instrument into the sample extracts, saving the cost to sterilise, and time to clean the instrument. In conclusion, pre-treatment with sonication followed by extracting C-phycocyanin using LBS was suggested to be the best way to obtain C-phycocyanin among the studied methods.

5.4.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of C-phycocyanin obtained using various pre-treatment methods and extracted by LBS was determined by SDS-PAGE analysis. In **Figure 5.5**, lane 1 represents the molecular marker, lane 2 represents the crude extract of C-PC which has pre-treated with sonication and lane 3 represents C-phycocyanin after pre-treatment and LBS. The C-phycocyanin showed two bands in at 18 kDa and 20 kDa from the SDS-PAGE. Deeper colour was observed in lane 2 compared to lane 3, indicating C-phycocyanin has been purified and more contaminant proteins have been removed to the bottom phase.

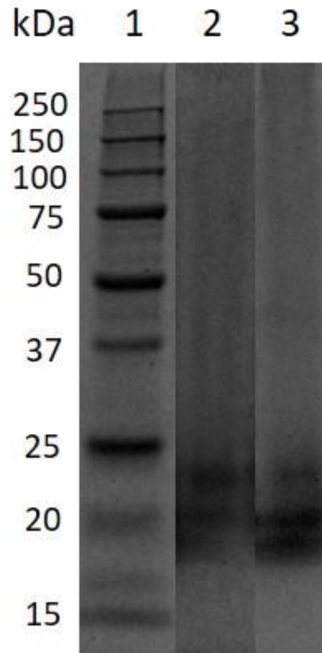
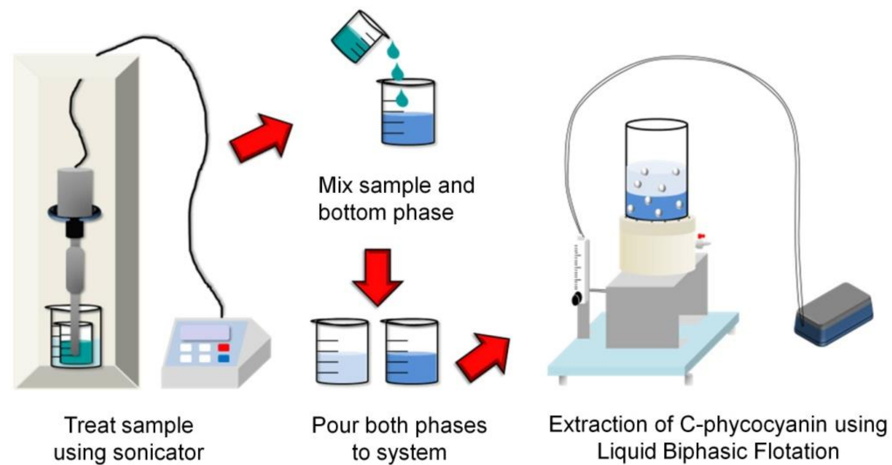


Figure 5.5: SDS-Page analysis for C-phycocyanin; Lane 1 is protein marker, Lane 2 is crude extract of C-phycocyanin (pretreated with sonication) and Lane 3 is C-phycocyanin after pretreatment and LBS.

5.5 Conclusions

C-phycocyanin was extracted and purified using LBS, with four pre-treatment methods to enhance the purity fold and recovery from *Spirulina* sp. The optimized parameters for each pre-treatment were: sonication – 20% power, 50% duty cycle and 7 min of irradiation time; microwave – 50% duty cycle and 2 min of irradiation time; homogenisation – 3 min of homogenisation and rotor's speed 3; and freeze-thawing – 8 h of freezing time. The highest recovery and purification fold of C-phycocyanin were obtained through sonication as the pre-treatment method, which was found to be 94.89% and 6.17, respectively.

CHAPTER 6 SONOPROCESSING-ASSISTED SOLVENT EXTRACTION FOR THE RECOVERY OF PIGMENT-PROTEIN COMPLEX FROM *SPIRULINA PLATENSIS*



This chapter presents the application of Liquid Biphasic Flotation assisted-with sonoprocessing for the extraction of the pigment-protein complex, C-phycocyanin. The optimized condition of sonoprocessing and Liquid Biphasic Flotation was investigated after confirming the most effective disruption method in previous chapter. The measurement of another component, allophycocyanin also evaluated along with C-phycocyanin with the condition optimizing to obtain maximal C-phycocyanin recovery and purification factor. This chapter is a thesis-version of the work published in the Chemical Engineering Journal (Chia S. R. et al., 2020, *Chemical Engineering Journal*, 125613).

6.1 Abstract

Current practice for C-phycocyanin (C-PC) extraction from fresh biomass is greatly perishable, so dried biomass is preferable for longer storage life and saving spaces for small scale industries. However, the resistance of dried biomass towards cell disruption is higher compared to fresh biomass. Therefore, this work aims to develop an effective technique for the extraction of C-PC from dried *Spirulina* sp. This study addresses the effect of sonoprocessing-assisted with liquid biphasic flotation (LBF) for the extraction and purification of C-PC and allophycocyanin (APC). The application of ultrasound was optimized by various parameters such as amplitude (20 to 30%), sonication time in pulse mode (5 to 25s), resting time in pulse mode (5 to 25s) and the total time of sonication (3 to 12 min). While for the liquid biphasic flotation, the studied parameters were air flowrate (75 to 175 cc/min), a volume ratio of both phases (1:0.5 to 1:1.5), flotation time (3 to 12 min), and weight of biomass (0.1 to 0.6 g). Results of C-PC and APC were determined using the optimized conditions and subjected to SDS-PAGE analysis. Total purification factor of 5.23 and recovery of 95.1% were obtained using 30% amplitude, 5s ON/5s OFF (pulse mode), 10 min sonication, volume ratio 1:1, 100 cc/min air flowrate, 7 min flotation time, and 0.45 g biomass. This study proves that the suggested method enhances efficiency in the recovery of C-PC and demonstrates the synergistic effect of sonoprocessing with LBF in extracting C-PC and other biomolecules from microalgae.

Keywords

Phycocyanins; *Spirulina* sp.; Ultrasound; Liquid Biphasic Flotation; Bioseparation; Extraction

6.2 Introduction

The demand for functional food and supplements has been increasing since the last decade as society values the importance of healthcare for a higher quality of life. The industries are focusing on the manufacture of these products to achieve a balance between the quality and primary cost due to the high demand. The primary cost of the products is often related to the capital and operating cost of the process. To compensate the cost required for both cultivation and manufacturing, the cultivation sites of microalgae can be located at rural areas, and the dried biomass can be transported to industries in urban places, which is also beneficial to both small and medium industries using dried biomass as a starting material (Tavanandi et al., 2018). Therefore, highly efficient cell disruption and extraction technique is required for obtaining biomolecules from dried microalgae biomass, to achieve high yields and purity of the targeted product.

Phycocyanin is a type of phycobiliproteins that acts as a significant accessory photosynthetic pigment in cyanobacteria (blue-green algae), which demonstrates a range of beneficial effects for human health. These include anti-inflammatory, antioxidant, antitumor, and anticancer properties, which make phycocyanin beneficial in pharmaceuticals, food, and cosmeceuticals industries (D. Kumar, Dhar, Pabbi, Kumar, & Walia, 2014). Phycocyanins are often used as natural dyes in cosmetics, food colorant, and fluorescent markers in biomedical research due to their natural blue colour and fluorescence. *Spirulina* sp. is one of the genus abundant in phycocyanins and is mainly produced for consumption as functional foods owing to their rich vitamin and mineral balance

(Herrera, Boussiba, Napoleone, & Hohlberg, 1989). Phycocyanins consist of C-phycocyanin, R-phycocyanin, and allophycocyanin. Among these, C-phycocyanin (C-PC) is the major phycobiliproteins in *Spirulina platensis*, which comprises up to 20% of the dry cell weight (Vonshak, 2014). The phycobilisomes of *Spirulina platensis* contain cores of allophycocyanins that are bound by C-phycocyanins on the periphery (Martelli, Folli, Visai, Daglia, & Ferrari, 2014). The absorption peak of C-phycocyanins can be detected between 610 and 620 nm, while the absorption peak of allophycocyanins can be identified between 650 and 655 nm. Earlier studies proved that phycocyanin constitutes a considerable portion of the biomass. Ajayan et al. (2012) stated that the content of phycocyanin in *Spirulina platensis* ranges from 99.2 $mg.g^{-1}$ to 148.1 $mg.g^{-1}$ depending on the nitrogen source with or without light reflector during cultivation (Ajayan, Selvaraju, & Thirugnanamoorthy, 2012), while Huang et al. (2007) claimed that phycocyanins were about 14.8% of the dry cell weight (Z. Huang, Guo, Wong, & Jiang, 2007).

