CULTIVATION OF MICROALGAE USING ORGANIC COMPOST AS NUTRIENT SOURCE FOR BIOMOLECULES EXTRACTION VIA MULTIPHASE PARTITIONING TECHNIQUES

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ABSTRACT

Microalgae have been gaining attention as a sustainable third-generation feedstock for biofuel and bioproducts production due to their high products yield and renewability compared to other plant sources. Nevertheless, the production of biofuels and valuable compounds from microalgae does not perform well in terms of an economic and environmental perspective. The cultivation of microalgae requires large amounts of freshwater and nutrients, leading to high operating costs. There is a need to find alternative sources which could potentially promote the recycling the nutrients for renewable feedstock cultivation. Utilization of these waste sources could alleviate the global resource crisis and contribute to better nutrient use efficiencies. Besides that, the conventional processes used for extracting biomolecules from microalgae are not efficient and requires evaluation on the feasibility. Most of the processes involves the isolation, concentration and purification steps that uses high amount of chemicals and expensive equipment, hence contributing to the high total cost of producing valuable compounds from microalgae. This raises the significance of developing approaches to reduce the cost and increase efficiency in both the cultivation of microalgae and extraction techniques for biomolecules production.

This thesis presents the ultimate goal of discovering and developing a sustainable processing for the cultivation and biomolecules extraction from microalgae. The sustainable approaches include the discovery of an alternative nutrient source for the cultivation of microalgae, utilization of uprising bioseparation techniques for effective biomolecules extraction and purification, as well as the subsequent value-added products generation from the

combination of various waste sources. This thesis discusses the use of food waste compost as an organic medium for microalgae cultivation. Food waste compost solution has the potential to partially substitute a portion of the inorganic medium and have shown to produce better biomass production rate (11.1% increase) and higher biochemical content (10.1% higher lipid and 2.0% higher protein content) in the biomass. This thesis also presents the use of two multiphase partitioning techniques, namely microwave-assisted three phase partitioning (MWTPP) and liquid biphasic flotation (LBF), for the extraction and purification of proteins and pigments, respectively. These techniques are simple and easy to operate, rapid processing, environmentally friendly, produce high yield and separation efficiency, and are potential to be scaled-up without difficulty. The utilization of these multiphase separation processes has shown promising extraction of valuable biomolecules from microalgae. MWTPP has successfully recovered 63.2% of proteins from *Chlorella* microalgae and LBF has purified 90.4% of C-phycocyanin from *Spirulina* microalgae. Moreover, this thesis illustrates the densification of food waste compost with dairy waste powder as an effective approach for producing value-added compounds using waste sources. The densification was done through pelletizing the compost with waste additive into pellets that are easier for handling, transportation and management. Lastly, the research achievement in these works and the future opportunities for further works are highlighted in the later parts of this thesis.

LIST OF PUBLICATIONS

Journal Publications

1. **Chew, K. W.**, Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., Lee, D.-J., Chang, J.-S., 2017. Microalgae biorefinery: high value products perspectives. *Bioresource Technology*. 229, 53-62. DOI: 10.1016/j.biortech.2017.01.006

2. Chia, S. R., Ong, H. C., **Chew, K. W.**, Show, P. L., Phang, S.-M., Ling, T. C., Nagarajan, D., Lee, D.-J., Chang, J.-S., 2018. Sustainable approaches for algae utilisation in bioenergy production. *Renewable Energy*. 129, 838-852. DOI: 10.1016/j.renene.2017.04.001

3. **Chew, K. W.**, Juan, J. C., Phang, S. M., Ling, T. C., Show, P. L., 2018. An overview on the development of conventional and alternative extractive methods for the purification of agarose from seaweed. *Separation Science and Technology*. 53, 467-480. DOI: 10.1080/01496395.2017.1394881

4. **Chew, K. W.**, Ling, T. C., Show, P. L., 2019. Recent Developments and Applications of Three-Phase Partitioning for the Recovery of Proteins. *Separation & Purification Reviews*. 48, 52-64. DOI: 10.1080/01496395.2017.1394881

5. Chia, S. R., **Chew, K. W.**, Show, P. L., Yap, Y. J., Ong, H. C., Ling, T. C., Chang, J. S., 2018. Analysis of economic and environmental aspects of microalgae biorefinery for biofuels production: a review. *Biotechnology Journal*. 1700618. DOI: 10.1002/biot.201700618

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Frontiers of Environmental Science & Engineering. 12, 2. DOI: 10.1007/s11783-018-1040-0

10. **Chew, K. W.**, Chia, S. R., Show, P. L., Ling, T. C., Arya, S. S., Chang, J.-S., 2018. Food waste compost as an organic nutrient source for the cultivation of Chlorella vulgaris. *Bioresource Technology*. 267, 356-362. DOI: 10.1016/j.biortech.2018.07.069

11. Din, S. S., **Chew, K. W.**, Chang, Y. K., Show, P. L., Phang, S. M., Juan, J. C., 2018. Extraction of agar from Eucheuma cottonii and Gelidium amansii seaweeds with sonication pretreatment using autoclaved method. *Journal of Oceanology and Limnology*. DOI: 10.1007/s00343-019-8145-6

12. Chia, S. R., **Chew, K. W.**, Show, P. L., Manickam, S., Ling, T. C., Tao, Y., 2018. Isolation of Protein from Chlorella sorokiniana CY1 using Liquid Biphasic Flotation assisted with Sonication through Sugaring-out Effect. *Journal of Oceanology and Limnology*. DOI: 10.1007/s00343-019-8246-2

13. **Chew, K. W.**, Chia, S. R., Lee, S. Y., Zhu, L., Show, P. L., 2019. Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique. *Chemical Engineering Journal*. 367, 1-8. DOI: 10.1016/j.cej.2019.02.131

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2. **Chew, K. W.**, Show, P. L., Yap, Y. J., 2018. Effects of moisture content and milk powder waste additives on the pelletization of food waste compost. *4th International Conference on Energy Materials and Environmental Engineering (ICEMEE)*. Kuala Lumpur, Malaysia.

3. **Chew, K. W.**, Show, P. L., 2017. Nutrient-enriched biowaste from food industry as organic fertilizer for improving growth and yield of crops. *4th Postgraduate Colloquium for Environmental Research (POCER)*. Melaka, Malaysia.

Book Chapter

1. **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 iii

ACKNOWLEDGEMENTS.....vii

LIST OF FIGURES xv

LIST OF TABLES..... xviii

LIST OF ABBREVIATIONS.....xx

CHAPTER 1 INTRODUCTION 1

1.1 Research Background 1

1.2 Problem Statement.....6

1.3 Research Objectives.....7

1.4 Research Contributions.....8

1.5 Outline of Thesis.....10

1.6 Flow Diagram of the Research 11

CHAPTER 2 LITERATURE REVIEW 12

2.1 Microalgae as a Third-generation Feedstock.....13

2.1.1 Valuable components of microalgae and processes.....14

2.1.1.1 Lipids 14

2.1.1.2 Proteins 15

2.1.1.3 Carbohydrates 16

2.1.1.4 Pigments.....17

2.1.2 Biological activity from microalgae 23

2.2 Effects of Culture Medium for Microalgae Cultivation 24

2.2.1 Freshwater microalgae 24

2.2.2 Saltwater microalgae 28

2.2.3 Wastewater microalgae 29

2.2.4 Organic mediums for microalgae cultivation 32

2.3 Microalgae Biorefinery for High Value Products 34

2.3.1 Biorefinery concept 34

2.3.2 Biorefinery of microalgae 37

2.3.2.1 Thermochemical conversion 39

2.3.2.2 Biochemical conversion 40

2.3.2.3 Transesterification 41

2.3.2.4 Photosynthetic microbial fuel cell 41

2.3.3 Applications and potentials of microalgae 42

2.4 Emerging Bioseparation Techniques for Biomolecules Extraction 43

2.4.1 Three-phase partitioning (TPP) 44

2.4.1.1 Basic principles 45

2.4.1.2 Key parameters affecting TPP system 49

2.4.1.3 Variants of three phase partitioning 56

2.4.2 Liquid biphasic flotation (LBF) 65

2.4.2.1 Basic principles 65

2.4.2.2 Key parameter affecting LBF system 66

2.5 Densification of Compost Pellets 70

2.6 Research Gaps 75

CHAPTER 3 FOOD WASTE COMPOST AS AN ORGANIC NUTRIENT SOURCE FOR THE CULTIVATION OF CHLORELLA VULGARIS 77

3.1 Abstract.....79

3.2 Introduction.....80

3.3 Materials and Methods 83

3.3.1 Microalgae strain and medium compositions.....83

3.3.2 Microalgae cultivation with food waste compost 83

3.3.3 Determination of microalgae cell concentration 85

3.3.4 Quantification of nitrate and phosphorus content in medium ..85

3.3.5 Microalgae lipid extraction 86

3.3.6 Cell disruption and protein extraction.....87

3.3.7 Determination of carbohydrate contents 87

3.3.8 Statistical analysis 88

3.4 Results and Discussion 88

3.4.1 Effects of nutrient concentrations on biomass yield 88

3.4.2 Nutrient removal efficiencies.....92

3.4.3 Biochemical composition.....96

3.4.3.1 Lipid content 96

3.4.3.2 Protein content 99

3.4.3.3 Carbohydrate content 101

3.4.4 Evaluation of biochemical content in microalgae 102

3.5 Conclusion 104

CHAPTER 4 ENHANCED MICROALGAL PROTEIN EXTRACTION AND PURIFICATION USING SUSTAINABLE MICROWAVE-ASSISTED MULTIPHASE PARTITIONING TECHNIQUE 105

4.1 Abstract 107

4.2 Introduction 108

4.3 Materials and Methods 111

4.3.1 Materials 111

4.3.2 Microalgae strain and medium compositions 111

4.3.3 Determination of nitrate content and microalgae cell concentration 112

4.3.4 Protein extraction with TPP system 112

4.3.5 Protein extraction with MWTPP system 113

4.3.6 Protein assay 115

4.3.7 Determination of recovery yield and separation efficiency 115

4.3.8 Cell disruption by microwave 116

4.4 Results and Discussion 116

4.4.1 Effect of type of salts 116

4.4.2 Effect of salt concentration 118

4.4.3 Effect of t-butanol ratio 120

4.4.4 Effect of microwave time 121
4.4.5 Effect of microwave duty cycle 123
4.4.6 Effect of microwave power 125
4.4.7 Effect of microalgae biomass 127
4.4.8 Comparison of MWTPP with conventional TPP technique 128
4.5 Conclusion 133
CHAPTER 5 LIQUID BIPHASIC FLOTATION FOR THE PURIFICATION OF C-PHYCOCYANIN FROM SPIRULINA PLATENSIS MICROALGA 134
5.1 Abstract 136
5.2 Introduction 137
5.3 Materials and Methods 140
5.3.1 Materials 140
5.3.2 Preparation of crude phycocyanin extract 140
5.3.3 Liquid biphasic flotation technique 140
5.3.4 Analytical procedures 142
5.3.4.1 Determination of C-phycocyanin concentration 142
5.3.4.2 Gel electrophoresis 143
5.4 Results and Discussion 143
5.4.1 Effect of PEG molecular weight 143
5.4.2 Effect of types of salt 145
5.4.3 Effect of polymer and salt concentration 146

5.4.4 Effect of volume ratio 149
5.4.5 Effect of pH 151
5.4.6 Effect of air flotation time 152
5.4.7 Effect of crude extract concentrations 154
5.4.8 SDS-PAGE 155
5.5 Conclusion 156
CHAPTER 6 DENSIFICATION OF FOOD WASTE COMPOST: EFFECTS OF MOISTURE CONTENT AND DAIRY POWDER WASTE ADDITIVES ON PELLET QUALITY 157
6.1 Abstract 159
6.2 Introduction 160
6.3 Materials and Methods 164
6.3.1 Physico-chemical characterization of food waste compost 165
6.3.2 Preparation of compost for pelletization 166
6.3.3 Physical characterization of compost pellets 167
6.3.3.1 Pellet production 167
6.3.3.2 Pellet density 167
6.3.3.3 Compression test 168
6.3.3.4 Wettability Index (WI) 169
6.3.3.5 FESEM analysis 169
6.4 Results and Discussion 170
6.4.1 Physico-chemical characterization of raw material 170

6.4.2 Effect of MC and AA on pellet production 171
6.4.3 Densification of compost pellets 174
6.4.4 Compressive strength of compost pellets 176
6.4.5 Wettability Index of compost pellets 178
6.4.6 FESEM analysis of binding mechanism 179
6.5 Conclusion 182
CHAPTER 7 CONCLUSIONS AND FUTURE WORKS 183
7.1 Research Achievements 183
7.2 Future Works 186
REFERENCES 189
APPENDIX 213

LIST OF FIGURES

Figure 1.1: Flow chart illustrating the flow of research works in this PhD thesis. 11

Figure 2.1: Applications of a fully integrated microalgae biomass biorefinery system. 37

Figure 2.2: Algal biomass conversion processes for biofuels and bioenergy production. 39

Figure 2.3: Phase diagram of the water/ammonium sulphate/t-butanol three component system at 25 °C (Kiss et al., 1998). 47

Figure 2.4: Schematic representation of TPP system. 48

Figure 3.1: Growth curve of *C. vulgaris* cultivated in BG11 and various food waste compost medium combinations. 89

Figure 3.2: Nitrate content during growth of *C. vulgaris* cultivated in BG11 and various food waste compost medium combinations. 94

Figure 3.3: Initial and final phosphorus concentrations and removal rates from the cultivation medium consisting of BG11 and organic food waste compost medium. 96

Figure 3.4: Biochemical composition of *C. vulgaris* in BG11 medium and various food waste compost medium combinations. Values shown are the mean of triplicate reading ± SD. 97

Figure 4.1: Effect of various types of salt (Ammonium sulphate, sodium sulphate, magnesium sulphate, magnesium acetate and dipotassium hydrogen phosphate) on the recovery yield and separation efficiency of proteins from *C. vulgaris*. 117

Figure 4.2: Effect of salt concentration (15, 20, 25, 30 and 35%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.....119
Figure 4.3: Effect of ratio of slurry to t-butanol (1:0.5, 1:1, 1:1.5 and 1:2) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.....121
Figure 4.4: Effect of microwave irradiation time (60, 90, 120 and 150 s) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.....123
Figure 4.5: Effect of microwave duty cycle (20, 40, 60, 80 and 100%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.....124
Figure 4.6: Effect of microwave power (100, 180 and 300 W) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.....126
Figure 4.7: Effect of microalgae biomass concentration (0.25, 0.5, 0.75, 1 and 1.25%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*. 128
Figure 4.8: FESEM visualization of *C. vulgaris* cell disruption: (a) microalgae cells without treatment; (b) microalgae cells with microwave-only treatment (Microwave time: 120s, duty cycle: 80%, power: 100W); (c) microalgae cells with optimized MWTPP process (AS concentration: 30 %w/w, ratio of slurry to t-butanol: 1:1, microwave time: 120s, duty cycle: 80%, power: 100W)...132
Figure 4.9: Enlarged FESEM visualization (Mag: 20000×) of *C. vulgaris* cell disruption: (a) microalgae cells without treatment; (b) microalgae cells with optimized MWTPP process. 133
Figure 5.1: Effect of various types of salt on the C-PC recovery yield and purification fold from *Spirulina platensis*.....146
Figure 5.2: Effect of polymer concentration (g/L) on the C-PC recovery yield and purification fold from *Spirulina platensis*.....147

Figure 5.3: Effect of salt concentration (g/L) on the C-PC recovery yield and purification fold from *Spirulina platensis*.....148
Figure 5.4: Effect of volume ratio on the C-PC recovery yield and purification fold from *Spirulina platensis*. 150
Figure 5.5: Effect of pH of the system on the C-PC recovery yield and purification fold from *Spirulina platensis*.....152
Figure 5.6: Effect of air flotation time (min) on the C-PC recovery yield and purification fold from *Spirulina platensis*.....153
Figure 5.7: Effect of crude extract concentration (%w/w) on the C-PC recovery yield and purification fold from *Spirulina platensis*.....155
Figure 5.8: SDS-PAGE of C-phycocyanin: Lane A indicates the molecular marker; lane B indicates the crude extract of C-PC; and lane C shows the C-PC after LBF process.....156
Figure 6.1: Effects of MC and additive addition on the pellet production. ...173
Figure 6.2: Effects of MC and additive addition on the pellet density of the compost pellets.....175
Figure 6.3: Effects of MC and additive addition on the compressive strength of the compost pellets.....177
Figure 6.4: Effects of MC and additive addition on the Wettability Index of the compost pellets.....179
Figure 6.5: FESEM images on the compost pellet (35%MC) surface. **(a)** Uncompressed pellet with 0%AA; **(b)** Uncompressed pellet with 20%AA, yellow dotted lines represent some of the clusters; **(c)** Compressed pellet with 0%AA; and **(d)** Compressed pellet with 20%AA.....180

LIST OF TABLES

Table 2.1: Extraction method and yield of lipids and carbohydrates from various microalgae species. 18

Table 2.2: Extraction method and yield of pigments from various microalgae species. 22

Table 2.3: Freshwater species description, nutrient content, scale-up potential and large scale application. 26

Table 2.4: Saltwater species description, nutrient content, scale-up potential and large scale application. 31

Table 2.5: Chromatographic operations and the physicochemical properties, characteristics and applications for purification of proteins. 46

Table 2.6: Enzyme extraction from various crude extracts using three-phase partitioning (TPP). 57

Table 2.7: Protein extraction from various crude extracts using liquid biphasic flotation (LBF) technique. 69

Table 3.1: Characteristics of the food waste compost and organic compost medium. 84

Table 3.2: Dry cell weight concentration (DCW), average biomass productivity (P_b) and specific growth rate (μ) of microalgae cultivated in various combinations of BG11 and compost medium. 91

Table 3.3: Lipid, protein and carbohydrate content (mg/g) in *C. vulgaris* cultivated in BG11 medium and various food waste compost medium combinations. 104

Table 4.1: Operating parameters of MWTPP system for protein extraction from *Chlorella vulgaris*. 114

Table 4.2: Comparison of the yield of protein and separation efficiency obtained using TPP, initial MWTPP and optimized MWTPP on microalgae.....129
Table 5.1: Operating parameters of LBF system for C-phycocyanin purification from *Spirulina platensis*.....141
Table 5.2: Effect of polymer molecular weight on the C-PC recovery yield and purification fold from *Spirulina platensis.*.....144
Table 6.1: Properties of food waste compost.....171

LIST OF ABBREVIATIONS

AA	Additive addition
AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
AS	Ammonium sulphate
ASABE	American Society of Agricultural and Biological Engineers
ATPE	Aqueous two phase extraction
$BmimBF_4$	1-butyl-3-methylimidazolium tetrafluoroborate
BmimTfO	1-butyl-3-methylimidazolium trifluoromethanesulfonate
BSA	Bovine serum albumin
C	Concentration
CHP	Combined heat and power
C-PC	C-phycocyanin
C/N	Carbon to nitrogen ratio
DCW	Dry cell weight
DHA	Docosahexaenoic acid
DHP	Dipotassium hydrogen phosphate
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
E	Separation efficiency of proteins
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FESEM	Field emission scanning electron microscopy
GHG	Greenhouse gas
IL	Ionic liquid
ILATPS	Ionic liquid aqueous two phase system
ILTPP	Ionic liquid three phase partitioning
K	Partition coefficient
LBF	Liquid biphasic flotation
LBS	Liquid biphasic system
MA	Magnesium acetate
MAE	Microwave assisted extraction
MC	Moisture content

MS	Magnesium sulphate
MW	Molecular weight
MWTPP	Microwave assisted three phase partitioning
N_{ea}	Total nitrogen % in microalgae
NTP	Nitrogen to protein conversion factor
N:P	Nitrogen to phosphorus ratio
OD	Optical density
OVAT	One-variable-at-a-time
P_b	Average biomass productivity
PBR	Photobioreactor
PEG	Polyethylene glycol
PF	Purification factor
PT	Purity of phycocyanin
PUFA	Polyunsaturated fatty acid
R	Recovery yield of proteins
RC	Recovery yield of C-phycocyanin
SC	Supercritical
SC-CO_2	Supercritical carbon dioxide
SD	Standard deviation
SFE	Supercritical fluid extraction
SS	Solvent sublation
SSE	Sodium sulphate
TAG	Triacylglycerol
TPP	Three phase partitioning
UATPP	Ultrasonic assisted three phase partitioning
USP	Upstream processing
UV-Vis	Ultraviolet-visible
V	Volume
VR	Volume ratio
WI	Wettability index
μ	Specific growth rate

CHAPTER 1

INTRODUCTION

1.1 Research Background

Microalgae have received much interest as a biofuel feedstock in response to the uprising energy crisis, climate change and depletion of natural sources. The current developments of microalgal biofuels from microalgae lacks the economic feasibility due to overwhelming capital investments and operations. Hence, high-value co-products have been produced through the extraction of a fraction of algae to improve the economics of a microalgae biorefinery. Examples of these high-value products are pigments, proteins, lipids, carbohydrates, vitamins and anti-oxidants, with applications in cosmetics, nutritional and pharmaceuticals industries (Chew et al., 2017). To promote the sustainability of this process, an innovative microalgae biorefinery structure needs to be implemented through the production of multiple products in the form of high-value products and biofuel. The operation costs of microalgae cultivation and biomolecules extractions needs to be reduced through employing sustainable methods and processes to highlight the feasibility of the microalgae biorefinery process.

The cultivation of microalgae in large scale requires a lot of water and nutrients, which results in high production costs that does not meet the economic growth requirements. Alternative waste sources such as wastewater from different industries have been utilized to cultivate microalgae (Cheah et al., 2016; Cheng et al., 2019; Daneshvar et al., 2019), but the wastewater sources

are prone to contamination and hazardous chemicals content which will affect the growth of microalgae. Food waste contains loads of nutrients which can be recycled to the environment. Instead of disposing them into landfills, these wastes can be converted into compost to contribute to a sustainable waste management system. Compost are stable organic mixtures that are a product of the breakdown of organic components. They are commonly used as organic fertilizer and soil amendments to improve soil fertility and crops growth (Cerda et al., 2018). Besides using food waste compost for fertilizing crops and plants for human consumption, there is a possibility of using the compost as a culture medium for aquatic plants such as microalgae. Food waste compost is derived from mainly food waste and green waste, making its nutrient contents less varying compared to wastewater. The prospects of replacing a portion of the inorganic medium used in commercial microalgae production with food waste compost is studied in this research. Food waste compost is also available in abundance and contains high nutrient content which makes it a promising culture medium for microalgae growth (Chew et al., 2018a).

The effective cultivation of microalgae then leads to the need for feasible downstream processing to obtain high value products from the biomass. The importance of downstream processing lies in the maintenance of the products quality while operating in the most cost effective and environmentally friendly manner. Microalgae biomass are harvested not only for biofuel production, but also for high-value products such as pigments, proteins, carbohydrates, anti-oxidants and vitamins (Chew et al., 2017). These biomolecules can be extracted from microalgae using various processes. In this research, our interest focuses on the extraction of proteins and pigments from microalgae.

Conventional methods such as crystallization, column chromatography, precipitation and ultrafiltration for the purification of proteins are deemed as costly processes. These techniques involve multiple steps processing, high operating cost, long working time and have difficulties in scaling up (Asenjo & Andrews, 2012). Hence, it is necessary to develop and apply a potential separation technique which can purify the proteins in an efficient and cost-effective manner. This research aims to explore the advanced multiphase partitioning mechanism for the extraction and purification of proteins. Multiphase partitioning applies the concept of two or more phases to recover the desired biomolecules to one phase for subsequent purification (Phong et al., 2017). Multiphase partitioning techniques are simple and rapid separation processes, with high recovery yields and separation efficiency. Besides that, the process is eco-friendly as organic solvents and salts from aqueous phases can be recovered and recycled. These techniques can also be incorporated with additional cell disruptive processes to improve the overall product quality (Kulkarni & Rathod, 2015; Sankaran et al., 2018a).

Two novel multiphase techniques studied in this research is microwave-assisted three phase partitioning (MWTPP) and liquid biphasic flotation (LBF) for the extraction and recovery of protein and phycocyanin, respectively. MWTPP is a combination of three phase partitioning (TPP) incorporated with microwaves assistance to disrupt the cell wall of microalgae to promote the release of valuable biomolecules. MWTPP proves to be an enhanced version of the TPP technique as it can recover higher yields in shorter span of time (Kulkarni & Rathod, 2014). Microwave assisted extraction have the advantages of quick heating, low solvent quantity consumption and reduction in energy

usage and pollution (Cheng et al., 2017). LBF utilizes the bubbles-assisted concept and incorporates it with the traditional liquid biphasic system (LBS), making it a combination of both LBS and solvent sublation. The adsorptive gas bubbles will adsorb the desired biomolecules onto the surface of the bubbles and transfer them from one liquid phase to another (Sankaran et al., 2018a). LBF is technically simple, energy efficiency and commercially scalable, besides that, it has the advantages of high recovery yield and separation efficiency, ease of processing, high concentration coefficient and is environmentally friendly (Leong et al., 2018). Both of these bioseparation techniques are very useful as a potential downstream process for biomolecules extraction and purification.

The utilization of organic composts medium for microalgae cultivation will generate solid wastes, which could lead to environmental impacts if disposed inappropriately. This creates the need to process the solid wastes in a sustainable manner which can contribute to the recycling of valuable sources. Hence, it is imperative that the organic sources are processed in the simplest way possible, using the least harmful chemicals, so that there will be a possibility of recycling these wastes into value-added products. The food waste compost used for the cultivation of microalgae in this research was processed only by diluting them in water (Chew et al., 2018a). The food waste residue in the form of soaked compost solids can then be dried and reused as compost for plants growth. However, these compost solids have released a portion of their nutrients to be used as a nutrient supplement. Hence, their nutrient content needs to be improved for the compost solids to have a better practicality in fertilization. The need for densification and the search for a suitable additive that can be added into the compost pellets then becomes vital. The compost

solids after drying would still retain some physicals properties of the original compost, particularly the powdery particle sizes. This creates difficulty in the handling, management and storage of compost in its powder form as they are weighty and bulky (Obidziński, 2012). Since the nutrient value of these compost solids are reduced, they can be densified to increase the nutrient content by several folds to be used in smaller proportions (Zafari & Kianmehr, 2013).

The production of compost pellets is highly dependent on the moisture content of the compost (Chew et al., 2018b). The adjustment of the moisture content to produce the best pellets is essential as too much moisture will lower the strength and durability of the pellets, while too little moisture will lead to problem in producing desirable pellets (Monedero et al., 2015). Additives can be added and are commonly used as binders in biomass densification to improve the physical and mechanical characteristics of the biomass by forming stronger bonds within the pellet. The utilization of suitable waste materials as a binder for compost pellets will also contribute to better waste management. Dairy waste products, which are compatible with humans and contain a lot of valuable compounds, is a great additive that can be used as a binder for pelletization. Dairy powder wastes have plenty of micronutrients which can benefit plants growth. The major content in these dairy wastes are usually lactose, where when dissolve in water, produces a sticky solution that can aid in the formation of good pellets (Silalai & Roos, 2010). The ability of lactose to crystallize will strengthen the pellets and at the same time, improve the nutritional content of the compost pellets. The conversion of both food waste compost and dairy waste products into a value-added product is performed in this research by pelletizing the compost and evaluating its physical and mechanical properties.

1.2 Problem Statement

The major issues faced by modern society is waste management and sustainability. The increasing amount of waste produced and lack of sustainable processing has led to accelerated damage to the planet and ecosystem. This has called for much efforts to reduce waste production, reuse waste and recycle them. Third generation renewable feedstock like microalgae have shown great prospects to produce biofuel and bioproducts (Chew et al., 2017; Chia et al., 2018a). However, the culturing of these biomass requires a lot of inorganic nutrients. Alternative sources of organic nutrients such as food waste compost are being searched for to substitute the use of inorganic nutrient mediums. Apart from that, the current bioprocessing of these biomass for bioproducts generation have also shown indications of potential improvement in terms of economic feasibility and environmental impacts (Asenjo & Andrews, 2012; Przybycien et al., 2004). The handling of food waste compost in its original form has also pose many hazards and difficulty especially in terms of storage, transportation and management. The reutilization of waste resources produced by incorporating them with other waste sources to create value-added products then become a necessity to promote sustainability.

Landfilled organic wastes that are transformed into value-added products, such as compost or organic amendments, have good nutrient content that can be recycled for plants growth. These organic wastes are available in abundance and have the potential to substitute chemical-based fertilizer. Moreover, the evolution of bioseparation processes such as liquid biphasic system has reported efficient and green processing of biomolecules from various sources. The simplicity and effective operation of this technique is

highly favourable for biomolecules extraction and purification. This PhD research aims at understanding the need to reutilize waste sources for microalgae cultivation as well as value-added products synthesis, followed by the development of novel efficient purification methods for biomolecules extraction from microalgae. Apart from that, this research also aims to incorporate valuable dairy powder waste into food waste compost for the densification into compost pellets.

1.3 Research Objectives

This research work was aimed at managing waste and resource recovery through microalgae cultivation and biorefinery. The objectives of the research were to identify a potential alternative nutrient source for microalgae cultivation as well as to apply greener and more economical techniques for the biorefinery of microalgae for high value products. The scopes of this research are stated as followed:

1) To substitute the inorganic nutrients in microalgae cultivation process with an alternative organic nutrient source.

2) To perform biomolecules extraction from microalgae using novel green bioseparation techniques.

3) To utilize the organic waste source generated from microalgae cultivation with combination of other waste sources for value-added products generation.

1.4 Research Contributions

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

1) Utilization of food waste compost as an organic nutrient source for the cultivation of microalgae.
    - Food waste compost medium was used as a nutrient supplement for the cultivation of *Chlorella vulgaris*. Various combination of compost mixtures were substituted in the inorganic medium during the cultivation. The algal growth and biochemical composition of the microalgae biomass were evaluated for the inorganic medium and organic medium combinations.

2) Development and implementation of microwave-assisted multiphase partitioning technique for protein extraction from microalgae.
    - A novel and efficient bioseparation method of microwave-assisted three phase partitioning (MWTPP) was developed for the extraction of proteins from *Chlorella vulgaris*. Various parameters including types of salts, concentration of salts, ratio of slurry to t-butanol, microwave time, microwave duty cycle, microwave power and microalgae biomass concentration were optimized to maximize the protein recovery yield. The protein yield and separation efficiency from MWTPP were evaluated and compared with the traditional TPP.

3) Development and implementation of liquid biphasic flotation system for the purification of phycocyanin from microalgae.

   - A rapid and efficient bioseparation method of liquid biphasic flotation (LBF) was developed for the purification of C-phycocyanin from *Spirulina platensis*. Various parameters including type of polymer and salt, concentrations of polymer and salt, volume ratio, pH, air flotation time and microalgae biomass were optimized to maximize the C-phycocyanin recovery yield. The recovery yield and purification fold obtained from LBF were evaluated.