Despite the high C-PC content in the biomass, the extraction of C-PC from the biomass is not standardized yet. C-PC is often extracted through cell disruption methods, such as freeze-thawing, chemical treatment, osmotic pressure, mechanical cell disintegration, etc. (C. C. Moraes, Luisa Sala, G. P. Cerveira, & S. J. Kalil, 2011). These methods are mainly applied to breakdown the cell wall of algal biomass, which is highly resistant to cell rupture, thereby promoting the release of C-PC from the biomass (Stewart & Farmer, 1984). C-PC is successfully extracted from the biomass using the abovementioned methods, as reported by Moraes et al. (2011). However, these methods lack

specificity as the impurities or cell debris released during the extraction led to lower purification factor and quality of the extracts, as well as affecting the total recovery of C-PC (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017). It is also reported that dried biomass is more resistant to cell disruption compared to wet biomass, due to the rigid cell wall and leads to lower extraction efficiency of C-PC (Tavanandi et al., 2018). Therefore, an effective and sustainable technique in obtaining high recovery and purification factor from dried biomass is necessary to be introduced to overcome the issues faced.

Liquid biphasic flotation (LBF) is a potential downstream processing technique which is an integration of liquid biphasic system and solvent sublation. Two-phase components are required to construct the system such that the combination of components can be alcohol/salt, alcohol/sugar, polymer/polymer, polymer/salt, or ionic liquid/salt. The unique properties of the system are low denaturation of the targeted components owing to high water content and similarity in the properties of the phases (Merchuk, Andrews, & Asenjo, 1998), as well as a simple operation that can concentrate, separate, and purify the targeted product from the biomass. The study of Sankaran et al. (2018) has shown a high separation efficiency of 92.29% and 95.73% of lipase yield using LBF incorporated with fermentation (Sankaran, Show, Lee, Yap, & Ling, 2018). Also, the upscale of the system has shown promising results such that 89.53% of separation efficiency and 93.82% of yield, presenting the possibility of large-scale production using LBF. The excellent recyclability of LBF also has been presented by Phong et al. (2017), where a total protein recovery of 88.86% was obtained at third recycling process (Phong et al., 2017). The extraction of

other biomolecules such as astaxanthin from microalgae has presented a maximum recovery of 78.38% and 99.86% of extraction efficiency using the alcohol and salt as phase components (Khoo et al., 2019). Since higher extraction efficiency of the bioproduct can be obtained using LBF, the recovery of the targeted compound can be further enhanced with the appropriate cell disruption method.

Ultrasonication is widely employed for a range of applications, such as dispersion, catalysis, extraction, nanoparticle synthesis, and graphene production (Muthoosamy & Manickam, 2017). The ultrasonication can improve the yield of compounds through cavitation, which is the formation, growth, and collapse of bubbles in a solution. The mass transfer between biomass or sample and solvent could be increased due to the mechanical effect of mixing and disruption exerted by ultrasonication. The cell rupture, on the other hand, occurs via the shock waves from the collapse of cavitation bubbles, which improves the release of intracellular components into the solution (Chia et al., 2019). A study by Tomšik et al. (2016) has predicted a total phenolic content of 1.60 g gallic acid equivalent/100 g dry weight which can be extracted from wild garlic using 79.8 min of irradiation time (RSM as a mathematical tool) (Tomšik et al., 2016). It is also reported that only an irradiation time of 25 min is required to yield high total phenol content (891. 76 $mg.lit^{-1}$) and antioxidant activity (2.17 $mmol.TE.lit^{-1}$) from lemon balm and peppermint extracts (Zlabur et al., 2016). Hence, due to the immense potential of ultrasonication as the cell disruption method, it was employed in the current study.

In the present work, the production of C-PC and allophycocyanin (APC) was examined using sonoprocessing coupled with LBF. The effect of sonoprocessing-assisted with LBF on dried microalgae biomass was investigated and evaluated to find the best condition for the highest recovery of C-PC. The biomass was firstly subjected to sonication for pretreating the biomass to release the inner cellular medium, where the sonication parameters such as amplitude, irradiation and resting time in pulse mode, and the total sonication time were investigated for optimum conditions. After sonication, the samples obtained using the optimized conditions of sonication were then subjected to LBF to extract C-PC and APC. The extraction was performed and studied for parameters including volume ratio of top and bottom phases, air flowrate of the system, flotation time, and biomass loading into the system. Optimized conditions were obtained based on the recovery of C-PC, and the extracts obtained were then characterised using SDS-PAGE. We believe that the current work contributes to investigating a potential technique in disrupting dried microalgae biomass effectively while releasing and obtaining maximal recovery and purity of C-PC from microalgae biomass; besides applying it to other biomolecules as well.

6.3 Materials and Methods

6.3.1 Materials

Polyethylene glycol 4000 (PEG 4000), dipotassium phosphate (K_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from R&M Chemicals (Malaysia). Bio-safe Coomassie Stain, 2x Laemmli Sample Buffer, and 10x Tris/Glycine/SDS were obtained from Canvio Sdn. Bhd. (Shah Alam, Malaysia). Dried *Spirulina platensis* powder was purchased from

Charming & Beauty Co. Ltd. (Taipei, Taiwan). All chemicals and reagents used were of analytical grades.

6.3.2 Ultrasound Treatment

Before subjecting to ultrasound treatment, 0.45 g of dried *Spirulina platensis* powder was dissolved in 50 mL of phosphate buffer. A stock solution of phosphate buffer was prepared using 1M K_2HPO_4 and 1M KH_2PO_4. The samples kept inside a larger glass reactor were stirred and subjected to sonication (Cole-Parmer Instruments, USA, 20 kHz, 750 W, 20% amplitude) for 7 min. During treatment to prevent local overheating, the temperature was controlled using cold water with temperature of 4 ^{o}C. After treatment, the samples were centrifuged to obtain supernatants and left overnight at 4 ^{o}C in a refrigerator. All the samples were vortexed before extraction. To examine the optimum conditions of sonication for the extraction of phycocyanin, various parameters were considered. All the experiments were performed in triplicate. The schematic diagram for the setup is shown in **Figure 6.1**.

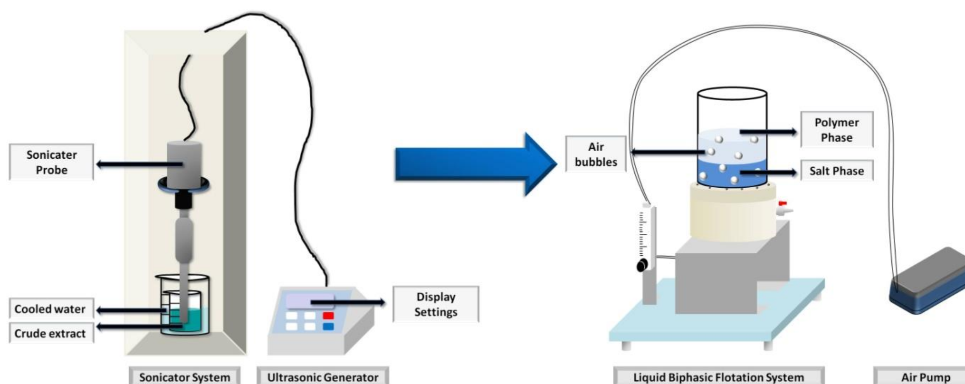


Figure 6.1: Schematic of the sonoprocessing-assisted solvent extraction method.