4) Densification of food waste compost by altering the moisture content and addition of dairy powder waste additives for pellet quality enhancement.

   - Food waste and green waste compost were pelletized using a roller rotating pelletizer. Various combination of moisture content and dairy waste powder additives were added to enhance the pellet quality during the pelletizing process. The physical and mechanical properties such as moisture content, bulk density, producibility, particle density, compressive strength and moisture resistance were evaluated for the pelletized compost products.

1.5 Outline of Thesis

The outline of this thesis is organized as followed: Chapter 1 introduces the background of this research study and the problems faced by the industries. In this chapter, the research objectives and contributions are also highlighted. Chapter 2 comprises of the thorough literature review on microalgae as a third-generation feedstock, microalgae cultivation systems, microalgae biorefinery for high value products, emerging bioseparation techniques for biomolecules extractions and compost pelletization and densification, which are part of the scopes in this research. Chapter 3 outlines the use of organic food waste compost as an alternative nutrient source for the replacement of inorganic fertilizers in microalgae cultivation (manuscript published in *Bioresource Technology*). Chapter 4 and Chapter 5 each describe a novel multiphase bioseparation process for the extraction and purification of biomolecules from different microalgae species. Chapter 4 depicts the multiphase partitioning mechanism with microwave assistance for protein extraction from *Chlorella* (manuscript published in *Chemical Engineering Journal*), while Chapter 5 depicts the liquid biphasic system with solvent sublation for phycocyanin purification from *Spirulina* (manuscript submitted to *Bioresource Technology*). In Chapter 6, the densification of food waste compost with dairy waste additives is proposed to utilize the waste products from the dairy industries to create value-added organic fertilizers (manuscript published in *Process Safety and Environmental Protection*). Lastly, the list of accomplishments of this research work are summarised in Chapter 7, along with potential future works and recommendations for the developed approaches.

1.6 Flow Diagram of the Research

The flow chart of illustrating the scopes in this research is shown in Figure 1.1. Food waste compost was used to cultivate microalgae and produce value-added compost pellets. Moreover, novel multiphase partitioning techniques were used for the extraction and purification of valuable compounds from microalgae.

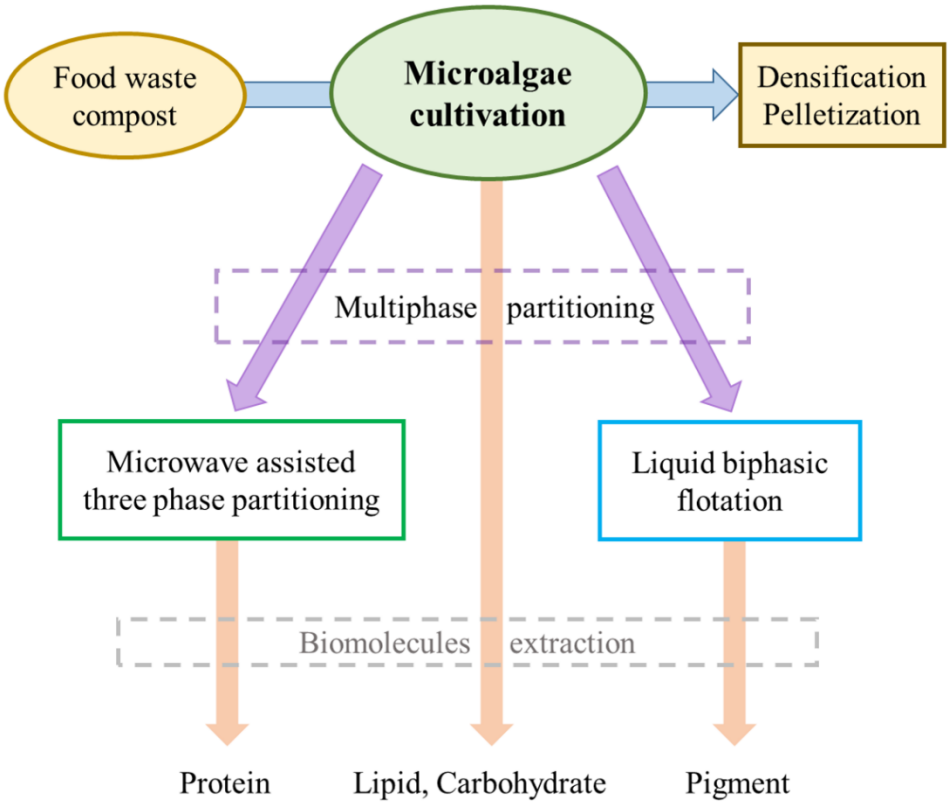


Figure 1.1: Flow chart illustrating the flow of research works in this PhD thesis.

CHAPTER 2

LITERATURE REVIEW

Renewable feedstocks will eventually replace sources of fossil origin (oil, gas and coal) which are rapidly depleting. These feedstocks will be used as fuel and raw materials for the chemical industries. Microalgae is a photosynthetic microorganism which has great prospects as a biomass due to its high production rate and rich nutritional content. Along with its ability to assimilate organic nutrients that can be derived from waste sources, the cultivation of microalgae will not only solve the issues of food and energy security, but also play a major role in waste management. This chapter presents the literature review related to the scopes of this research. Section 2.1 discusses the overview of microalgae as a third-generation feedstock, which includes the valuable components and biological activity from microalgae. Section 2.2 examines the effects of different culture medium such as freshwater, saltwater, wastewater and organic medium, for microalgae cultivation. Section 2.3 presents the microalgae biorefinery concept and applications and prospects of microalgae biorefinery. Section 2.4 discusses on the emerging bioseparation techniques, such as microwave three-phase partitioning and liquid biphasic flotation, for biomolecules extractions and purification from various sources. Section 2.5 provides the review on the densification of compost pellets and potential addition of additive for pellet properties enhancements.

2.1 Microalgae as a Third-generation Feedstock

Microalgal biomass has recently become increasingly significant as an alternative source for renewable fuels. The search for renewable fuels has gained attention due to the higher energy demand and increasing world population over decades. Certain approaches have been suggested for the use of microalgal biomass for hydrothermal liquefaction as well as for the extraction of valuable compounds, which could widen the market opportunities of microalgae products and open up further possibilities of coupling production of algae for biofuels and high value compounds (Barreiro et al., 2014). There has been renewed attention towards the utilization of microalgae in various sectors including food, chemical feed and pharmaceuticals. The high value products that can be obtained from microalgae include proteins, carbohydrates, lipids, pigments, anti-oxidants, polyunsaturated fatty acids, and vitamins.

Microalgae contains high amount of proteins, lipids and carbohydrates which could be the feedstock for different products. They are grown in open ponds or closed systems that involves mixing and concentrating processes. These processes are energy intensive and the maximum exploitation of microalgae biomass while using minimum energy remains the primary focus (Vanthoor-Koopmans et al., 2013). Extracted microalgal lipids can be utilized as a potential feedstock for biodiesel production while microalgal carbohydrates can be used as a carbon source in fermentation industries to replace conventional carbohydrate sources like simple sugars or treated lignocellulosic biomass. Furthermore, long chain fatty acids found in microalgae have important functions as health food supplements, while proteins and pigments found in microalgae exhibit properties desired in the pharmaceutical industries

to treat certain diseases (Yen et al., 2013). The important role of microalgae in the production of biofuels and bio-based chemicals makes it promising to be considered as an alternative to many natural components and sources.

The advantage of using microalgae for the extraction of high value products is that it can be cultivated by utilizing only water and atmospheric CO_2, which may be available at minimum cost. It does not create the competition for land and food crops as microalgae can grow on degraded land. However, a culture medium rich in nutrients and salts is required for the microalgae cultivation (Baicha et al., 2016). Besides that, microalgae biomass also has high photosynthetic efficiencies, which when coupled with bio-energy production systems, has the capability to be a sustainable pathway to obtain renewable energy sources for the future (Khoo et al., 2013). In addition, microalgae are able to bio-sequester CO_2 from flue gases generated from power plants and this will contribute to the tremendous reduction of greenhouse gases emissions (Cheah et al., 2015).

2.1.1 Valuable components of microalgae and processes

2.1.1.1 Lipids

Microalgae can accumulate a high percentage of lipids, in which the lipids usually account for approximately 30–50% of their content by weight. The lipid content is dependent upon culture conditions, such as high carbon to nitrogen (C/N) ratio in culture medium or under stress conditions. In order to increase the lipid content, higher C/N ratio is needed in the microalgae cultivation. The earlier lipid accumulation will start when the earlier the nitrogen source is exhausted due to the lower amounts of nitrogen in the

fermentation media (Fakas et al., 2009; Zhang et al., 2011a). Stress conditions such as nitrogen starvation, high temperature, pH shift and high salt concentration are required to enhance lipid productivity (Kwak et al., 2016). Microalgae lipids are able to obtain higher lipid productivity compared to other lipid-based energy crops and this makes it attractive as a raw material for biodiesel production and health food supplements (Yeh & Chang, 2012). Different techniques have been used for lipids extraction from microalgae, including solvent extraction (Kochert method and Soxhlet method), ultrasonic extraction, microwave assisted extraction and electroporation (Biller et al., 2013; Hernández et al., 2014). The main drawbacks of the techniques mentioned is that they require energy intensive processing, high operation temperature and use of organic solvents, which has low selectivity and high flammability.

2.1.1.2 Proteins

Proteins are part of the main constituents of microalgae, comprising of 50–70% of the microalgae composition. Protein is one of the important products of microalgae biorefineries and can be used for human or animal nutrition. Although some microalgae contain toxic proteins, analyses can be performed to identify the safe proteins for utilization. Conventional techniques are used to separate cellular material from the soluble components in liquid medium by using a centrifugation or filtration step, resulting in cellular protein loss. Nevertheless, proteins had been successfully recovered by using solvent extraction, which solubilizes the proteins in organic solvents with surfactants while maintaining their functional properties. The factors influencing the extraction process include pH, ionic strength and also type of salt used in bulk

aqueous phase (Vanthoor-Koopmans et al., 2013). SC-CO_2 extraction had also been attempted to extract protein from microalgae without the use of flammable organic solvents (Bjornsson et al., 2012).

2.1.1.3 Carbohydrates

Microalgae typically has a high carbohydrate content which is about 50% higher than its dry weight as it has a relatively high photo conversion efficiency and can easily store carbohydrates (Yen et al., 2013). Algal carbohydrates mainly compose of glucose, starch, cellulose and various kinds of polysaccharides. Among these, algal glucose or starch is conventionally used for biofuel production like bioethanol and hydrogen, while polysaccharides have biological functions as storage, protection and structural molecules (Aikawa et al., 2012; John et al., 2011). Microalgal polysaccharides are able to modulate the immune system and inflammatory reactions, making them highly favorable as sources of biologically active molecules, such as cosmetic additives, food ingredients and natural therapeutic agents. Carbohydrates are normally extracted via chemical hydrolysis from microalgae. A chemical pretreatment (e.g. hydrogen sulfide and sodium hydroxide) has to be carried out in order to convert the carbohydrates into fermentable sugar before the extraction (Karemore & Sen, 2016).

Table 2.1 shows the extraction of lipids and carbohydrate in microalgae species. Different approaches had been adapted for the extraction process. The species involved were *S. almeriensis*, *N. oculata*, *P. ellipsoidea*, *C. vulgaris* and *C. infusionum*. Chemical-based lipid extraction methods normally have low

energy consumption and potential to be scaled-up. However, there are several issues to be addressed such as bio-toxicity, chemical cost and lipid degradation.

2.1.1.4 Pigments

Three basic classes of natural pigments in microalgae should be emphasized: carotenoids, chlorophylls and phycobiliproteins. These pigments have been applied as precursors of vitamins in food and animal feed, additives, cosmetics, pharmaceutical industries, food colouring agents and biomaterials (Krupa et al., 2010; Nobre et al., 2013; Tamiaki et al., 2014; Zhou et al., 2015).

Carotenoids are fat-soluble pigments which give colour to parts of plants and are considered as accessory pigments (Chen et al., 2016). Traditionally, extraction methods of carotenoids from microalgae utilized organic solvents and Soxhlet extractions (Chen et al., 2016), where the selection of solvent is significant to determine the degree of affinity and promote the release of carotenoids. However, low selectivity and excessive solvent requirements lead to the alternative of using supercritical fluid extraction (SFE) (Nobre et al., 2013). A co-solvent modified supercritical carbon dioxide (SC-CO_2) method was also used by Liau et al. (2010) to extract carotenoids. It was deduced that the addition of co-solvents increased the efficiency of carotenoid extraction.

Besides that, an anti-solvent precipitation process was also suggested to enhance the amount of carotenoids in the particulates (Liau et al., 2010). Singh et al. (2013) performed various cell disruption methods to test for the maximum extraction of zeaxanthin, a type of carotenoid. For chemical cell disruption, the microalgae biomass was immersed in dimethyl sulfoxide (DMSO) and then centrifuged, followed by the use of strong inorganic acids such as hydrochloric

Table 2.1: Extraction method and yield of lipids and carbohydrates from various microalgae species.

Species	Products	Extraction/Purification methods	Yield	Remarks	References
S. almeriensis	Lipids	Solvent extraction [Kochert: methanol–chloroform 1:2 (v/v)]	N/A	Need for organic solvents	(Hernández et al., 2014)
S. almeriensis	Lipids	Solvent extraction [Soxhlet: methanol–chloroform 2:1 (v/v)]	8.0 DW%	Need for organic solvents	(Hernández et al., 2014)
N. oculata	Lipids	Ultrasonic extraction	25.0 DW%	Poor product quality.	(Adam et al., 2012)
P. ellipsoidea	Lipids	Microwave extraction (140 °C)	37.5 wt.% (lipid)	Energy intensive. High extraction yield.	(Biller et al., 2013)
S. almeriensis	Lipids	SC-CO_2	10.1 DW%	High operating condition. High extraction yield.	(Hernández et al., 2014)
S. almeriensis	Lipids	Microwave SC- CO_2 (80 °C)	3.1 DW%	Low extraction yield.	(Hernández et al., 2014)
Chlorella sp.	Lipids	Isotonic extraction	19 wt.% (lipid)	Energy intensive. High capital cost.	(Vanthoor-Koopmans et al., 2013)
C. vulgaris	Lipids	Enzyme extraction	5.27%	High materials (enzyme) cost.	(Liang et al., 2012)
C. infusionum	Carbohydrate	Chemical hydrolysis (chemical pretreatment)	89.6 % (sugar)	Low cost.	(Karemore & Sen, 2016)

acid and sulfuric acid or mild organic acids such as citric acid and acetic acid for the treatment of freeze-dried cells before extraction. It was reported that in chemical cell disruption, the use of stronger acids gave a lower yield while the extraction efficiency was increased with mild acids, where a 30-fold increase in carotenoid yield was observed (Singh et al., 2013). As for mechanical cell disruption, the ultrasonication process achieved the highest carotenoid yield, reaching almost a 40-fold increase over direct extraction method.

The sonication process not only assists in cell disruption, but also helps to disperse the small particles of cell debris (Gerde et al., 2012). This indicates that chemical and mechanical disruption is significant to disrupt the cell wall and improve carotenoid extraction. Furthermore, light-related strategies are attractive to be applied to promote cell growth and carotenoids productions such as lutein (Ho et al., 2014).

Chlorophylls are lipid-soluble pigments with low polarity which are widely present in vegetables and fruits as primary photosynthetic pigments. Similar to carotenoids, the traditional method of extracting chlorophyll requires several extraction steps with organic solvents. There are a few factors in solvent extraction that may affect the yield of pigments, namely the type of solvent used, cell wall disruption technique, time for extraction and use of different empirical correlations (Henriques et al., 2007). The use of methanol and ethanol as solvents was found to show better extraction efficiency compared to acetone (Henriques et al., 2007). SC-CO_2 has also been regarded as a suitable solvent for the extraction of chlorophyll and carotenoids as these compounds have low polarity. However, a temperature range of 50-60 °C and high-pressure range of 300-500 bar are necessary for SC-CO_2 extraction operation.

Phycobiliproteins are the major photosynthetic accessory pigments in microalgae. The major applications of phycobiliproteins include natural dyes in food industry and pharmaceuticals products. It exhibits antioxidant, antiviral, anticancer, anti-allergic, anti-inflammatory and neuro protective properties which makes it a promising material in health related applications (Chen et al., 2013; Spolaore et al., 2006). Phycobiliproteins can be obtained through centrifugation, drying, homogenization, repeated freeze-thaw process consecutively (Hemlata et al., 2011).

Separation and purification methods have been developed for phycobiliproteins but many of them are tedious, time consuming, involves large number of steps and produce low yields. A potential alternative to the purification technique is aqueous two phase extraction (ATPE) (Patil et al., 2008), whose application to the extraction of phycobiliproteins was studied under various process parameters, such as type of salts, tie line length and volume ratio, on the final product purity. An integration of membrane process with ATPE also showed that the separation of phase forming components was associated with an elevation in product purity (Patil et al., 2008).

Pigments such as astaxanthin are potent antioxidants among carotenoids as they have strong anti-aging, sun proofing, anti-inflammatory and immune system boosting effects which are very useful in nutraceutical, food and feed supply industries (Cheng et al., 2016). Astaxanthin is highly sensitive to heat, light and oxygen, causing it to degrade easily when exposed to oxidative stress during conventional organic solvent extraction processes (Thana et al., 2008). Applying SC-CO_2 with a co-solvent such as vegetable oils were found to increase the astaxanthin extraction efficiency due to the higher solubility of

astaxanthin in the SC-CO_2 and oil mixture. The enhanced mass transfer rate caused by the swelling of biomass pores will also encourage release of the pigments. Pigment recovery processes from various species of microalgae are summarized in Table 2.2.

Table 2.2: Extraction method and yield of pigments from various microalgae species.

Species	Product	Extraction/ Purification method	Yield	Remarks	Reference
Chlorella pyrenoidosa	Chlorophyll	Solvent extraction	2.9 % (11.4 mg/g)	Organic solvent needed	(Bai et al., 2011)
Isochrysis galbana	Chlorophyll	Solvent extraction	5.6 %	Organic solvent needed	(Bai et al., 2011)
C. saccharophila	Zeaxanthin	Ultrasonication and cell disruption	72.2 % (11.3 mg/g)	Improved extraction method	(Singh et al., 2013)
C. saccharophila	B-carotene	Ultrasonication and cell disruption	37.3 % (5.1 mg/g)	Improved extraction method	(Singh et al., 2013)
Spirulina platensis	Phycocyanin	Photobioreactor with CO_2 fixation	92 mg/g	Enhanced by engineering strategies	(Chen et al., 2013)
Anabaena NCCU-9	Phycocyanin	Repeated freezing and thawing	128 mg/g	Optimization of culture conditions	(Hemlata et al., 2011)
Haematococcus pluvialis	Astaxanthin	Solvent extraction	46 mg/l	Highest yield obtained with 6% CO_2	(Cheng et al., 2016)
H. pluvialis	Astaxanthin	Cell disruption and solvent extraction	32.5 pg/cell		(Kim et al., 2016)
H. pluvialis	Astaxanthin	Physical disruption and solvent extraction	35 % (35.1 mg/g)	Grinding cell wall disruption	(Jaime et al., 2010)

2.1.2 Biological activity from microalgae

Natural pigments such as chlorophyll, carotenoids and their derivatives from microalgae have been extensively studied to identify the effectiveness of these compounds as a cancer chemopreventive agent (Ferruzzi & Blakeslee, 2007). These compounds also show anti-mutagenic and anti-tumorigenic effects, which assists in suppressing the activity of cancer cells formation. Apart from that, phycobiliprotein pigments were also found to contain antioxidant and anticancer activities. Cytotoxicity assay was carried out to determine the anticancer activity of the pigments, where the anticancer activity was found to be higher under nitrogen stressed conditions (Shanab et al., 2012). These findings imply that natural pigments from microalgae are potentially able to be chemopreventive agents to prevent carcinogenesis.

Components such as PUFAs and pigments from microalgae have also demonstrated the ability to reduce inflammation and this suggests the potential for dietary ingredients to cure chronic inflammatory diseases (Robertson et al., 2015). The in vivo studies conducted by various researchers verified that ingredients such as carotenoids in microalgae contain anti-inflammatory activities (Deng & Chow, 2010). This enforces the capability of microalgae to treat inflammatory and other related diseases. Besides that, Kyadari et al. (2013) found that anti-angiogenic and anti-proliferative activity were present in the organic extract of green alga. The extract was tested using experimental models of neovascularization and MTT assay, where MTT assay was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extracts (Kyadari et al., 2013). The microalgae extract showed promising chemo preventive activity against carcinogenesis.

2.2 Effects of Culture Medium for Microalgae Cultivation

The benefits of microalgae over fossil fuels and field crops are that it can produce a greater oil yield per hectare of land, and it can be cultivated in different environments such as sea water, fresh water or even polluted water (Zuorro et al., 2016). Wastewater contains high amount of nutrients, phosphorus and nitrates which can promote the growth of microalgae, resulting in its usage for water purification and remediation along with reduction of global greenhouse gases. The growth of microalgae requires high amount of CO_2, 1 kg of dry algal requires approximately 1.8 kg of CO_2 for survival. Currently there is a wide variety of algae that can be used, where the diversity of species and the large volume of wastewater to be treated makes overall scale up process rather difficult. Strain selection here is important considering that wastewater is not a simple environment for microalgae to survive in (Li et al., 2010). Wastewater colour is also one of the limiting factors here that can justify this statement. The species selected needs to survive under limited light conditions while being able to degrade the compounds responsible for the colour and at the same time withstand the very high nutrient concentrations. The following section elaborates further on the different water culture mediums such as freshwater, saltwater and wastewater for microalgae cultivation.

2.2.1 Freshwater microalgae

The freshwater habitat comprises mainly of low salt and can be split into many different types: ponds, lakes, streams, rivers, and wetlands. Ponds and lakes can sustain a diverse amount of aquatic flora and fauna which include many different types of algae such as microalgae and cyanobacteria species.

The nutrients available in open ponds as well as the exposure to sunny and warm conditions can lead to algal bloom. As for streams and rivers, the water is different at varying river sections. Some areas are fast flowing and have higher oxygen levels but fewer algae can be found there. This is because fast flowing area accumulate less nutrients which inhibits the growth of algae. Freshwater systems usually do not contain much nitrogen and phosphates unless in urban areas where the contribution of nutrient may be derived from treated wastewaters or sewage treatment plants, and also from fertilizer runoff. This limiting condition can thus be modified to suit the needs of large scale cultivation. It is apparent that growing algae in limited nitrogen condition allows for greater growth and productivity (Kalana et al., 2016).

Each species of freshwater algae has different growth performance and a different biological composition compared to other species. This makes isolation and selection of species important to maximize large scale cultivation profits (Duong et al., 2012). Table 2.3 details several freshwater species and provides accurate descriptions of the algae along with other details that focus towards large scale cultivation. *Chlorella sp.* has attracted a lot of interest in both research and commercial sectors due to previous success in a variety of cultivation methods for human nutrition and animal needs. This species can rapidly uptake nutrients and assimilate them for growth. This allows the algae to grow rapidly and hence is good for large scale cultivation and to be made into a variety of different products (Liu & Hu, 2013). Currently the *Chlorella* species is cultivated on a large scale in several locations such as United States, Germany and Japan.

Table 2.3: Freshwater species description, nutrient content, scale-up potential and large scale application.

Species	Descriptions	Nutrient composition	Suitability for scaling up	Large scale production purpose	Reference
Ankistrodesmus falcatus	Green microalgae, forms colonies, described as long needles but curved with sharp edges	Limited phosphorus; high ratio of protein to lipids; high amount of carbohydrates	Possible due to potential in wastewater growth	Possible alternative wastewater treatment to remove nitrogen and phosphorus	(Kilham et al., 1997)
Botryococcus braunii	Green microalgae, oval/egg shaped; widely spread in different habitats	Amount of carbohydrates vary; Usually high lipid composition	Good results for growth in freshwater	Hydrocarbon production and animal waste treatment	(Yamaguchi et al., 1987)
Chlamydomonas reinhardtii	Green microalgae, spherical, large basal pyrenoid within cell	Extract contains good amount of protein and carbohydrates	Difficult to scale up, more for use as model cell and research	-	(Kightlinger et al., 2014)
Chlorella emersonii	Green microalgae; spherical and normally found solitary or in groups; pyrenoid found within cell	High protein content and tend to accumulate lipids and carbohydrates under stressful conditions	Known to produce high amount of nutrients and have good growth and productivity; good potential especially once scaled up for mass cultivation	Possible use in biofuel production, wastewater treatment, CO_2 reduction	(Guccione et al., 2014)

Species	Descriptions	Nutrient composition	Suitability for scaling up	Large scale production purpose	Reference
Chlorella protothecoides	Similar properties to *Chlorella emersonii*				(Al Hattab et al., 2015)
Chlorella sorokiniana	High reproduction rate; tolerant to radiation, temperature and high CO_2 concentrations	Similar properties to other *Chlorella sp.*			(Al Hattab et al., 2015)
Chlorella vulgaris	Close to spherical shape; ellipsoidal pyrenoid with surrounding starch grains; Tolerant to high CO_2 conditions	Similar properties to other *Chlorella sp.*			(Guccione et al., 2014)
Scenedesmus obliquus	Green microalgae, number of cells linearly arranged; straight with sharp edges	Crude protein ranges from 20-40% while lipids range from 10-20%	Good growth in wastewater and inexpensive media; good potential for scale up	Production of biodiesel due to suitable fatty acid profiling	(Álvarez-Díaz et al., 2017)

2.2.2 Saltwater microalgae

Taking into consideration that the most abundant water supply is saltwater from the ocean, this makes saltwater a very economical option for microalgae cultivation to reduce cost. Currently the ocean houses a diverse number of algae in macro, micro form along with cyanobacteria. The majority of the marine algae is represented by different kelp and seaweed but there are some microalgae that can thrive in seawater due to certain adaptations (Wei et al., 2013). Saltwater is usually suitable for microalgae resistant to high salinities as that is the main parameter that will affect the growth in these conditions. The saltwater habitat itself is usually very suitable for salt resistant algae but due to a larger amount of predators in these waters, overall growth outlook is reduced (Mata et al., 2010). Therefore during the cultivation of marine microalgae, the habitat must be closely monitored at all times to ensure that parameters remain optimum to allow for maximum growth and productivity (Kumar et al., 2015).

Compared to freshwater microalgae, the diversity of saltwater species is less as most algae in the ocean are usually of macro type and many of the saltwater microalgae have toxic or harmful properties. Since the ocean receives plenty of sunlight in most of the areas, the algae that grow within have developed adaptations over freshwater algae. The marine algae have higher photo conversion and can therefore make better use of the abundant sunlight to rapidly synthesize biomass. Moreover, the algae lack hemicellulose or lignin which allows them to be processed easily into other products (Wei et al., 2013).. Table 2.4 shows some examples of saltwater microalgae currently under research for large scale cultivation.

There has been the introduction of a recent cultivation technology that utilizes a hybrid approach, whereby the microalgae are initially grown in closed photobioreactors to ensure successful starting growth (Greene et al., 2016), and then proceeds with moving the algae to raceway ponds for short term cultivation until high concentrations are achieved to avoid contamination. This facility is frequently used for demonstrations and experiments where the results demonstrate that algal biofuels produced can compete with fossil fuels when co-products are produced as well. Marine algae can perform similarly to freshwater algae but the cost is offset slightly due to the nutrient content of saltwater.

2.2.3 Wastewater microalgae

Wastewater represents a high potential water source for cultivation of microalgae. This method is known as bio-remediation and can be used as wastewater treatment. By applying algae into wastewater, the algae uses nutrients in the wastewater which purifies the water which can then be discharged back into the water sources. The types of wastewater can be split into domestic, leachate, agricultural, refinery and industrial (Gonçalves et al., 2017). Even palm oil mill effluent can be applied as a culture medium since it typically contains a lot of nitrogen and other nutrients from fertilizers and fruit bunches (Cheah et al., 2016). Wastewaters have roughly similar organic contents, but the inorganic contents of the water differ. Three quarters of organic carbon in sewage are present as carbohydrates, fats, proteins, amino acids, and volatile acids. Inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals. This makes wastewater a good substitute for

ideal culture mediums which are too expensive to use on a large scale. These wastewaters also usually contain very high amounts of nitrogen and phosphates which if discharged to local water sources will cause eutrophication and loss of aquatic life. Since microalgae use these nutrients for growth, they can be employed as a secondary water treatment method (Abdel-Raouf et al., 2012).

Although the high amounts of nutrients favour algal growth, it can be detrimental as well since not all algae species can adapt to the very high nutrient conditions. Wastewater can also contain pathogens and inhibitory substances that can cause the loss of growth and productivity in algae. Therefore, careful selection of microalgae must be done and the microalgae should be allowed to slowly adapt to the wastewater to ensure maximum growth and productivity. A more robust strain will have a higher tolerance towards the environmental conditions and will have lower susceptibility towards predators (Chen et al., 2010). This ensures that the algae selected have the highest chances of adapting and surviving the harsh conditions of wastewater. *Nannochloropsis oculata* and *Tetraselmis suecica* are both marine species that are known to grow well in wastewater. To ensure a good probability of success for large scale cultivation, the algae grown in wastewater should preferably have the properties such as high oil content, high saturated fatty acid, rapid growth rate, tolerance to salinity, good sinking and settling capability, good structural properties, resistance to high temperature and shearing and many more (Duong et al., 2012).

Table 2.4: Saltwater species description, nutrient content, scale-up potential and large scale application.

Species	Descriptions	Nutrient composition	Suitability for scaling up	Large scale production purpose	Reference
Tetraselmis suecica	Found in tidal pool or open sea; motile chlorophyte and contains high lipid content	High amounts of fatty acids	Very suitable and currently used for aquaculture	Aquaculture and biofuel production due to high lipid content	(Otero & Fábregas, 1997)
Phaeodactylum tricornutum	Diatom type algae; exists in different shapes and morphology	High amounts of fatty acids produced; large amounts of C16 fatty acid	Difficult as currently no research done towards scale up of this type	Nutritional products and biofuel production	(Siron et al., 1989)
Chaetoceros calcitrans	Small, fast-growing diatom; usually elliptical in valve view	*Similar to above two species*	Unsuitable as cultures have tendency to fail	-	(Kaspar et al., 2014)
Isochrysis galbana	Small oval green algae	High amounts of carbohydrates present	Unsuitable as cultures have tendency to fail	Used in aquaculture to feed juvenile fish and crustaceans, as well as bivalve larvae in mollusc hatcheries	(Yu et al., 2010)
Nannochloropsis oculata	Unicellular free floating algae, contains yellow-green chloroplast	High amounts of Omega 3 fatty acids	High growth rates can be achieved making it suitable to be scaled up	Used as nutritional products, for aquaculture and biofuel production	(Al Hattab et al., 2015)

The growth of microalgae in wastewater can be limited due to a number of growth factors and operational conditions which include biotic factors, abiotic factors and operational factors (Gonçalves et al., 2017). In all types of wastewater, the main parameters that require close attention are light quality and quantity, presence of pathogens or toxic components, and different nutrient profiles. As compared to freshwater and saltwater, there are similarities regarding temperature, pH and salinity where there is an optimum that needs to be achieved for maximum growth. Different wastewaters will usually differ in nutrient ratios such as the Nitrogen to Phosphorus ratio (N:P) which can strongly influence microalgal biomass production and nutrient uptake. The current trends of wastewater used states that the most appropriate wastewater with suitable N:P are from the swine industry, domestic wastewaters, dairy industry and municipal sewage after anaerobic digestion (Gonçalves et al., 2017). The challenges to be faced in this approach include maintenance of long term cultivation and effectiveness, ensuring stable performance and fine tuning of its performance over time.