6.3.3 Liquid Biphasic Flotation System

500 mL of the Liquid Biphasic Flotation system (Donewell Resources, Malaysia) was used for the extraction of C-PC. The system was equipped with a sintered disk of G4 porosity (approximate pore size of 10 µm) at the bottom of the column, and a pump was connected to the system to generate air bubbles from the bottom of the column. A rotameter (Dwyer, USA) was used to regulate the air flowrate of the system from 25 to 250 cc/min. 150 mL of 250 g/L PEG solution was added into 150 mL of 250 g/L potassium salt solution and 50 mL of crude phycocyanin extract (containing 0.45 g of biomass). The mixture was aerated at 100 cc/min for 7 min and transferred to a beaker to initiate the process of settling. After settling down for 1 h under dark condition, the mixture was separated into two phases. The concentrations of C-PC and allophycocyanin (APC) in the top and bottom phases were subjected to analysis using a UV-vis spectrophotometer. The effect of various parameters was determined using a one-factor-at-a-time (OFAT) approach. The studied initial conditions, as well as operating parameters, are stated in **Table 6.1**.

Table 6.1: Operating parameters and initial conditions.

No.	Parameters	Initial	Variables	Unit
1.	Amplitude	20	22, 25, 27, 30	%
2.	Sonication time in pulse mode	20	5, 10, 15, 25	s
3.	Resting time in pulse mode	20	5, 10, 15, 25	s
4.	Total sonication time	7	3, 5, 10, 12	min
5.	Volume ratio	1:0.75	1:0.5, 1:1, 1:1.25, 1:1.5	-
6.	Air flowrate	100	75, 125, 150, 175	cc/min
7.	Flotation time	7	3, 5, 10, 12	min
8.	Biomass loading	0.45	0.1, 0.2, 0.3, 0.4, 0.5, 0.6	g

6.3.4 Determination of the Recoveries of C-PC and APC

The concentrations of C-PC and APC recovered from the biomass could be calculated using Equations (1) and (2) (Bennett & Bogorad 1973), respectively, as follows.

$$PC = \frac{[OD_{615}-0.474 \times OD_{652}]}{5.34} \quad (1)$$

$$APC = \frac{[OD_{652}-0.208 \times OD_{615}]}{5.09} \quad (2)$$

where PC is the concentration of C-PC in mg.mL^{-1}, and APC is the concentration of APC in mg.mL^{-1}. In the case of OD_{615} and OD_{652}, OD stands for optical density, and the subscript indicates the specific wavelength used to examine the sample. The purities of C-PC and APC were calculated using Equations (3) and (4), respectively:

$$Purity_{C\text{-}PC} = \frac{OD_{620}}{OD_{280}} \quad (3)$$

$$Purity_{APC} = \frac{OD_{652}}{OD_{280}} \quad (4)$$

where the absorbance of 280 nm represents the total concentration of proteins in the extract, while the absorbances of 620 nm and 652 nm represent the concentrations of C-PC and APC, respectively. Equations (3) and (4) indicate the purities of C-PC and APC for various forms of contaminating protein, respectively. The purification factors of C-PC and APC were calculated by the following Equations (5) and (6), respectively.

$$P_{FC} = \frac{Purity_{PC}}{Purity_{C}} \quad (5)$$

$$P_{FA} = \frac{Purity_{APC}}{Purity_{C}} \quad (6)$$

where P_{FC} and P_{FA} are the purification factors of C-PC and APC, $Purity_{PC}$ and $Purity_{APC}$ are the purities of C-PC and APC in the samples after LBF, respectively and $Purity_{C}$ is the purity of the crude extract. Recovery of C-PC and APC (%) was calculated by the following Equations (7) and (8), respectively.

$$R_{CPC}(\%)=\frac{[PC_{Phase}\times V_{Phase}]}{(PC_{TOP}\times V_{TOP})+(PC_{BTM}\times V_{BTM})}\times 100\% \quad (7)$$

$$R_{APC}(\%)=\frac{[APC_{Phase}\times V_{Phase}]}{(APC_{TOP}\times V_{TOP})+(APC_{BTM}\times V_{BTM})}\times 100\% \quad (8)$$

where PC_{Phase} is the concentration of C-PC in the selected phase, PC_{TOP} is the concentration of C-PC in the top phase, PC_{BTM} is the concentration of C-PC in the bottom phase, V_{Phase} is the volume of the selected phase, V_{TOP} is the volume of the top phase, and V_{BTM} is the volume of the bottom phase.

6.3.5 SDS-PAGE Analysis

The recovered samples were purified using ultrafiltration units (Amicon Ultra, Millipore) to remove PEG before analysis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using 12% Mini-Protean TGX Precast Protein Gels system (Bio-Rad) and was run at 60 V for 3-4 h. The gel was stained with Coomassie Brilliant Blue R250 for 1 h and destained with deionized water for 30 min.

6.3.6 Statistical Analysis

All the obtained results were analyzed by one-way analysis of variance (ANOVA) using SPSS statistical program (SPSS version 23.0 for window; IBM) to evaluate the significant differences ($p<0.5$) and the mean differences were

compared using Tukey HSD *post-hoc* test. All the experiments were conducted in triplicate, and the results are shown as mean ± standard error.

6.4 Results and Discussion

6.4.1 Mechanism of Liquid Biphasic Flotation

The Liquid Biphasic Flotation (LBF) integrates the mechanisms of both Liquid Biphasic System (LBS) and solvent sublation. Involvement of equilibration and phase separation in LBF is related to the mechanism of LBS. The process of phase-forming compositions and partitioning of target compounds achieving equilibrium state occurs through mass transfer mechanisms in mixing process is equilibration (Leong, Chang, et al., 2019). The phase-forming components are usually soluble to water and only form two phases when they exceed certain concentration. The partitioning of the target compounds to both phases is manipulated by the characteristics of the substances and the interaction of both phases in the system. LBS has a phase diagram that consists of specific conditions (e.g. concentration of phase-forming components and pH) to define the working area for these selective phase-forming components (Kaul, 2000). The equilibrium relationship of top and bottom phases can be evaluated through partition coefficient, K, of the system. The combination of polymer and salt is used as the phase components in this study as polymer-based system possesses low ionic environment for the biomolecules that are sensitive to ionic condition, where appropriate control of ionic strength is required during phycocyanin extraction (Chaiklahan, Chirasuwan, & Bunnag, 2012; Khoo et al., 2020). The unique properties of biphasic system are the large portion of water contained in both phases creates a favourable environment for

biomolecules in avoiding denaturation while separating both phases through low interfacial layer (Khoo et al., 2020). Besides, LBF also possesses the mass transfer mode of solvent sublation. The facilitation of biomolecules in biphasic system is performed to concentrate or enrich the targeted compounds through the adsorption of targeted compounds onto the surface of bubbles. The flotation of bubbles from bottom to top phase brings the targeted compounds to top phase and the targeted compounds are released through the bubble rupturing process. Therefore, most of the targeted products can be obtained at the top phase rather than bottom phase (M. Li & Dong, 2010; Sankaran, Show, Lee, et al., 2018). The mass transfer mechanisms in LBF can be achieved through several ways, such as by gas bubbles, molecular diffusion or water droplets. In the current study, mass transfer is achieved by gas bubbles, as shown in **Figure 6.1**.

6.4.2 Effects of Sonication on C-PC and APC

6.4.2.1 Ultrasonic amplitude

Preliminary studies were conducted to determine the range of appropriate amplitudes that do not lead to the temperature of the system higher than 40 ºC. The control of temperature is vital, as the thermal effect through sonication could cause temperature to rise more than 40 ºC and lead to less yield of extraction, as indicated by Dey and Rathod (Dey & Rathod, 2013). The measurement of the temperature of the extracts was carried out after sonication. The range of amplitudes from 20 to 30% was performed to check the purification factor and recovery of C-PC and APC. The amplitude indicates the intensity of sonication or cavitational effects, and hence its optimization is crucial in the case of sonication-assisted extraction (Wen et al., 2018).

The purification factor of C-PC obtained using the studied range, 20 to 30% amplitude, is shown in **Figure 6.2(a)**. The lowest purification factor of C-PC, about 3.11, was observed using a 22% amplitude, and the highest purification factor was obtained using a 30% amplitude. A slight increment was noted by employing amplitude from 22 to 27%, while the purification factor of C-PC was gradually increased by applying amplitude from 27 to 30%. This study of the effect of amplitude was studied only until 30% owing to the increasing temperature of the extract. The extract is overheated if 30% amplitude was applied for more than 7 min, even when the extraction was performed using a cooling water jacket at 3 °C. A temperature of more than 47 ºC results in the degradation of phycobiliproteins, as reported by Chaneva et al. (Chaneva et al., 2007). Several studies have shown that the optimum amplitude for the extraction of phycocyanin ranges from 50 to 80% in a much shorter sonication time of about 2 to 4 min (Manisha B Bachchhav, Kulkarni, & Ingale, 2017; Manisha B. Bachchhav, Kulkarni, & Ingale, 2019; Tavanandi et al., 2018). Therefore, adjusting the sonication parameters between amplitude and time is necessary for controlling the temperature of the extract, where a higher range of amplitude could be considered only by using short sonication time. Alternatively, a low range of amplitude could be considered for longer sonication time. The purity of C-PC as reported by Tavanandi et al. (2018) using a 50% amplitude and 2.5 min of sonication was 0.62, which is much lower compared to the current study that utilized a 22% amplitude, 7 min of sonication time and led to a purity of 1.07 (Tavanandi et al., 2018). This indicates that the utilization of a higher amplitude reduces the purification factor of C-PC due to the release of other components from the biomass. A marginal difference in the recovery of C-PC has been noted

by applying different amplitudes, as shown in **Figure 6.2(a)**. The total recovery of C-PC ranged from 57.2 to 64%, with the highest recovery was observed using a 27% amplitude. The C-PC recovered was similar by employing amplitudes from 20 to 25% and were increased by 27 and 30% amplitude. The results showed that the tested range of amplitude was sufficient to rupture the cell wall for releasing a considerable amount of C-PC from the inner medium of biomass (above 55% of C-PC). Since the recovery of C-PC obtained using a 30% amplitude was similar with 27% amplitude, and the purification factor of C-PC gained using a 30% amplitude was the highest, the optimum was chosen as 30% amplitude.

For APC, the purification factor obtained was fluctuating along with the increment of amplitude. The highest purification factor of 6.02 was gained using a 30% amplitude, whereas the lowest purification factor was around 4 using a 22% amplitude, as shown in **Figure 6.2(a)**. The purification factor obtained using 20 and 25% amplitude was similar, falling in a range of about 5. The overall purification factor of APC extracted from the biomass was higher than the purification factor of C-PC extracted using the same range of amplitude, in which the obtained purification factor was ranged from 4.26 to 6.02. Higher amplitude equates to higher intensity of vibration, in other words, higher cavitation intensity within the sample. Hence, a high purification factor of APC was obtained using high amplitude as more APC was released compared to the impurities released from the biomass. The total recovery of APC from the biomass was noted in the range from 20 to 30% amplitude and was seen to increase up to 70% recovery gradually and then decreased to around 50%

recovery at 30% amplitude. As shown in **Figure 6.2(a)**, the highest recovery of APC was obtained at a 25% amplitude, which was slightly different from the optimal amplitude obtained in recovering C-PC from the biomass. Although both the purification factor and recovery of APC were higher than C-PC, the optimum amplitude was chosen according to the results of C-PC due to the market price of C-PC being much costlier compared to APC.

6.4.2.2 Sonication time for pulse mode

Investigation on the pulse mode of sonication was done by adjusting the sonicator to be pulsed on and off in a specific sonication time. The sonication was pulsed off for 20s and pulsed on from 5 to 25s with an interval of 5s while the total sonication time was fixed at 7 min for every sample.

Figure 6.2(b) displays the purification factor and recovery of C-PC obtained using pulse mode for various sonication times. The purification factor of C-PC was found to be the highest using 20 and 5s of sonication in pulse mode, in which both led to a similar purification factor. Lower purification factor was observed using other sonication times, 10s, 15s, and 25s. From the work of Sørensen et al. (2013), the highest purity of C-PC obtained using extraction solely was 2.7 ± 0.1 without performing ultrafiltration (Sørensen, Hantke, & Eriksen, 2013). By comparing the results obtained from this study, even the lowest purity (2.98) gained is higher than the results obtained by Sørensen et al. (2013), proving the high efficiency of sonication in extracting C-PC from biomass. On the other hand, the trend of C-PC recovery showed a decreasing trend from the lowest sonication time (5s) to the highest sonication time (25s).

The recovery of C-PC from samples was within 60 to 70%, with only a difference of about 10% between the highest and lowest recovery. Higher sonication time in pulse mode increases the temperature of the extracts, leading to the possible degradation of C-PC. Thus, the optimum sonication time of 5s was selected as the time of extraction, as the recovery obtained was the highest despite the purification factor being slightly lower.

The purification factor of APC, as displayed in **Figure 6.2(b)** shows a significant difference of 3.42 between the highest and lowest purification factor. The trend observed is similar to the one obtained for the purification factor of C-PC, indicating the capability of this extraction method in purifying C-PC and APC was the same. The other reason could be due to the release of more impurities from the biomass due to higher sonication time utilized for breaking the cell wall of algae. In **Figure 6.2(b)**, the recovery of APC obtained decreased from 5 to 20s of sonication time and increased again at 25s. The highest recovery of APC was observed at 5s, and the lowest recovery of APC was obtained using 20s.

6.4.2.3 Resting time

The resting time is the time for which sonication was pulsed off, and a range of sonication pulse off from 5 to 25s was investigated. The pulse on time was fixed at 5s, as it was the optimum result obtained from the studies of previous parameter. The resting time in pulse mode allows samples to be cooled down due to the rise in temperature caused by the collapse of cavitation bubbles. The conversion of sound energy to kinetic energy heats the cavitation bubbles to

extreme temperatures (Rae et al., 2005). The collapse of cavitation bubbles then imposes a rapid explosion of liquid to cell surfaces, rupturing the cell, and enhancing the mass transfer between the medium and inner product of cells (Soares Melecchi et al., 2006).

The optimization of resting time in pulse mode is necessary to determine the most efficient route for saving the power and cost, at the same time, to obtain the highest recovery and purification factor of the targeted product. Purification factor and recovery of C-PC gained using the resting time in the range from 3 to 4 are illustrated in **Figure 6.2(c)**. It could be observed that the purification factor of C-PC obtained using 25s of resting time is slightly lower compared to the purification factor of C-PC obtained using other resting times. The recovery of C-PC at different resting times follows a decreasing trend, along with an increase in the resting time. The highest recovery of C-PC was 81.7%, which was found at 5s of resting time, while the lowest recovery of C-PC was 67.72%, which was obtained at 25s of resting time. Longer resting time/pulse off time in sonication resulted in a lower purification factor and recovery of C-PC, as shown in the observed results.

Sonication time was further applied to the samples when the resting time was shorter, giving the samples sufficient cavitational effect to rupture the cell wall of *Spirulina* sp. to transfer the C-PC from the inner cell to the medium. Studies by Tavandi et al. (2018) and Pan-utai and Iamtham (2019) fixed the resting time in pulse mode with a 50% amplitude, where a pulse on/off time was 1s/1s for 2 min and 60s/30s for 5 min (Pan-utai & Iamtham, 2019; Tavanandi et

al., 2018). The longer total resting time requires longer total sonication time to rupture the biomass for releasing the inner components effectively. Hence, the appropriate resting time has to be examined using the total sonication time for the initial condition to gain a maximal recovery of C-PC. The highest recovery of C-PC and considerably a high purification factor of C-PC were observed using 5s of resting time, which was considered for the rest of the studies.

In **Figure 6.2(c)**, the purification factor of APC was observed to be the highest using 20s of resting time, while the lowest purification factor was obtained using 25s of resting time. The purification factor of APC was varying depending on resting times. This is due to the optimization being performed according to the purification factor and recovery of C-PC instead of APC. On the other hand, the recovery of APC using the same range of resting time in pulse mode is displayed in **Figure 6.2(c)**. It could be noted that the recovery obtained using 5s and 20s is similar, while others are lower as compared to the APC recovery obtained at the indicated resting time. The lowest recovery of APC was observed at the longest resting time in pulse mode, which is similar to the obtained recovery of C-PC.

6.4.2.4 Total sonication period

To determine the required contact time of the samples to achieve the maximum recovery of C-PC, different sonication times were performed using pulse mode of sonication (5s ON/5s OFF). Sonication time proves to be an influencing factor of yield and rate of mass transfer in the extraction of compounds (Tomšik et al., 2016; J. Wu et al., 2001). The optimization of

sonication time is necessary as it could positively or negatively influence the extraction process. Excessive sonication time may waste energy and increase the cost of operation, as well as affect the quality of the end product (Tomšik et al., 2016). In contrast, while insufficient sonication time may not succeed in rupturing the cell wall completely and lead to a low yield of biomolecules.