2.2.4 Organic mediums for microalgae cultivation

The prospects of using microalgae biomass to achieve a significant energy and greenhouse gas (GHG) reduction would require an enormous quantity of biomass. To achieve this biomass production, substantial amounts of freshwater and chemical fertilizer would be needed, resulting in high operating costs for microalgae production. Taking this into account, the realization of replacing fossil fuels with microalgae can only be accomplished if an environmentally sustainable and less energy intensive replacement for

chemical fertilizer can be found (Clarens et al., 2010). Substituting the inorganic mediums with organic nutrient sources are of particular interest to provide a circular economy, where valuable nutrients are recycled and the requirements for freshwater can be reduced for the production of microalgae biomass (Markou et al., 2018). The specific organic nutrient source of interest is food waste compost.

Food waste contains high compositions of proteins, carbohydrates, lipids and other micronutrients that makes it a valuable and sought-after raw material for many biotechnological processes (Lin et al., 2013). Recovering these nutrients from food waste sources can avoid these organic matters from being disposed in landfills. One of the effective methods of utilizing food waste is through composting. Composting has been described as one of the best approaches to reuse in agricultural production (Boldrin et al., 2009). The decomposition of organic matter creates a rich soil amendment which are compost. Compost can encourage the production of beneficial microscopic bacteria and fungi which breaks down organic matter to produce nutrient-rich humus. It also contains micronutrients which can act as a soil amendment to help soil fertility and retain moisture of the soil. The composting of organic waste will lessen the negative impacts on soil properties caused by urbanization, and can promote a better environment through developing green infrastructures in cities, such as nutrient recycling, carbon storage, nutrient use efficiency, water capacity of soil and biocontrol of pathogenic microorganisms (Bertoncini et al., 2008).

Whilst food waste compost has not been used for the cultivation of microalgae, various food waste mediums have been successfully applied for the

microalgae cultivation to enhance the biomass growth and biochemical composition. Dilute food waste hydrolysate made using solid-state fermentation and hydrolysis was used in the heterotrophic cultivation of *Chlorella vulgaris* (Lau et al., 2014). It was reported that the growth of *C. vulgaris* in 20 (%v/v) with modified basal medium showed improvements in the exponential growth rate of microalgae and a balanced biochemical composition. Another similar study using food waste hydrolysate made through food waste hydrolysis with fungal stated that the microalgae, *Schizochytrium mangrovei* and C*hlorella pyrenoidosa*, grew well on the complex food waste hydrolysate. The biomass obtained using food waste hydrolysate showed high specific growth rate, lipid and protein content, but lower carbohydrate content (Pleissner et al., 2013). Furthermore, a study using three main substrates of food waste, namely waste lipid, waste protein and waste carbohydrate, for the production of hydrogen and lipids was performed by Ren et al. 2018 (Ren et al., 2018). The results indicated that waste carbohydrates were the most suitable for energy production, yielding the highest cumulative hydrogen volume. These studies demonstrate the feasibility of cultivating microalgae using food waste mediums for the sustainable production of microalgae biomass. The recovery and recycling of organic nutrient sources are inevitable and needs to be implemented in order to secure a sustainable food and feed production in the long term.

2.3 Microalgae Biorefinery for High Value Products

2.3.1 Biorefinery concept

The concept of biorefinery is similar to traditional petroleum refinery, such that biomass is converted into marketable chemicals, fuels and products

(Pérez et al., 2017). The main difference between biorefinery and petroleum refinery is in terms of the raw materials (biomass or crude oil) and the technology employed. Different existing and emerging technologies are utilized in both concepts to obtain petrochemical products or bioproducts, respectively. Biorefineries are found in widespread sectors at industrial scale, and this allows the biorefineries to concentrate on multiple products processing. Integration of the production configurations can be applied in biorefineries to produce value-added products (Moncada et al., 2015). These designs usually employ maximum product output and profit from a single raw material source, though there are considerations in terms of scarcity of materials and environmental impact.

Biorefinery is a process to obtain biofuels, energy and high value products through biomass transformation and process equipment. The biorefinery concept is a promising way to mitigate greenhouse gas emission as fossil fuels emissions have contributed heavily towards global warming (Juan et al., 2011). The main bottleneck of a biorefinery approach is the separation of different fractions without causing harm to the other fractions. This can be overcome through the use of simple, low energy consumption, cost effective and scalable separation processes. Microalgae are classified as potential candidates in biorefinery processes because they are capable of producing multiple products (González-Delgado & Kafarov, 2011). They are considered as renewable sources of biomass, which is beneficial in terms of rapid growth, decreased competition with food industry and composition that are selective.

Biorefinery is gaining popularity as the first-generation biofuels, which are produced from vegetable oils and residual oils or fats, are unsustainable due

to the potential stress caused by their production on food commodities. Second-generation biofuels, which are produced from plant biomass, are seen as an attractive alternative due to its abundance and cheap source, however, the present production processes are not cost effective and there are many technical barriers that need to be overcome to fully realize their potential (Naik et al., 2010). Hence, the current trend has advanced towards applying microalgae biorefinery as microalgae is a potential third generation feedstock for biofuels and high value products development. Figure 2.1 shows the applications of a fully integrated aquatic biomass cultivation processing system (González et al., 2015). The oil, minerals, carbohydrate and protein fractions can be used for the production of chemicals, fuels, feed, biogas and value-added products. Process residues such as glycerine and digestate can also be transformed into value-added products. Components of less value from protein and carbohydrate fraction will have the potential to be transformed into power for the combined heat and power (CHP) production in the refinery (Figure 2.1).

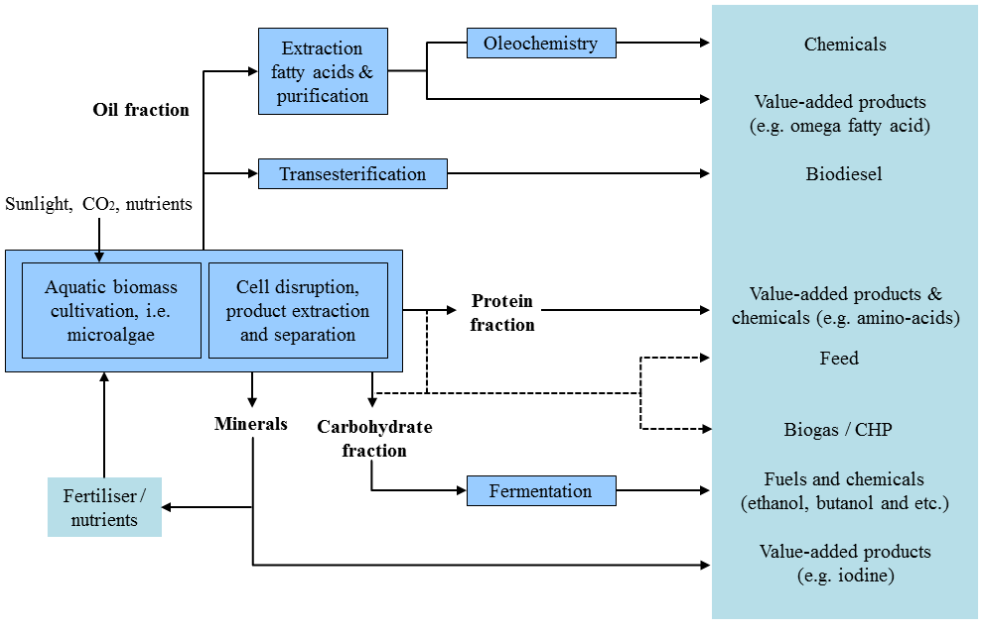


Figure 2.1: Applications of a fully integrated microalgae biomass biorefinery system.

2.3.2 Biorefinery of microalgae

Many investigations have successfully utilized microalgae for the production of bioproducts. Upstream processing (USP) and downstream processing (DSP) are the main stages of the microalgae biorefineries. The efficiency of the USP involves four important factors, namely: microalgae strain, supply of carbon dioxide (CO_2), nutrient source such as nitrogen and phosphorus, and source of illumination (Vanthoor-Koopmans et al., 2013). The nutrient sources are vital for microalgae production as they provide the necessary conditions to support the growth of microalgae, for example, a greater growth rate was observed when cultivating *Chlorococcum* sp. under artificial light conditions instead of direct sunlight (Aravantinou & Manariotis, 2016). This proves that light intensity and the source of light are important as they can directly influence the photosynthesis rate of microalgae.

Conventional DSP processes are the unit processes that occur within the photobioreactor like extraction and purification methods to obtain valuable compounds from microalgae. Conventional methods like bead beating, homogenizers, high pressure heating and chemicals, can incur high cost of production and the lack of an economical process has induced the need for the integration of multiple steps (Jacob-Lopes et al., 2015). The harvesting of biomass followed by biorefinery techniques has been developed as part of the integration of biomass conversion processes. The use of mild separation technologies, which do not involve high pressure and solvents, are significant for the production of the desired compounds without damaging other fractions (Vanthoor-Koopmans et al., 2013). Moreover, selection of appropriate technologies for microalgae biorefineries depends on the required energy input and the availability of existing technology. The integration of enhanced techniques should also improve the downstream processes in terms of economic, simplicity and ease of subsequent processing steps.

The conversion technologies utilized in for macroalgae and microalgae biomass can be divided into four categories, namely thermochemical conversion, biochemical conversion, transesterification and photosynthetic microbial fuel cell (Figure 2.2) (Naik et al., 2010; Posten & Schaub, 2009). Apart from that, the main factors affecting the choice of conversion process are the quantity and type of biomass feedstock, economic considerations, specification of projects and the end form of the desired product.

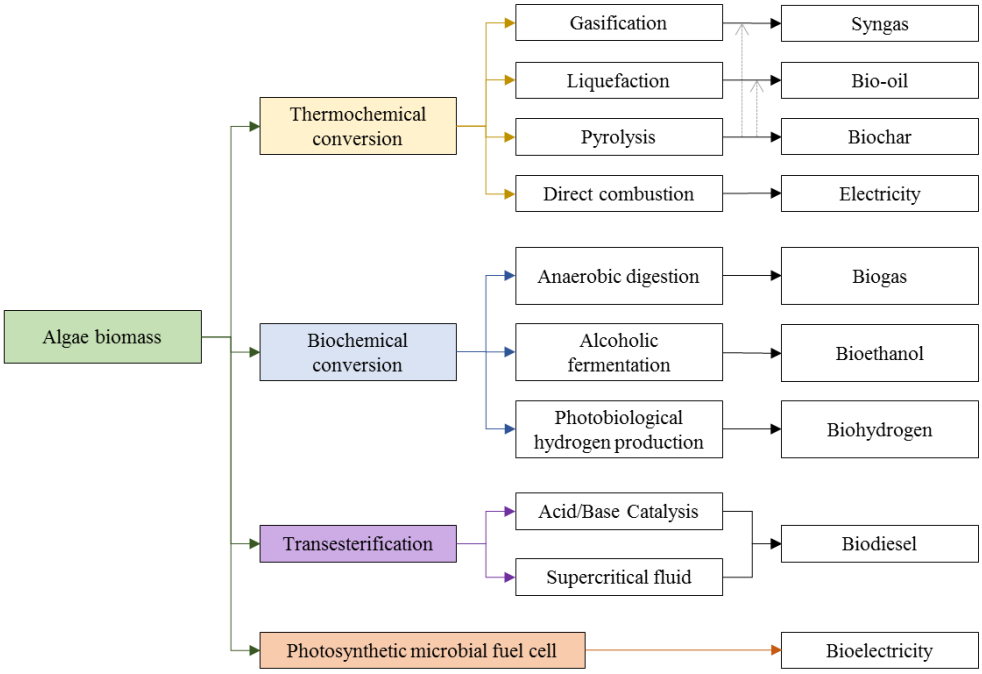


Figure 2.2: Algal biomass conversion processes for biofuels and bioenergy production.

2.3.2.1 Thermochemical conversion

Thermochemical conversion applies the principle of thermal decomposition of organic materials in biomass to extract fuel products. Examples of thermochemical conversion processes include gasification, thermal liquefaction, pyrolysis and direct combustion (Figure 2.2). Gasification is the chemical process where carbonaceous materials are converted to synthesis gas (syngas), which are used to make a wide range of fuels and chemical intermediates or can be directly used as a fuel for gas engines. For thermal liquefaction, the algal biomass will undergo liquefaction to decompose the biomass into smaller molecules with higher energy density. On the other hand, pyrolysis depicts the thermal degradation of biomass without the presence of oxygen. This process has potential for large scale production and can generate biofuels with medium-low calorific power (Brennan & Owende, 2010). Direct

combustion involves the chemical reaction between a fuel and oxygen with the presence of air to produce carbon dioxide, water and heat as products (Goyal et al., 2008). Energy is generated through the combustion of biomass and higher efficiencies can be achieved with co-combustion techniques in coal fired power plants.

2.3.2.2 Biochemical conversion

Biological processing of biomass into biofuels for energy conversion can be done through various biochemical conversion processes which include anaerobic digestion, alcoholic fermentation and photobiological hydrogen production (Figure 2.2). Anaerobic digestion involves the conversion of organic wastes like algal biomass into biogas. The biogas produced contain high energy value and the recovery rate is comparable to that of the extraction from cell lipids, making the process attractive as an alternative for fuel production (Suganya et al., 2016). As for alcoholic fermentation, an organic substrate will experience the metabolic process, where chemical changes will occur as a result of enzymes activities produced by microorganisms. The biomass materials which contain sugars, cellulose or starch are converted into ethanol by yeast (Brennan & Owende, 2010). The photobiological hydrogen production applies the conversion of water to hydrogen ions and oxygen by the algae. Algae are grown photosynthetically in normal conditions, and subsequently cultured by inducing anaerobic conditions to stimulate hydrogen and oxygen gas production where these gases will be spatially separated.

2.3.2.3 Transesterification

Transesterification is the reaction of triglycerides with alcohol in the presence of a catalyst to produce fatty acid chains and glycerol. This process can produce fatty acid methyl esters (FAME) by using methanol and ethanol. However, transesterification may be limited by the oil impurities and nature of catalyst. Determining the reaction conditions such as temperature and time are also a factor that might affect the transesterification process (Parra-Saldivar et al., 2014). The reactions of triglycerides to glycerol are usually catalyzed by an acid or base, using either a homogeneous or heterogeneous catalytic process (Suganya et al., 2016). Besides that, conducting transesterification in a supercritical condition can weakened the hydrogen bond of the alcohol. This would enable the complete conversion of triglycerides to esters rapidly as the chemical kinetics is accelerated under supercritical conditions.