The purification factor of C-PC obtained using different total sonication time is shown in **Figure 6.2(d)**. The C-PC purification factor ranges from 3.52 to 4.89, and hence a difference of 1.37 between the highest and lowest purification factor was noted. The highest purification factor of C-PC was found using 10 min of sonication before extraction. Further sonication (12 min) resulted in a lower purification factor of C-PC in which C-PC was denatured during sonication. The purification factor of C-PC was found to be the same when the applied sonication time was less than 10 min, only with a small difference of 0.13. C-PC recovered gradually increased as the sonication time prolonged and then decreased after 10 min of sonication. The least C-PC was obtained as expected using the lowest sonication time due to insufficient sonication to break down the cell wall completely. The highest purification factor and recovery of C-PC were found using 10 min of sonication. Mittal et al. (2017) extracted phycobiliproteins from *Gelidium* sp. using a similar working range from 1 to 10 min, as a further increase in sonication time led to the increment of the temperature, and consequently affected the stability of phycobiliproteins (Mittal et al., 2017). The results obtained by Mittal et al. (2017) are in agreement with this study that 10 min is the optimum sonication time to recover the most phycobiliproteins.

However, the purification factor of APC was found to be the highest using 3 min of sonication, and a similar purification factor was obtained using 7 min of sonication too. The purification factor of APC was different from C-PC, in which a lower purification factor was observed at longer sonication time, and a purification factor of about 3 was obtained at both 10 and 12 min of sonication. This could represent that more impurities release from the cell at longer sonication time and lower the purification factor of APC in the samples. The highest APC recovery of 70% was obtained using 7 min of sonication but drastically decreased to 27.76% at 10 min of sonication. The amount of APC recovered at long sonication time was lesser compared to the recovery obtained at shorter sonication time. Hence, an optimum sonication time of 10 min was considered.

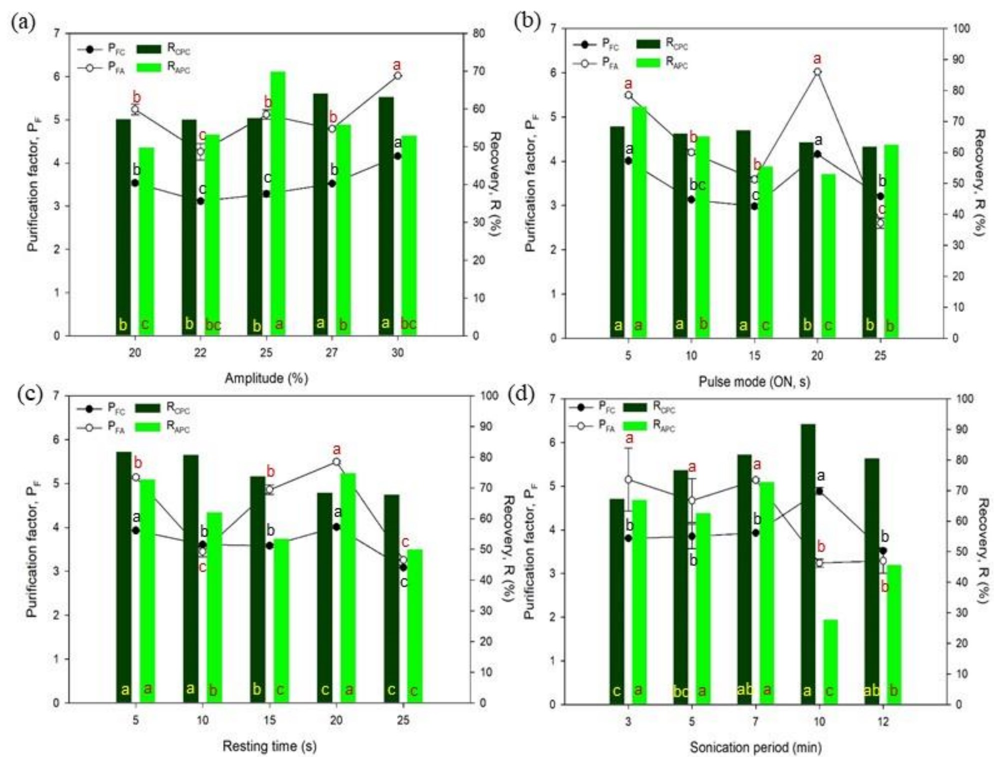


Figure 6.2: Effects of sonication parameters towards the purification factor and recovery of c-phycocyanin and allophycocyanin (a) Amplitude (b) Pulse mode

(c) Resting time (d) Total sonication period. Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). The indication of black and yellow letters: P_{FC} and R_{CPC}, respectively; red letters: P_{FA} and R_{APC}.

6.4.3 Other Effects on the Extraction of C-PC and APC

6.4.3.1 Volume ratio

In **Figure 6.3(a)**, the purification factor of C-PC was examined using various volume ratios, where the ratio of the top phase and bottom phase was varied. The studied ratios of the bottom phase to the top phase were 1 to 0.5, 1 to 0.75, 1 to 1, 1 to 1.25, and 1 to 1.75. The amount of the top phase used varied due to the favorability of C-PC to both phases. Most of the biomolecules studied prefer the top phase (polymer phase), compared to the bottom phase (salt phase), which is similar to this study. Several studies proved that the soluble components often partition to the top phase depending on their charge-charge interaction (Chew, Chia, Lee, et al., 2019; Chia et al., 2019). As mentioned by Nagaraja and Iyyaswami (2015), the difference in electrical potential caused by the non-uniform distribution of salt in both phases also contributes to the preference of proteins to one of the phases through electrostatic repulsion or attraction (Nagaraja & Iyyaswami, 2015). The optimum volume ratio to obtain the highest C-PC purification factor was 1:1, where a total purification factor of 5.23 was obtained. The lowest purification factor of C-PC was achieved using a ratio of 1:0.5, followed by the ratio of 1:1.75. C-PC recovered using the same range of volume ratio is displayed in the figure as well. The highest recovery of C-PC was obtained at the same volume ratio as the highest purification factor of C-PC, which was the ratio of 1:1. The C-PC was recovered in a range of 72.74 to 95.1%,

presenting a difference of 22.36% between the studied volume ratios. The trends of C-PC recovery and purification factor were similar, where the obtained results gradually increased to the highest point and then gradually decreased. A low amount of top phase was not able to fill and extract all C-PC from the extracts since there was less free volume for C-PC to occupy (Chew, Chia, Krishnamoorthy, et al., 2019). Besides, a high amount of top phase would extract more C-PC and impurities from the extracts, resulting in a lower purification factor of C-PC. The recovery of C-PC was reduced as well due to the increment of viscosity for the polymer phase at high volume.

On the other hand, the highest purification factor of APC was 4.75, while the lowest purification factor was 2.71, using a ratio of 1:1 and 1:1.75, respectively. The purification factor of APC was varied around 2 between the highest and lowest purification factor with the moderate purification factor of APC recovered (3.24 and 3.42) using ratios of 1:0.75 and 1:1.25, respectively. The recovery of APC was found to be the lowest using a ratio of 1:0.75, where only about 28% of APC was recovered. In contrast, the highest recovery was obtained using a ratio of 1:0.5, obtaining a total recovery of 59%. Other ratios, such as 1:1, 1:1.25, and 1:1.75, recovered around 30% to 40% of APC from the biomass. Thus, an optimum volume ratio of 1:1 was considered for the subsequent studies.

6.4.3.2 Air flowrate

In LBF, air flowrate is one of the significant parameters as the rate of air bubble assists in upbringing the targeted compounds from the bottom to the top

phase. The targeted compounds were selectively adsorbed on the surfaces of air bubbles and released in the top phase. Also, the area of air-water interface per unit volume of an aqueous solution with time was affected by the air flowrate and flotation time (Sankaran, Show, Lee, et al., 2018). The flowmeter with a range of 0 to 200 cc/min was installed in the system, considering the amount of C-PC available in the system, and recovering the biomolecules using a low air flowrate such as <75 cc/min was unlikely to show efficient results while an extremely high airflow rate could impose more impurities to the top phase. Hence, the studied range of air flowrate was set at 75 to 175 cc/min.

The purification factor of C-PC obtained using various air flowrates is shown in **Figure 6.3(b)**. It could be found that the purification factor increased from the lowest air flowrate to 100 cc/min and then gradually decreased from 100 cc/min to the highest air flowrate. The least purification factor of 3.36 was obtained among the tested air flow rate, while the highest purification factor was 5.23. Increasing air flowrate enhances the transfer of C-PC to the top phase, but the higher air flowrate also increases the transportation of impurities to the top phase. A total C-PC recovery of 95.10% was obtained using an air flowrate of 100 cc/min. The overall trend for the recovery of C-PC was similar to the pattern observed in the purification factor of C-PC obtained. The amount of C-PC recovered using 75 cc/min, and 150 cc/min of air flowrates was comparable, which were 75.97% and 76.33%, respectively, with only a slight difference of 0.44%. A similar trend was observed in the work of Sankaran et al. (2018), where the yield of lipase dropped after an air flowrate of 100 cc/min (Sankaran, Show, Lee, et al., 2018). This is due to the higher number of air bubbles within the

system, causing C-PC to not properly attach on the surface of air bubbles as there is less space for the air bubbles to float to the top phase at a specific time.

Alternatively, the purification factor of APC using different air flowrates of the system was observed in a decreasing manner as the air flowrate increases. A high level of APC purification factor was found using the lowest air flowrate, while for other flow rates similar purification factor of APC was obtained that ranged from 4.87 to 3.92. The recovery of APC (82.24%) was found to be the highest at the lowest air flowrate as well. The recovery obtained using other air flowrates was similar except at 125 cc/min, where recovery of 10% excess was obtained as compared to 100, 150, and 175 cc/min of air flowrates. The lowest recovery of APC (48.82%) was obtained using 100 cc/min. A significant difference of 34% was observed between the highest and lowest APC recovery. Based on this, the optimum air flowrate for the following study was 100 cc/min.

6.4.3.3 Flotation time

The flotation time has the same influence as that of the flotation rate towards extraction. Hence, flotation time was varied to examine the desired purification factor and recovery of the targeted compound. The flotation time was studied in the range from 3 to 12 min with other optimized parameters. As shown in **Figure 6.3(c)**, the highest purification factor of C-PC was obtained at 7 min of flotation time. The purification factor of C-PC was increased significantly from 3 to 5 min and then decreased from 10 to 12 min. Using 5 to 10 min, a similar value was obtained with a difference of only 0.34. Longer flotation time can enhance the mass transfer between the top and bottom phases.

However, the purification factor can be reduced if a longer flotation time was used for the extraction. The extended flotation time allows more bubbles to bring the targeted compounds from the bottom phase to the top phase, but more impurities or proteins are brought up to the top phase as well (Chew, Chia, Krishnamoorthy, et al., 2019). Therefore, less purification factor was obtained at the longest flotation time. The C-PC recovered using 7 min of flotation time had the highest recovery while the lowest recovery was obtained using 3 min of flotation time. The least recovery was obtained at 3 min due to the insufficient time for more C-PC to be carried to the upper phase at the fixed rate of air bubbles. The recovery of C-PC was found to steadily increase to 95.10% and subsequently lowered to 82.13%. As the highest purification factor and recovery of C-PC were obtained at the same flotation time, the optimum flotation time selected for the following study was 7 min.

In **Figure 6.3(c)**, the purification factor of APC obtained was in the range from 3.96 to 4.75, in which the highest purification factor was obtained using 7 min, and the lowest purification factor was obtained using 12 min of flotation time. The difference between the purities of all APC obtained was considerably low, i.e., less than 1. The trend observed in the recovery of APC was quite different from the trend obtained for the purification factor of APC, as shown in **Figure 6.3(c)**. The lowest amount of APC was recovered using 7 min of flotation time while the highest was recovered using 3 min of flotation time. This figure demonstrates a decreasing trend of the APC recovery, and the recovery of APC increased from 7 to 10 min, followed by a slight decrease in the recovery at 12 min of flotation time. It could be observed that the amount of C-PC recovered is

in contrast to the amount of APC obtained using a similar flotation time, where the system has limited capability to extract both the components with high recovery yield and purification factor at the same time.

6.4.3.4 Biomass loading

The amount of biomass loaded into the system was optimized as the partitioning behavior of the targeted product, and the interfacial balance between both phases is affected by this parameter (Chia et al., 2019). The higher the weight of biomass loaded for pre-treatment, the more viscous is the extract when it is used for extraction. The flotation of air bubbles used to extract the targeted product is getting influenced if the bottom phase is too viscous, as it is more difficult to separate the targeted product from the extract. However, loading of lower biomass may result in lowering the total recovery of desired biomolecules as there can only be a large amount of compounds within a given amount of biomass.

Figure 6.3(d) shows that the purification factor of C-PC obtained using various weights of biomass ranged from 0.1 to 0.6 g with an interval of 0.1 g for the initial biomass of 0.45 g. The highest purification factor of C-PC was observed using 0.1 g of biomass while the purification factor of C-PC obtained using other biomass loading was comparable. Most of the purification factor obtained was about 5.5, with the lowest purification factor was 5.23. The results showed that more loading of biomass did not exhibit a higher purification factor as the system was only capable of recovering similar purification factor using a biomass weight of more than 0.1 g. The higher biomass weight used may result

in more impurities being extracted or the incomplete disruption of biomass cells that restrict the release of more C-PC. Higher biomass also produces higher C-PC, which oversaturates at the top phase and causes an imbalance in the concentration of C-PC between both phases. From **Figure 6.3(d)**, the recovery of C-PC was obtained in a range of 74.6 to 95.1% using the tested range of biomass weight. It was observed that the recovery of C-PC gradually increased to 95.1% and slightly decreased by employing a higher biomass weight. It was shown that higher biomass weight led to higher recovery of C-PC, but the purification factor was low as more impurities were extracted to the top phase. The C-PC was preferably partitioned using 0.45 g to obtain a moderate purification factor with higher recovery.

On the other hand, the purification factor of APC obtained using different biomass weight was plotted, and a different trend was observed. The highest purification factor was obtained using 0.3 g of biomass while the lowest was observed using 0.5 g, in which the difference of purification factor can be as high as 4. With less biomass weight, a higher purification factor of APC was recovered compared to higher biomass weights. Besides, the highest recovery of APC (62%) was obtained using 0.3 g of biomass, as shown in **Figure 6.3(d)**. The lowest recovery was observed using the least biomass weight of 0.1 g and gradually increased to the highest recovery percentage before slowly decreased to 42.3%. The obtained results from the current work are compared with the open literature and shown in **Table 6.2**.

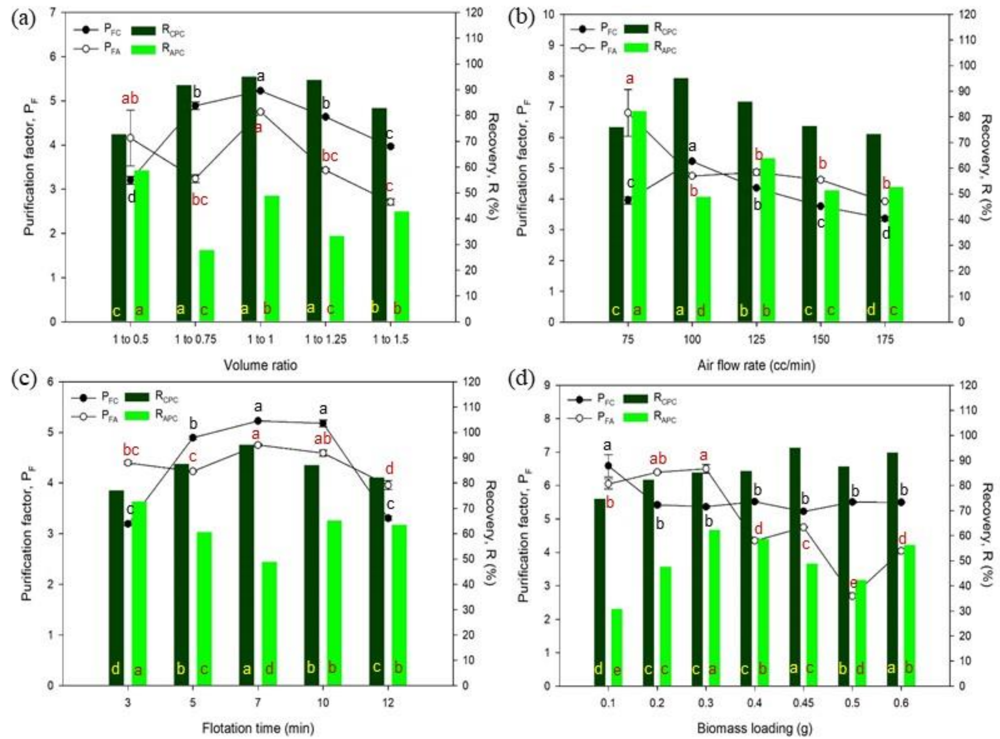


Figure 6.3: Effects of the parameters of liquid biphasic flotation system towards the purification factor and recovery of c-phycocyanin and allophycocyanin (a) Volume ratio (b) Air flowrate (c) Flotation time (d) Biomass concentration. Means with the same letter are not significantly different (One-way ANOVA followed with Tukey's test). The indication of black and yellow letters: P_{FC} and R_{CPC}, respectively; red letters: P_{FA} and R_{APC}.