2.3.2.4 Photosynthetic microbial fuel cell

Microbial fuel cells are bio-electrochemical devices that can generate electricity from the biodegradation of organic matter under anaerobic conditions. The integration of microalgae with photosynthetic microbial fuel cells has shown potential in the production of an oxygen rich environment as well as the removal of CO_2 through the photosynthetic activity of algae (Uggetti & Puigagut, 2016). The photosynthetic microbial fuel cell consists of an anode and a cathode separated by an ion exchange membrane. The bacteria in the anode oxidize the organic compounds and produces electrons, which are transferred to the cathode electrode through an external circuit to produce electricity. The benefit of this system is that bacteria in the anode can also treat

biodegradable wastes. Besides that, microalgae in the cathode can perform CO_2, nitrogen and phosphorus fixation simultaneously with the production of bioelectricity (Gouveia et al., 2014).

2.3.3 Applications and potentials of microalgae

Microalgae biorefineries have developed the progress of transformation of biomass into fuels, cosmetics, chemicals, food, feed and value-added compounds (Christenson & Sims, 2011; Wang et al., 2015). The microalgae-based carbohydrates consist mainly of cellulose and starch without lignin, making them useful as readily available carbon sources for the fermentation industry as well as biobutanol and bioethanol productions. Some microalgae, such as *Nannochloropsis, Tetraselmis, Isochrysis, Thalassiosira and Chaetoceros,* can also produce long chain fatty acids, such as DHA and EPA, which are valuable as health food supplements. In addition, proteins and pigments from microalgae also have considerable prospective for various medical and pharmaceuticals applications. However, when the products are targeted for medical biotechnology purpose, the cultivation of microalgae should be performed under well controlled conditions to avoid microbial contamination or the presence of impurities to meet the regulatory requirement. From this aspect, open cultivation systems, such as open pond or raceway pond, may not be suitable for growing microalgae that would be used for medical or pharmaceutical applications, despite that they are more economically feasible for large scale cultivation (Chisti, 2007; Milano et al., 2016). In contrast, closed culture systems (e.g., photobioreactors) (Costa & De Morais, 2011) with higher degree control on the cultivation conditions (e.g., temperature, pH and CO_2

concentration and so on) would be more feasible for this purpose, whereas the capital and operating cost will be higher.

Microalgae exhibits great potential in generating energy and valuable products from renewable sources through biotechnological processes which does not compromise on food agriculture and security. This reduces the need for a high yield per area of land compared with other crops and ability to be cultivated in arid lands. Major interest has been placed in microalgae for biofuel production as well as in chemical, feed and pharmaceutical sectors (Jacob-Lopes et al., 2015). Moreover, recent studies have indicated that the current developments in market conditions and production technology have made the production of microalgae biofuels and high value products economically feasible.

2.4 Emerging Bioseparation Techniques for Biomolecules Extraction

The process of extracting biologically important proteins in an efficient fashion has been the major effort in the biochemistry field. The separation and purification processes for protein and enzyme production often account for a major proportion of the total production cost (Sadana & Beelaram, 1994). Considerable efforts are being directed towards the development of biotechnology processes, where recombinant therapeutic proteins, food proteins and industrial enzymes are having an increasing impact on the global market (Kelley et al., 2009). The global market for industrial enzymes was estimated to be about $4.2 billion in 2014 and is expected to acquire a compound annual growth rate of 7% to reach $6.2 billion by 2020 (Singh et al., 2016). The production of specific proteins is highly dependent on the purpose of the

proteins, where the purified proteins are extracted to suit the applications it was meant for. If the design of the process is not accurately perceived, this may cause infeasibility in the production for economic and technical reasons. Steps in the operation of protein production include isolation, concentration and purification (Leser & Asenjo, 1994). Several processes for protein purifications are shown in Table 2.5. The recovery of proteins involves processes that uses high amount of chemicals and expensive equipment that contributes to the high total cost of the purified products. Hence, great effort and interest has been placed to develop new separation and purification techniques which can reduce the processing time, more cost effective, lower energy input, non-toxic and possess environmentally friendly characteristics. Examples of these methods used for protein extraction includes aqueous two-phase separation (Ding et al., 2014; Grilo et al., 2016; Lee et al., 2017b), precipitation with smart polymers (Gautam et al., 2012a; Gautam et al., 2012b), magnetism (Fischer et al., 2013), micellar extraction (Wang et al., 2013) and perfusion chromatography (Garcia et al., 2000; Regnier, 1999).

2.4.1 Three-phase partitioning (TPP)

Three-phase partitioning (TPP) is rapidly developing as a novel bioseparation technique for the separation and purification of biomolecules. TPP has been applied in a wide range of applications including enzyme stability and enhancement of its catalytic activity (Gagaoua & Hafid, 2016; Panadare & Rathod, 2017a; Thomson & Ward, 2002). This partitioning system consists of three phases and mainly utilizes high concentrations of well-buffered aqueous solutions with the addition of equal volumes of alcohol. The three-phase

mixture will form when the two liquid layers separate and an intermediate layer of precipitate filled with the desired products will be present (Yan et al., 2017). This novel bioseparation procedure is effective to be used in both upstream and downstream production of proteins. The physico-chemical parameters such as kosmotropic action, conformational tightening, electrostatic forces and the protein hydration shifts have been recommended as the basis for proteins to be extracted (Dennison & Lovrien, 1997; Nadar et al., 2017).

2.4.1.1 Basic principles

Three-phase partitioning (TPP) employs the use of well-buffered aqueous salts with an equal volume of water-miscible aliphatic alcohol to form three phases. Although alcohols such as methanol, ethanol, 1-propanol, 2-propanol and t-butanol can form a homogenous solution with water, none of them are miscible with the aqueous solutions of the anti-chaotropic salt, ammonium sulphate ($(NH_4)_2SO_4$). As a result, two liquid phases will be formed, where the alcohol floats to the top while the salty water remains below. For example, t-butanol and $(NH_4)_2SO_4$ are used to precipitate proteins from the aqueous solution. With the addition of salt, the t-butanol will form a two phase solution (i.e. a lower aqueous phase and an upper t-butanol phase) (Rachana & Jose, 2014). The proteins present in the aqueous solution will then form a third phase in between the two phases, forming a three-phase solution. Figure 2.3 shows the phase diagram of the three components system consisting of water, ammonium sulphate and t-butanol. One liquid phase is observed under the tieline near the water corner, two immiscible phases can be seen in the composition area with tielines and above that area the excess salt will be present

Table 2.5: Chromatographic operations and the physicochemical properties, characteristics and applications for purification of proteins.

Operation	**Physicochemical properties**	**Characteristic**	**Application**
Adsorption	Van der Waals forces	Good to high resolution Moderate capacity Moderate to high speed	Sorption from crude feedstock, fractionation
Ion exchange	Charge (titration curve)	High resolution High speed High capacity	Initial sorption, fractionation
Hydrophobic interaction	Surface hydrophobicity	Good resolution speed High capacity	Partial fractionation
Affinity chromatography	Biological affinity	Excellent resolution High speed High capacity	Fractionation, adsorption from feedstocks
Reversed phase liquid chromatography	Hydrophilic and hydrophobic interactions	Excellent resolution Intermediate capacity May denature proteins	Fractionation
Gel filtration	Molecular size	Moderate resolution Low capacity Excellent for desalting	Desalting, end polishing, solvent removal

in solid form (Kiss et al., 1998). The numbers 1 to 9 represents different compositions of the partitioning systems. The effectiveness of the systems with shorter tielines was lower (70-80% protein yield) compared to longer tielines (95-100% protein yield) as the systems with longer tieline would contain more t-butanol or $(NH_4)_2SO_4$ and also create higher interfacial tension. The composition range which is useful for partitioning experiments lies within area of the triangle with the tielines. The formation of this protein layer is dependent on the salt concentration added. Pigments, lipids and enzymes inhibitors are collected on the upper phase while polar components like saccharides are concentrated in the lower phase. It was recommended that the composition of the partitioning system be selected based on its effectiveness on the system, since several compositions along a tieline would result in equilibrium liquid phases with the same composition. This would allow the same accumulation ratio to be obtained within various systems (Kiss et al., 1998).

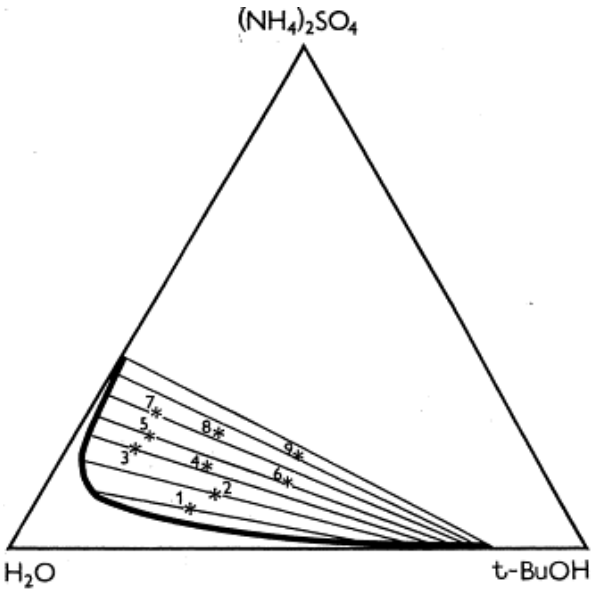


Figure 2.3: Phase diagram of the water/ammonium sulphate/t-butanol three component system at 25 °C (Kiss et al., 1998).

The addition of t-butanol to $(NH_4)_2SO_4$ precipitates the protein from the solution, where an interfacial precipitate will form between the lower aqueous and upper organic layers as the alcohol attaches to the proteins. The co-solvent will bind to the hydrophobic parts of the protein to reduce the protein molecules density, allowing it to float at the interface of the aqueous and organic solvent phases (Choonia & Lele, 2013). The protein precipitates would then continue to accumulate on the interface of the aqueous phase, creating a dense layer which would ease the floatation of proteins to the aqueous layer (Tschesche, 2011). T-butanol is commonly chosen as the organic solvent as it has been generally reported to produce the best purification results for separation of proteins, owing to its size and branched structure which does not allow it to permeate easily into the folded protein molecules (Pike & Dennison, 1989). These characteristics will prevent the t-butanol from denaturing the proteins. The schematic representation of TPP is shown in Figure 2.4.

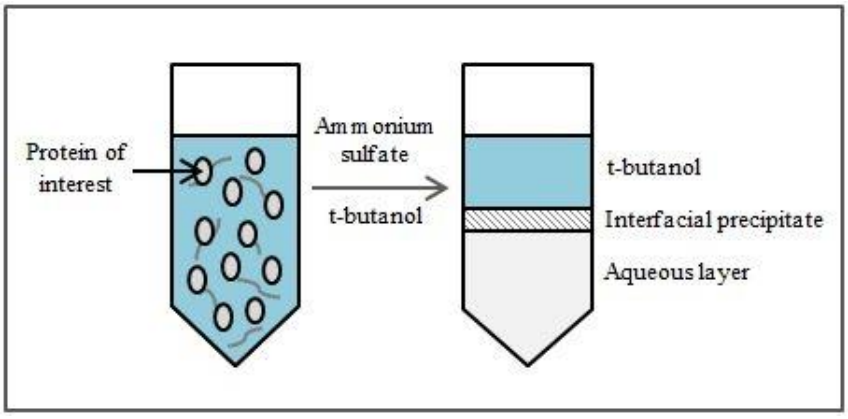


Figure 2.4: Schematic representation of TPP system.

The presence of protein/t-butanol coprecipitates resembles the structure of t-butanol bounded to TPP precipitated proteins. These proteins remain above

the denser aqueous salts due to the buoyancy elevation by the bound t-butanol. The principle of TPP resembles a hybrid of salting out and alcohol precipitation steps due to its promising separation efficiency during the extraction. However, there are several indications that TPP is incomparable to simple combinations of the aforementioned steps. Firstly, TPP requires less $(NH_4)_2SO_4$ concentration compared to salting out procedures. Secondly, TPP precipitation is dependent on the initial protein concentration and the precipitating salt concentration (Pike & Dennison, 1989). Moreover, TPP can be conducted at room temperature, whereas organic solvent precipitations are usually carried out at low temperatures (Mondal et al., 2006). Hence, TPP provides a more conducive process environment for the maintenance of the native protein structure.

2.4.1.2 Key parameters affecting TPP system

Various parameters such as type of alcohol and salt, concentration of salt, volume ratio, pH, temperature, and many more can affect the partitioning of proteins in the TPP system. The concentration of salt is crucial in TPP as it can significantly affect the solubility of proteins. Salts at low concentrations will attract various charged groups from the protein molecules to the solution, making it dissolve more easily into the solution. However, the addition of salts will eventually reach a saturation level, such that the maximum protein solubility will be achieved. Further increase in salt concentration will then create a shortage of solvent available to solubilize the proteins and the proteins will precipitate (Zhang, 2012). One of the most common salts used in TPP is ammonium sulphate. In a simple solution of $(NH_4)_2SO_4$, the density increases linearly with increasing concentration of salts. But in TPP, the densities of the

phases are derived from a complex function, whereby the proteins would float on the aqueous phase rather than sink to the bottom like in a simple $(NH_4)_2SO_4$ solution (Garg & Thorat, 2014). The sulphate anion will bind to cationic sites in the protein by utilizing the synergistic effect of both the salt and t-butanol, allowing for lower consumption of salts compared to the conventional salting out procedures (Dennison & Lovrien, 1997). The salt concentration can be optimized to obtain the maximum recovery of biomolecules such as proteins.

The effect of salt concentration has been studied with the use of $(NH_4)_2SO_4$ and t-butanol for the extraction of a milk-clotting enzyme (zingibain) from *Zingiber officinale Roscoe rhizomes* (Gagaoua et al., 2015). In this work, salt concentrations of 30 -80% (w/v) were used on the TPP for the crude enzyme extract at a constant crude extract to t-butanol ratio of 1.0:1.0. It was found that the maximum purification fold of 14.91 and 208% recovery of zingibain was achieved with 50% (w/v) $(NH_4)_2SO_4$. On the other hand, the lowest 30% (w/v) and highest 80% (w/v) salt concentrations yield very low percentages of recovery, at 51% and 42%, respectively. Further increase in salt concentration decreases the recovery percentage. This might be attributed to the irreversible denaturation of protein (Dennison & Lovrien, 1997; Pike & Dennison, 1989). At lower ratios, the recovery is less due to inadequate synergizing with $(NH_4)_2SO_4$. Generally in TPP, it is common to start at minimum salt concentrations of 20% (w/v) to optimize the partitioning conditions (Gagaoua et al., 2015). Another supporting research is the purification of invertase from tomato. A maximum purification fold of 4.4 and 300% recovery of invertase was achieved with the use of 50% (w/v) $(NH_4)_2SO_4$ saturation. The increasing amount of salt yields a higher amount of protein in

the midlayer, but the selectivity of extraction and degree of purification were reduced (Özer et al., 2010). $(NH_4)_2SO_4$ is largely used in TPP as the kosmotropic salt for salting out protein as it has the necessary properties like good ionic strength effects, surface tension enhancement, ability to bind sulphate ions to cationic sites in proteins and it is a good exclusion crowding agent (Dennison & Lovrien, 1997).