Table 6.2: Comparison of the current work with results reported from literature.

Method	**Recovery of C-PC**	**Purity (P) /Purification factor (P_F)**	**Reference**
Aqueous two phase extraction (3 times)	85%	P: 4.05	(G. Patil & Raghavarao, 2007)
Ionic liquid-based aqueous two-phase system	99%	P_F:5.8	(Chang et al., 2018)
Liquid Biphasic Flotation	90.4%	P_F: 3.49	(Chew, Chia, Krishnamoorthy, et al., 2019)
Microwave-assisted extraction	4.54 $mg.mL^{-1}$	P:1.27	(Vali Aftari, Rezaei, Mortazavi, & Bandani, 2015b)
Pulsed electric fields	100 mg/g dry weight	-	(Martínez et al., 2017)
Salt precipitation	80%	P: 1.5	(D. Kumar et al., 2014)
Salt precipitation	2.67 $mg.mL^{-1}$	P: 0.79	(F. S. Antelo, A. Anschau, J. A. V. Costa, & S. J. Kalil, 2010)
Sonoprocessing-assisted with liquid biphasic flotation	95.1%	P_F: 5.23	Current work
Stirring with activated carbon and chitosan followed by salt precipitation	6.1% (present in dry biomass)	P: 1.1	(Gantar, Simović, Djilas, Gonzalez, & Miksovska, 2012)
Ultrasonic bath with glass pearl	43.75 mg/g dry weight	-	(C. C. Moraes et al., 2011)
Ultrasound-assisted extraction	2.84 $mg.mL^{-1}$	P: 0.65	(Vali Aftari et al., 2015b)
Ultrasonication with freeze-thawing	92%	P: 0.8	(Tavanandi et al., 2018)

6.4.4 SDS-PAGE

Figure 6.4 shows the molecular weight of the obtained C-PC using SDS-PAGE, in which lane 1 indicates the protein marker, lane 2 represents the crude extract of C-PC before any treatment, and lane 3 describes the molecular weight of C-PC after extraction and ultrafiltration. The estimation of the C-PC size resolved by gel electrophoresis was performed using a protein marker (lane 1). The molecular weight of C-PC was observed around 18~20 kDa as it's alpha and beta subunits from **Figure 6.4** (indicated by the red circle). The contaminant proteins are defined as other types of proteins except for the presence of targeted product (C-PC) in the samples within all molecular weights except 18~20 kDa in lane 2 and 3. It is observed that the crude extract (lane 2) consists of the majority of contaminant proteins compared to the extract after extraction and ultrafiltration (lane 3). In **Figure 6.4**, several bands (around 100, 75 and between 75 to 25 kDa) are observed in lane 2 compared to lane 3, where no visible bands are observed. No visible bands in lane 3 represent that no other type of proteins is detected with the mentioned molecular weight. This result proves that ultrafiltration is required to purify the obtained C-PC and remove other contaminant proteins.

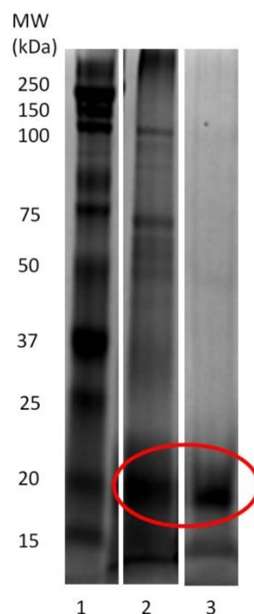


Figure 6.4: SDS-PAGE analysis for the obtained C-PC (Lane 1: Molecular marker; Lane 2: Crude extract; Lane 3: C-PC after processing and ultrafiltration).

6.5 Scaling-up and Technical Feasibility of the Process

The sonoprocessing-assisted LBF can be scaled-up for industrial production. Various studies showed that scaling up the total working volume from laboratory to pilot scale of LBF does not pose a significant effect on the recovery of targeted products (Mathiazakan et al., 2016; P. L. Show et al., 2013). Show et al. (2013) have demonstrated similar yield of lipase using different working volumes of LBF system ranging from 200 mL to 1L with the optimized conditions (P. L. Show et al., 2013). The extraction of polyphenols from Camu-camu also showed comparable results using a semi-pilot scale, and a total working volume of 2.5L with a laboratory scale (de Araújo Padilha et al., 2018). The usage of ultrasonic probe in large scale is applicable, where the disruption of algae can be performed in the flow rate ranging from 0.8 to 8 m^3/h (Hielscher Ultrasound Technology, 1999). However, the performance of current work

should be investigated in larger scale prior to industrial scale to observe the homogeneity of cell extracts after sonoprocessing.

Besides, the polymer utilized in the studies of Show et al. (2013) was recycled up to 75%, and no significant change was observed (using both recycled polymer and salt phase) compared to the extraction using fresh forming components (P. L. Show et al., 2013). In this study, salt recovery can be achieved using phosphoric acid to convert the phosphate to KH_2PO_4 and dilution crystallization of methanol can be applied to crystallize some amount of phosphate (C. C. Moraes et al., 2011). It shows that the consistency of results obtained using the suggested technique and the recyclability of the phase components is possible to sustain the process. The reduction in the usage of fresh chemicals often decreases the process cost, where 60% of the price is reduced to recover phosphate in the study of Li et al. (2011) (Z. Li, Teng, & Xiu, 2011). Positive results were obtained even using the recovered salt from the previous study where the effect of recycling both phases for four cycles have indicated the potentiality of the process as cost-saving and promising approach for industrial application (Sankaran, Show, Yap, et al., 2018).

A potential limitation of the current work on the industrial scale is on the energy consumption. Although the utilization of recycled phase components can aid in reducing the total production cost, the overall cost for total energy consumption for industrial scale remains unknown. Furthermore, the application of ultrasound to process the biomass requires a considerable amount of energy, although LBF does not require as high energy as ultrasound. If the recycling

approach is applied in the process, the recycling process of large-scale phase components has to be well-designed. An economical analysis should be performed further to investigate the feasibility of the process for commercial application.

6.6 Conclusions

In this study, the extraction of phycocyanin from microalgae, *Spirulina platensis* was performed using sonication and LBF system, which integrates bubbling effect with LBS process. The influence of sonication and bubbling effects on C-PC extraction were investigated. The best recovery of C-PC was obtained using an ultrasonic amplitude of 30% which was operating for 10 min of total sonication time at 5s ON/5s OFF in pulse mode. For the effect of other variables related to LBF system, the maximal amount of C-PC was obtained using 1:1 volume ratio for phase components, 0.45 g biomass, along with operating the system with 100 cc/min of air flowrate for 7 min of flotation time. The optimization of C-PC extraction through sonoprocessing with solvent extraction is proved to be an effective method for the extraction and recovery of the pigment-protein (phycocyanin) as high purification factor (5.23), and recovery of C-PC (95.1%) were obtained through the current study compared to the literature reports. The characterization analysis, SDS-PAGE further affirmed that the C-PC content was extracted from biomass using the performed technique. However, the removal of the polymer has to be performed before analysis. This work enhances prospects of sonoprocessing with solvent extraction as a potential technology in downstream processing of phycocyanins to be upscaled for industrial production.

CHAPTER 7 CONCLUSIONS AND FUTURE WORKS

In this chapter, the findings of each scope will be summarized and presented to conclude this research work and the recommendations for future works are included. All the research works accomplished are reported and possible future works are discussed in this chapter.

7.1 Conclusions

The development of sonoprocessing assisted techniques for biomolecules recovery from microalgae biomass has been achieved in this research work. The identification of sonoprocessing as effective pre-treatment method or cell disruption technique to be carried out simultaneously with Liquid Bi- and Tri-phasic System is performed. The large-scale system to extract and purify high value-added products, phycocyanins and proteins are studied as well. The conclusion of each research scope is stated as below:

(1) **Isolation of protein from *Chlorella sorokiniana* CY1 using liquid biphasic flotation assisted with sonication through sugaring-out effect**: The development of an efficient and rapid extraction method, Liquid Biphasic Flotation (LBF) assisted with sonication was investigated as single unit operation for protein extraction. Various parameters were studied and the optimized conditions to obtain highest recovery and separation efficiency of protein are: 200 g/L of glucose, 10 s of resting time and 5 s of sonication time (in pulse mode), volume ratio with 1:1.25 (volume of bottom phase: volume of top phase) and 0.25 g

of microalgae load. A total 80% of protein recovery and 49% of separation efficiency has been obtained, showing the effective recovery of protein from microalgae biomass within short period of time (5 min) via proposed technique.

(2) **Microalgal protein extraction from *Chlorella vulgaris* FSP-E using triphasic partitioning technique with sonication**: A novel emerging technique, ultrasonic-assisted three phase partitioning (UATPP) in extracting protein from *Chlorella* sp. is presented. The best protein recovery and separation efficiency of 56.57% and 74.59% are obtained using the optimized parameters as shown: 50% of salt saturation, ratio of 1: 2 (slurry to t-butanol), 100% sonication power, 10 min irradiation time, 35 kHz of frequency, 80% duty cycle and 0.75 wt% of biomass loading in the system. UATPP has enhanced the recovery and separation efficiency by around 15% and 3%, respectively with significant decrement of processing time. The results obtained in scaled up system has shown promising results which has comparable yield with the lab scale system and the surface of microalgae biomass were shrunk after the sonication treatment, indicating the breakdown of microalgae cell wall. This research has presented the potentiality of UATPP in producing protein in industrial scale.

(3) ***Spirulina platensis* based biorefinery for the production of value-added products for food and pharmaceutical applications**: Several pre-treatments techniques, namely freeze-thawing, homogenisation,

microwave and sonication were investigated for the feasibility in treating the microalgae biomass for pigment-protein complex extraction using Liquid Biphasic System (LBS). Highest recovery and purification fold of 94.89% and 6.17, were obtained respectively, using sonication (20% power, 50% duty cycle and 7 min irradiation time). The effectiveness of pre-treatments is arranged in the ascending order of sonication, homogenisation (3 min processing time and speed of 3), microwave (50% duty cycle and 2 min irradiation time) and finally freeze-thawing (8 h freezing time and 20 min thawing time). The extract containing phycocyanins are characterized using SDS-PAGE. This work provides insights to the next scope which sonication as an effective technique to rupture biomass for biomolecules production.

(4) **Sonoprocessing-assisted solvent extraction for the recovery of pigment-protein complex from *Spirulina platensis***: Sonoprocessing of phycocyanins has been performed and the optimized conditions are as followed: 30% amplitude, 5 s ON/ 5 s OFF in pulse mode, 10 min of total irradiation time, 1:1 volume ratio, 100 cc/min of flotation rate, 7 min of flotatition time and 0.45 g of biomass loading have recovered 95.1% of C-phycocyanins with purity factor of 5.23. The obtained results have made a great improvement of phycocyanins compared to conventional processing technique and literatures reports, presenting great prospects of sonoprocessing-assisted solvent extraction technique for commercializing valuable compounds.

7.2 Future Works

The research scopes addressed in this thesis are accomplished, yet some future works are recommended to improve the techniques of downstream processing or commercializing the production of biomolecules in a greener and sustainable way. First, the proposed techniques are suggested to be designed and studied for the extraction and purification of biomolecules in pilot scale study prior to industrial scale. Although the lab scale of system has similar performance for a slightly larger scale of system, the performance of pilot scale system and industrial scale system are uncertain. The feasibility of the proposed techniques for industrial scale production can only be observed with scaled-up study. Secondly, the phase components used in the systems such as sugars, salts, polymers and alcohols are proposed to be recycled for next usage to save operating cost and reduce the usage of fresh chemicals for environmental purposes. The potentiality of salting-out in biomolecules extraction especially proteins are acknowledged with the advantages of preventing protein denaturation, no mechanical mixing for inducing efficient extraction and lesser solvent consumption. However, the major problem to be faced in large-scale study such as equipment corrosion due to reaction between salt solution and metal and the pH environment is altered according to the pH of salt used (Poole, 2020). The mentioned issues can be overcome by using sugaring-out where sugar solution does not contribute to pH change of system and react with metal, reducing the probability of equipment corrosion and saving the maintenance cost. Hence, recycling sugar is expected to be a better option compared to recycling salt. The storage of recycled solution can be an issue as well for salting out methods before removing or filtering the contaminants in the solution prior

to the next cycle of extraction, which is related to the reaction of salt solution with the equipment. Both sugar and salt can be recycled through different methods such as evaporation or addition of solvent, so the cost reduction by recycling raw materials is feasible. More studies in recycling of salt solution were performed compared to recycling of sugar solution, and often showed convincible results compared to the first extraction performed. Although the recycled studies of salt have showed good results, it is more practical to recycle sugar when the process is upscaled to industrial production in maximizing the profit of industry. The quality of the targeted compounds obtained using the recycled phase components has to be investigated as well to compared those obtained using first cycle of extraction. The design of equipment may alter if recycling large volumes of solution are considered. Lastly, it is suggested to improvise the proposed methods for phycocyanin extraction and purification to minimize the unit operation for reducing the processing time. The simultaneous sonoprocessing techniques are not workable for phycocyanins at which bubbling was observed in the polymers phase and resulted in loss of products. A suitable phase component for the system is required to be discovered particularly for phycocyanin as it is not soluble in alcohol (as top phase).

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APPENDIX

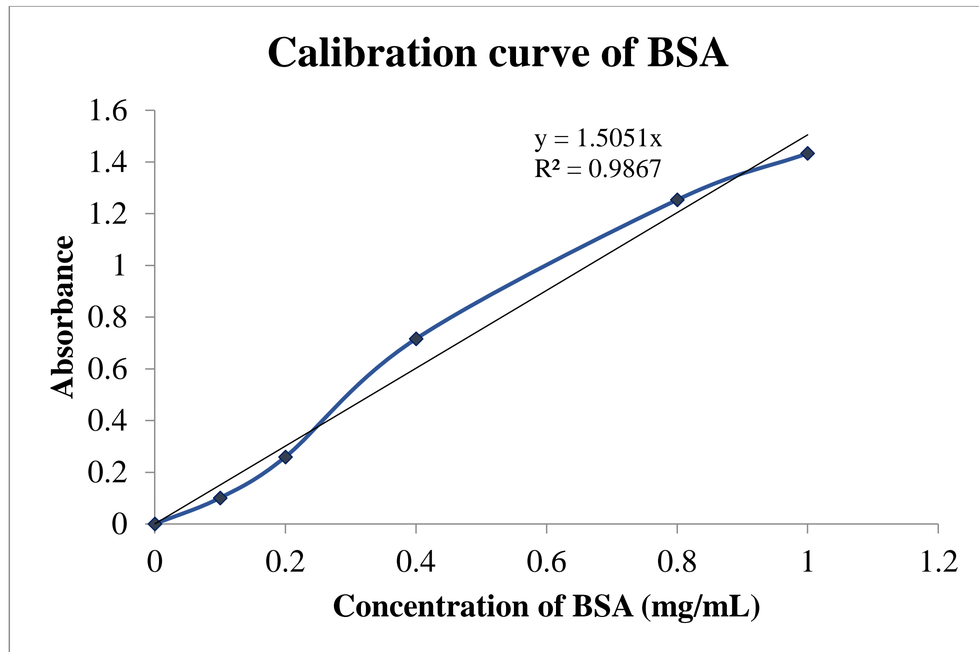

Figure A 1: Calibration curve of bovine serum albumin (BSA).