The use of different alcohols such as n-propanol, n-butanol, isobutanol and t-butanol for the TPP system will affect the overall yield of proteins. T-butanol is considered to be the best solvent for TPP as it can easily bind to the proteins, hence increasing their buoyancy and helping in the floatation of proteins to the upper aqueous salt layer. The utilization of larger amount of alcohol assists in the enhancement of protein buoyancy and stability at the interface, resulting in more precipitated proteins floating to the upper layer which is desired. Özer, *et al.* (2010) optimized the ratio of the volume of crude extract to t-butanol for the recovery of invertase from tomato. The highest recovery of around 350% was obtained at the ratio of 1.0:1.0, but the recovered product was reported to decrease with subsequent increase in crude extract to t-butanol ratios. The amount of t-butanol used must also be sufficient as less t-butanol will hinder its synergization with $(NH_4)_2SO_4$ (Özer et al., 2010). In another study of catalase recovery (Duman & Kaya, 2013b), the highest catalase activity of 183% and purification fold of 12.4 were recorded at the 1.0:1.0. The synergetic effects of increasing t-butanol concentration and decreasing $(NH_4)_2SO_4$ saturation simultaneously reduced the recovery activity and purification fold compared to performing these parameter's changes individually (Narayan et al., 2008). An increase in the yield of peroxidase was

also observed at the crude extract to t-butanol ratio of 1.0:1.0. A 22-fold purification with 52% activity recovery was achieved at the crude extract to t-butanol ratio of 1.0:1.0 after two cycles of TPP. The second cycle of TPP was performed to recovery the remaining protein at the interface. During the second cycle, the $(NH_4)_2SO_4$ content was decreased because at higher concentrations of the salt, the solubility limit of $(NH_4)_2SO_4$ will be exceeded and it will remain insoluble in the presence of t-butanol. The decrease in $(NH_4)_2SO_4$ led to the decrease in activity yield for the crude extract to t-butanol ratio of 1.0:0.75, whereas an increase of yield was observed for 1.0:1.0, due to the synergistic effects of increasing t-butanol concentration since the $(NH_4)_2SO_4$ concentration at 1.0:1.0 ratio was higher compared to 1.0:0.75 ratio (Narayan et al., 2008).

It is believed that the optimum ratio of crude extract to t-butanol of 1.0:1.0 may be selected due to two factors: When the t-butanol content is lower, the synergistic effect of $(NH_4)_2SO_4$ required for the recovery of proteins will be less. In contrary, if the t-butanol content is higher, the denaturation of the protein occurs more often (Sharma & Gupta, 2001). It is also worthy to note that for the industrial potential of TPP, it is usually recommended to use the mixture of salts and solvents which will incur the least production cost and obtain the highest recovery. This would result in the economic advantage for the commercialization of TPP process.

Furthermore, the alteration in pH of TPP system causes the ionization of amino acids present in the protein (Gagaoua et al., 2014; Wati et al., 2009). This parameter is considered to be significant in TPP as the distribution and partitioning of proteins is significantly affected by pH due to the electrostatic interactions between the phases and charged proteins (Gagaoua et al., 2014).

TPP systems often abruptly change near the isoelectric point (pI) of the proteins and it is highly dependent on the pH of the system. Proteins tend to precipitate most readily at their pI. Below the pI, proteins will be positively charged and precipitated out by the TPP, whereas negatively charged proteins will be more soluble in the TPP (Dennison & Lovrien, 1997). The change in pH will facilitate the changes in net charge of the targeted protein and thus affect the partitioning of the protein. In the study of β-amylase extraction and purification from stems, a good activity was present between pH 4.5 and pH 7.5 with the maximum activity of close to 100% between pH 6.0 and pH 6.5 (Sagu et al., 2015). A considerable loss of activity was observed at extremely acidic or basic pH. This is because lower pH corresponds to the presence of more H^+ ions which compete with the enzyme for the interaction with solvent molecules. Moreover, the shift in pH affects the net charge of a target protein and thus its partitioning due to the electrostatic interactions between the sulphate anion and the protein (Dennison & Lovrien, 1997).

Another study on the TPP of alkaline proteases explores the effect of pH on their partitioning. Five different pHs ranging from 6 to 10 were studied in the case of crude extracts containing proteins with various pI. A maximum activity recovery of 155% was obtained at pH 8.0 but the maximum purification fold (23-fold) of proteases was achieved at the system pH of 10.0. This might be due to the electrostatic component in the reactions leading to the partition of most contaminant proteins into the aqueous bottom phase above the pI. The increase in recovery at pH 8.0 is most likely due to the conformational stability of alkaline proteases towards t-butanol at this pH. This indicated that alkaline proteases precipitate more readily into an interfacial phase at its pI and is more

soluble above its pI value (Ketnawa et al., 2014). Hence, the effect of pH should be cautiously examined to enhance the purification fold and recovery of the target enzymes as they may be damaged or denatured by moderate or severe pH conditions. Besides that, pH stability is also significant during the industrial applications of proteins and it is important to evaluate the pH stability profiles for specific proteins to apply the most suitable pH conditions for optimum protein production.

The reaction rate of enzymes also increases with increasing temperature, however, this theory applies only up to a certain temperature. At high temperatures (above 60°C), the activity declines radically due to denaturation (Gagaoua et al., 2014; Hashim et al., 2011; Kumar et al., 2012). To sustain minimal protein denaturation, low temperatures are usually used during TPP to dissipate the heat more rapidly. The optimum temperature range for obtaining high recovery in TPP usually lies within 20 to 40°C, although there are ambiguities in the requirement for this selected range being reported in literature. The effect of temperature on the purification of α-galactosidase and invertase was evaluated. An activity recovery of 98% and purification fold of 12 were achieved at the temperature of 40°C. It was found that lowering the temperature decreased the activity yield but did not affect the specific activity (Dhananjay & Mulimani, 2008). The effect of temperature on another α-galactosidase activity was also studied and a relative activity of about 100% was achieved near the temperature range of 50°C. The thermal stability test conducted showed that the relative activity of the enzymes decreased sharply when the temperature was increased to 55°C, indicating that the TPP separation is not efficient at high temperatures (Şen et al., 2011). This temperature range

compares well with other reports which show high α-galactosidase activity in the temperature range of 37 - 60°C depending on their source and incubation time (Çalci et al., 2009; Şen et al., 2011). When the temperature was increased consecutively, the activity of the enzymes reduced significantly. Thermal stability of the purified α-galactosidase was conducted by incubating the enzyme in the absence of substrate at various temperatures. The results showed that the α-galactosidase was very stable between temperature ranges of 4 - 60°C (Bayraktar & Önal, 2013).

An experiment on the extraction or peroxidase from orange peels also found that the suitable operational temperature was at 30°C, which is close to room temperature. The further increase in temperature lead to lower peroxidase purification and recovery, and the reason for this results is the thermal deactivation of enzymes at higher temperatures (Vetal & Rathod, 2015). The requirement for low temperatures in the operation of TPP is favorable as the heat generated from the solvent or salt precipitation can be dissipated quicker, causing minimal protein denaturation (Şen et al., 2011). Lower temperature condition also requires less energy consumption and are able to avoid protein dissolution at the liquid-solid interface (Tan et al., 2015). Hence, new strategies for the thermostability enhancement of enzymes are being developed to explain the thermal deactivation mechanism and to discover methods to increase the productivity of enzymes at high temperatures.

The introduction of the TPP technique has gained reputation for its high recovery efficiency of protein molecules, from enzyme extracts or fermentation broths, at low cost and ease of operation. Some of these enzymes which are utilized in food processing industries include amylase, pullulanase,

galactosidase, polyphenol oxidase, protease, laccase, catalase, pectinase, naringinase, invertase, and many more. The extraction of various proteins and their optimum condition are stated in Table 2.6.

2.4.1.3 Variants of three phase partitioning

There are several advanced variants of TPP with have been modified to improve the extraction yield and purity of enzymes. These variants include ultrasound assisted TPP (UATPP), microwave assisted TPP (MWTPP) and ionic liquid TPP (ILTPP). Ultrasound assisted extraction generates a reduction in mass transfer resistance, which will achieve higher yield in less time (Dey & Rathod, 2013; Ketnawa et al., 2017). The applicability of ultrasound on TPP is viable as TPP is dependent on the mass transfer phenomenon. The mass transfer is enhanced due to the cavitation phenomenon which is affected by parameters such as temperature, power consumption, type of solvent, solute to solvent ratio and others (Kulkarni & Rathod, 2014). Gas bubbles are formed through ultrasonic waves and these bubbles collapse to create shock waves and mechanical shears to the surrounding environment. The change in local energy densities and mechanical shears incurred due to the cavitation phenomenon will hasten the mass transfer across the different phases (Pakhale & Bhagwat, 2016). The effect of ultrasound assisted TPP compared to conventional TPP is promising as it brings many advantages. In a study of selective partitioning of fibrinolytic enzyme in the bottom phase, TPP showed a 7.98-fold purity after 1 hour conventional stirring while UATPP showed a 16.15-fold purity at the optimized condition of irradiation for 5 minutes (Avhad et al., 2014). The ultrasound irradiation not only doubled the purity fold, but also reduced the time

Table 2.6: Enzyme extraction from various crude extracts using three-phase partitioning (TPP).

Crude extract	**Enzyme**	**Ammonium sulphate (%)**	**Extract to solvent ratio**	**pH**	**Temp (°C)**	**Recovery (%)**	**Reference**
Aloe vera	Aloe protein	26.35	1:0.25	6.5	30	92.78	(Tan et al., 2015)
Wheat germ	α-amylase	30	1:1	7	20	85	(Sharma & Gupta, 2001)
Phaseolus vulgaris L.	α-amylase	30	1:1	5.25	25	81	(Wang et al., 2011)
-	α-chymotrypsin	55	1:2	7.8	25	>100	(Rather et al., 2012)
Aspergillus oryzae	α-galactosidase	60	1:1	-	37	92	(Dhananjay & Mulimani, 2009)
Aspergillus oryzae	α-galactosidase	30	1:1	4.3	40	50	(Dhananjay & Mulimani, 2008)
Solanum muricatum	α-galactosidase	50	1:1.5	5.25	50	127	(Şen et al., 2011)
Citrullus vulgaris	α-galactosidase	50	1:1	6	60	76.7	(Bayraktar & Önal, 2013)
Lycopersicon esculentum	α-galactosidase	50	1:1	4	37	80	(Çalci et al., 2009)
Cicer arietinum	β-galactosidase	60	1:0.5	2.8	50	133	(Duman & Kaya, 2013a)

Crude extract	Enzyme	Ammonium sulphate (%)	Extract to solvent ratio	pH	Temp (°C)	Recovery (%)	Reference
Lactobacillus acidophilus	β-galactosidase	30	1:0.5	7	25	78	(Choonia & Lele, 2013)
Trichoderma citrinoviride	B-1,3-glucanase	55	1:0.5	6	50	98.4	(Periyasamy et al., 2017)
Abrus precatorius	Amylase	49.46	1:1.15	5.2	70	156.2	(Sagu et al., 2015)
Capsicum annuum L.	Asparaginase	50	1:0.75	8.5	40	567.4	(Shanmugaprakash et al., 2015a)
Solanum tuberosum	Catalase	40	1:1	7	35	262	(Duman & Kaya, 2013b)
Cucumis melo	Cucumisin	60	1:1.25	8	20	156	(Gagaoua et al., 2017)
Aspergillus sojae	Exo-polygalacturonase	30	1:1	6.6	25	25.5	(Dogan & Tari, 2008)
Bacillus sphaericus	Fibrinolytic enzyme	80	1:2	9	30	65	(Avhad et al., 2014)
Ficus carica L.	Ficin	40	1:0.75	6.5	60	167	(Gagaoua et al., 2014)
Aspergillus oryzae	Invertase	20	1:2	5	-	54	(Dhananjay & Mulimani, 2008)

Crude extract	Enzyme	Ammonium sulphate (%)	Extract to solvent ratio	pH	Temp (°C)	Recovery (%)	Reference
Baker's yeast	Invertase	50	1:0.5	6	60	363	(Akardere et al., 2010)
Lycopersicon esculentum	Invertase	50	1:1	4.5	50	190	(Özer et al., 2010)
Solanum tuberosum	Invertase	20	1: 1	4.5	35	121	(Duman & Kaya, 2014)
Coriolopsis trogii	Laccase	40	1:1	5	20	75	(Liu et al., 2015)
Ganoderma sp.	Laccase	20	1:0.5	7	35	95.8	(Rajeeva & Lele, 2011)
Pleurotus ostreatus	Laccase	50	1:1.8	-	42	184	(Kumar et al., 2011)
Trametes hirsuta	Laccase	40	1:1	4.5	30	53.9	(Patil & Yadav, 2018)
Litopenaeus vannamei	Lipase	50	1:1	8	40	87.4	(Kuepethkaew et al., 2017)
Aspergillus brasiliensis	Naringinase	30	1:1	6	28	106	(Shanmugaprakash et al., 2015b)
Bacillus natto	Nattokinase	30	1:1.5	8	37	129.5	(Garg & Thorat, 2014)

Crude extract	Enzyme	Ammonium sulphate (%)	Extract to solvent ratio	pH	Temp (°C)	Recovery (%)	Reference
Citrus sinenses	Peroxidase	50	1:1.5	6	30	93.96	(Vetal & Rathod, 2015)
Ipomoea palmata	Peroxidase	30	1:1	9	37	160	(Narayan et al., 2008)
Momordica charantia	Peroxidase	20 (Na-citrate)	1:0.75 (dimethyl carbonate)	7	30	177	(Panadare & Rathod, 2017b)
Carica papaya	Proteases	20	1:0.5	7	-	253.5	(Chaiwut et al., 2010)
Fish viscera	Proteases	50 (Na-citrate)	1:0.5	8	25	220	(Ketnawa et al., 2014)
Papaya peels	Proteases	20	1:0.5	7	-	253.5	(Chaiwut et al., 2010)
Serratia marcescens	Serratiopeptidase	30	1:1	7	25	96	(Pakhale & Bhagwat, 2016)
Bacillus oceanisediminis	Xylanase	50	1:1.5	8	10	107	(Boucherba et al., 2017)

Crude extract	**Enzyme**	**Ammonium sulphate (%)**	**Extract to solvent ratio**	**pH**	**Temp (°C)**	**Recovery (%)**	**Reference**
Trichoderma citrinoviride	Xylanase	55	1:0.5	5	50	99.8	(Periyasamy et al., 2017)
Zingiber officinale	Zingibain	50	1:1	7	60	215	(Gagaoua et al., 2015)

of operation by 90%. Apart from that, another study on the recovery of oxidase using UATPP showed that the optimized UATPP yielded a purity fold of 19.7 and 98.3% recovery, with process time of 5 minutes compared to conventional TPP, which yield a 6.3-fold purity and 70% recovery with process time of 40 minutes (Niphadkar & Rathod, 2015). The use of ultrasound assistance compared to conventional methods can reduce process operating time, increase the recovery and purification fold. Lower ultrasound frequencies are preferred for higher extraction since the scattering and attenuation of sounds waves will be reduced and it would be easier for the cavitation to occur (Kulkarni & Rathod, 2014).

Another type of advanced TPP variant is MWTPP. Microwave assisted extraction (MAE) is a relatively new method which utilizes microwave energy to heat polar solvents in contact with solid samples. Depending on the polarity and temperature of the solvent, the release of valuable compounds from the plant cell will be accelerated due to the increase in localized temperature and pressure condition caused by the heating. This enables more rapid partitioning of desired compounds between the sample and the solvent, leading to a reduction of extraction time and solvent consumption (Dahmoune et al., 2015; Rahath Kubra et al., 2013). MAE has attracted significant research attention for its exceptional heating mechanism, moderate cost and great performance. This would improve the penetration of solvent into the matrix and boost the release of bioactive compounds.

A recent study done by Patil and Yadav (2018) tested on the application of microwave assisted TPP for laccase purification and found that the MWTPP used considerably less processing time (7 minutes) compared to the

conventional TPP (60 minutes). The maximum yield and purity of laccase was found at the microwave irradiation time of 5 minutes, where this was explained by the combining effects of heat generation and ionic conduction which promotes the separation. Besides that, the duty cycle on MWTPP was tested and the optimized value for duty cycle was found at 20%. Using additional cycles will lead to higher doses of irradiation which would create the molecular friction for a longer period, and this will generate heat and denature the protein. The effect of power input is also significant as power input which is too high will elevate the temperature of the mixture and this will affect the stability of the enzyme (Patil & Yadav, 2018). This shows that the utilization of microwave irradiation can compete favorably with the conventional TPP techniques.

Lastly, the use of ILATPS for the recovery of proteins has been actively ongoing (Lee et al., 2017b), but the studies concerning ILATPS are lacking in terms of biomolecules recovery and this poses a major barrier in the reassurance of its feasibility (Alvarez-Guerra et al., 2014). Ionic Liquid Three-Phase Partitioning (ILTPP) combines the benefits of both TPP and ILATPS. The desired proteins will accumulate at the intermediate phase and no addition steps such as back extraction steps will be needed. However, one of the significant challenges in the utilization of ionic liquid (IL) for extraction is the ability to recover the IL after each cycle of process. The recyclability and reusability of the IL will determine the efficiency of the extraction process. Several works by Alvarez-Guerra exploits the use of ILTPP for the recovery of various proteins molecules (Alvarez-Guerra et al., 2014; Alvarez-Guerra & Irabien, 2015; Alvarez-Guerra et al., 2014). It has been reported that the recovery of ionic liquid in ILTPP can be enhanced by concentrating the salt-rich phase by means

of vacuum evaporation. Vacuum evaporation can be conducted in room temperature and this will avoid the degradation of the ionic liquid and protein in salt-rich phase. This technique was able to recover 100% of the IL and salt under almost all operating conditions, which makes it very potential for usage in ILTPP to reduce the consumption of reagents (Alvarez-Guerra et al., 2014). Besides that, the addition of extra salt was performed to reduce the concentration of IL in the salt-rich phase. The increasing feed stream will indirectly reduce the water feed to the TPP system and hence, a high fraction of the salt-rich phase can be recycled without the need to increase the overall salt concentration of the system. This indicates that the addition of extra salts produces limited effect at high protein concentrations and is not viable from an economic point of view (Alvarez-Guerra et al., 2014).

The selection of suitable IL is also important to determine its feasibility in TPP. The ILTPP consisting of 1-butyl-3-methylimidazolium tetrafluoroborate ($BmimBF_4$), NaH_2PO_4 and water was used to accumulate lactoferrin and was successfully able to extract between 74 to 99% lactoferrin at the interface (Alvarez-Guerra & Irabien, 2014). Several other ILs were also tested for the extraction of proteins but some combinations with different salts were not able to form an intermediate protein layer at the liquid-liquid interface. Another research successfully used 1-butyl-3-methylimidazolium trifluoromethanesulfonate (BmimTfO) with NaH_2PO_4 to extract lactoferrin, and this system was able to avoid the risk of hydrolysis that is experienced by the latter system ($BmimBF_4/NaH_2PO_4$) when it is in contact with water (Alvarez-Guerra et al., 2014; Alvarez-Guerra & Irabien, 2015). The BmimTfO TPP system was able to recover between 83 to 99% proteins at the interface with the

advantage of chemical stability. Since the probability of hydrolysis of IL in water can disrupt the efficiency of the ILTPP process, it is essential to find alternative IL which can avoid this risk. Another risk associated with ILTPP is the conformational changes endured by the protein molecules and more researches are needed to resolve this challenge. The development of a rapid and straightforward technique such as TPP for the extraction of these proteins will certainly be beneficial in terms of operating cost reduction and simplification of operation for obtaining the proteins.

2.4.2 Liquid biphasic flotation (LBF)

2.4.2.1 Basic principles

Liquid biphasic flotation (LBF) as its name suggests, is a process that is generated from the partitioning of two liquid phases coupled with flotation effects. LBF is also known as aqueous two-phase flotation and is an imminent novel technique for the extraction and purification of biomolecules (Phong et al., 2017). This process is the combination of liquid biphasic system (LBS) and solvent sublation (SS) system, where gas bubbles would aid in the transport of desirable biomolecules from the bottom aqueous phase to the top organic phase of the LBS, thereby enhancing the total recovery yields. The biomolecules transportation is mainly caused by the adsorption of surface-active compounds of the biomolecules on the surface of the ascending gas bubbles (Sankaran et al., 2018a). The advantages of LBF over the traditional LBS is its enhanced separation efficiency, simple processing with less intensive conditions, and higher concentration coefficient (Lee et al., 2016). LBF has been successfully applied for the separation of various biomolecules such as lipase from

Burkholderia cepacia (Show et al., 2013), betacyanin from *Hylocereus polyrhizus* and proteins from microalgae (Phong et al., 2017).

2.4.2.2 Key parameter affecting LBF system

Apart from the typical parameter such as types and concentration of alcohol and salt, volume ratio, pH, temperature and biomass concentration, that affects the traditional LBS system, three unique parameters that can affect the performance of LBF are air flow rate, air flotation time and air bubble size. These three parameters are derived from the addition of gas bubbles in the flotation system, where they can lead to changes in the phase partitioning and overall yield recovery. The air flow rate will influence the area of air-water interface per unit volume of aqueous solution in a specific time. By increasing the air flow rate, the interfacial area and turbulence caused by the gas bubbles will increase (Sankaran et al., 2018a). This would result in a better interaction of the gas bubbles with the desired biomolecules and increase the partitioning of the biomolecules to the top phase. However, air flow rate which are too high will create intense turbulence that will likely cause the re-dissolution of the biomolecules into the bottom phase. The air flow rate can also affect LBF systems which are incorporated with other forms of cell disruptive technique, for example, ultrasound. The cavitation phenomenon resulting from ultrasound creates and collapses bubbles during the rarefaction phase of sound waves, where introducing air into the flotation system could affect the cavitation resulting from sonication and affect the biomolecules separation (Sankaran et al., 2018a).

The air flotation time also plays a part in the recovery of biomolecules. Short flotation duration will not generate sufficient transport of the desired biomolecules from the bottom to the top, as these biomolecules require time to adsorb onto the surface of the bubbles and be brought to the top phase. The increasing air flow time was reported to improve the yield, but until a certain period the yield remain the same for additional flotation time (Leong et al., 2018; Show et al., 2011). This indicates that extending the flotation time for the separation process becomes unnecessary as the yield achieved remains nearly constant. The study of the air flow time is hence required to estimate the shortest duration that can achieved the maximal yield without the need to prolong the LBF. Moreover, the study by Show et al. 2013, explores the effect of bubble size in a flotation system, and suggests that bubble size determines the interfacial area for gas-liquid mass transfer (Show et al., 2013). The size of the bubbles is generated based on the porosity of the glass disc in a flotation system, where larger porosity will give out larger bubbles. Small bubbles are unable to enter the hydrophilic organic solvent phase as they need to coalesce into larger bubbles to overcome the interfacial tension at the intermediate phase. Therefore, the porosity of the glass disc needs to be carefully selected to obtain the best yield and separation recovery (Show et al., 2013).

LBF is a simple single step process which is economical and efficient for separations. It has an outstanding potential as an alternative over the conventional separation and purification methods for the recovery of biomolecules. The extraction and purification of various proteins and their optimum conditions using liquid biphasic flotation are stated in Table 2.7. This shows the potential of LBF to be used for the extraction of various biomolecules

from microalgae. Optimization steps can also be performed to further improve the recovery yield and purity of the biomolecules extracted. This concludes the effectiveness of multiphase partitioning systems as a promising technique for the separation and purification of biomolecules from various sources.

Table 2.7: Protein extraction from various crude extracts using liquid biphasic flotation (LBF) technique.

Crude extract	Protein/Enzyme	Flotation time	Flotation rate	Purification factor	Recovery (%)	Reference
Burkholderia cepacia	Lipase	20 min	1.5 cc/min	4.75	80	(Sankaran et al., 2018c)
Bacillus cereus	Cyclodextringlycosyl-transferase	80 min	5 L/min	21.8	97.1	(Lin et al., 2015)
Red-purple pitaya - *Hylocereus polyrhizus*	Betacyanins	15 min	20-30 cc/min	95*	96	(Leong et al., 2018)
Chlorella sorokiniana	Proteins	10 min	4 mm^3/min	97.4*	88.9	(Phong et al., 2017)
Escherichia coli BL21	Lipase	10 min	20 ml/min	4.05, 99*	96	(Tan et al., 2014)
Chlorella vulgaris FSP-E	Proteins	10 min	100 cc/min	86.4*	93.3	(Sankaran et al., 2018a)
Burkholderia cepacian ST8	Lipase	30 min	30 ml/min	14.4	99.2	(Show et al., 2013)
Chlorella sorokiniana CY-1	Proteins	5 min	10 ml/min	49*	80	(Chia et al., 2019)

*Separation efficiency (%)

2.5 Densification of Compost Pellets

Organic composts are beneficial for agricultural purposes and are produced in large quantities to reduce the disposal of valuable food waste products into landfills. Composts are usually grinded to enable a better surface area, avoid any smell and to blend the materials together in a more uniform way. This poses a challenge to the handling, management and storage of compost in its powder form as they are weighty and bulky (Obidziński, 2012). The composts have low density and can cause environmental issues like odours and dust. Since the nutrient value of compost is lower than that of chemical fertilizer, they are required in larger portions. The mass production of compost would need a large storage area and suitable transportation means. To overcome this, the densification of compost can be performed to transform the bulky composts into pellets (Zafari & Kianmehr, 2013).

Compost pellets are a densified form of the powdery compost and can contribute to the reduction in technical limitations associated with storage, loading and transportation (Monedero et al., 2015). The main benefits of compost pellets are (Zafari & Kianmehr, 2014):

(1) Reduction of conservation and storage space.

(2) Safe for residential places due to dust-free products.

(3) Ease of transportation to long distances.

(4) Suitable for mechanization and implanting.

(5) Higher precision with spreader and reduces compost consumption.

(6) Able to sustain long time conservation period.

(7) Availability to add chemical additives to increase quality of pellets.

The production of uniform pellets can be done using densification systems such as pellet mill, cuber, screw extruder, roller press, briquette press, tablet press, roller rotating pelletizer and agglomerator (Tumuluru et al., 2011). Each type of systems will result in different effects on the feedstock material and physical properties of the pellet products. A screw press consumes the highest energy as it shears and mixes the material in addition to compression while a briquette press is more flexible for various feedstock where higher moisture content and larger particles can be transformed into good quality briquettes. The roller rotating pelletizer is a more commonly used pelletizer as it has a simple structure, adjustable pressure and applications for different materials. The structure of a roller rotating pelletizer consists of a disk with many holes and a roller. Compost is fed between the roller and the disk will turn rapidly, creating a shear force that will push the compost through the holes or to the rollers, which will rotate at high speed to mould the compost into the holes. As the compost are being forced through the holes, there will be a rotating cutter to slice the outcoming densified compost into pellets (Zafari & Kianmehr, 2014).

The compost pellets properties and quality are assessed by its pellet production, pellet density, compressive strength, pellet durability and moisture sorption (Alemi et al., 2010; Zafari & Kianmehr, 2012). Pellet production identifies the suitable process conditions of the pelletizer to produce as much pellets as possible. The pellet density measures the densification intensity of the compost and compares it with the initial compost density. The compressive strength is an indication of the pellet resistance during storage while pellet durability represents the measure of shear and impact forces that can be

withstand by a pellet during the storing, handling and transportation process (Kaliyan & Morey, 2009). The moisture absorption of the pellets designates the resistance of the pellets to moisture and its hydrophobicity in nature. This property can determine whether the pellets can be safely stored for a long duration with risk of biological deterioration (Zaini et al., 2017). The quality of pellets is affected by both the pelletizing equipment and the compost properties. For the pelletizer equipment, the process conditions such as die temperature, rotating speed, die length, etc can influence the properties of the pellets produced. Thus, it is essential to select a suitable pelletizing process and conditions depending on the type of biomass materials to be pelletized.

The physical properties of the biomass such as moisture content, particles size, raw material type, bulk densities, biomass shape and size (Yilmaz et al., 2017; Zafari & Kianmehr, 2012) can also affect the production of pellets. The key factor that influences the operational aspects of the pelletizer is moisture content (MC). During the densification process, water acts as the film-type binder to strengthen and promote bonding between the particles (Mani et al., 2003). Different materials require different ranges of moisture content to process the pellets smoothly, for example, 40% MC for composted manure (Hara, 1998), 21% MC for wheat straw pellets (Mišljenović et al., 2016), 35-45% MC for sewage sludge (Yilmaz et al., 2017), 10% MC for hydrochar (Zaini et al., 2017) and 21-25% MC for pig solid fractions (Pampuro et al., 2017). As for food waste compost, the most suitable range of moisture content falls within 35-45% MC (Zafari & Kianmehr, 2014; Zafari & Kianmehr, 2013). The density and durability of pellets will increase with moisture content until an optimum is achieved. Too high moisture content will result in compost pellets with lower

strength and durability. On the contrary, too low moisture content will pose difficulties for the pelletizer to produce pellets of decent quality (Monedero et al., 2015). The adjustment and evaluation of suitable moisture content of compost is hence necessary to produce good and uniform quality pellets.

Binders are used in biomass densification to improve the cohesive characteristics of the biomass by forming a gel with water, leading to the production of more durable products. The wear and tear of the pelletizer can also be reduced as biomass with binder will be pelletized more easily and smoothly. Commonly used binders in pellets making include lignosulphonates, sulfonate salts, bentonite and natural proteins (Tabil et al., 1997). The utilization of waste materials as a binder for compost pellets will be helpful towards the waste management process. Since compost are organic fertilizers which are used for plants and crops growth, the selection of waste material needs to be compatible for plants, in other words, chemical binders would not be suitable as they may cause harm to the crops, and subsequent side effects to human during the consumption of these crops. A potential waste that is compatible for humans and plants is dairy waste products, where in this study, dairy powder waste is explored in detail.

Dairy powder wastes are derived from the excess powders from dairy production, expired dairy powders and disposed powders in household or industries. These dairy wastes are expensive and contain valuable micronutrients which brings good health to humans. They contain high contents of proteins, fats and lactose and are potential to act as binders for improving compost pelletization. Casein from proteins found in dairy powder has been used as a binder for coating materials in the paper industry, textile industry,

leather industry, packaging films and biomaterials (Audic et al., 2003). Casein coating can promote abrasion resistance, enhance pigment binding and create a moisture barrier to the coated products. Other than that, lactose is also a major constituent of dairy powder. Lactose is a disaccharide of glucose and galactose and is often used as a sweetener to milk. Lactose contain many reactive sites that can be chemically or enzymatically modified. It has been used in various industries including, food ingredients, filler, coating agent for tablets in the pharmaceutical industry and raw materials for lactose derivatives (Audic et al., 2003; Gutiérrez et al., 2012; Vromans et al., 1987). The application of lactose in food products is limited due to its low digestibility and poor solubility (Zadow, 2012). Hence, the dairy industry needs to find a usage for these wastes to dispose of them in an environmentally acceptable way. This in turn brings an advantage for lactose as a binder to compost. Its ability to crystallize can aid in the binding of the pellets in order to produce pellets of higher quality. The rich content in dairy powder waste will also increase the overall micronutrient content in the compost pellets which will enhance the growth of crops. This enables the sustainable utilization of dairy waste products as a binder for food waste compost, where the transformation of both these wastes into valuable products can contribute tremendously to the environment.

2.6 Research Gaps

Microalgae has featured valuable nutritional values with their major organic components comprising of proteins, lipid, carbohydrate and pigments. These microorganisms have demonstrated practicality as a sustainable source of biofuel and functional food ingredients. The main research gaps exist in the effective cultivation of microalgae and the subsequent extraction of these valuable compounds. Despite the good bioremediation properties of microalgae in wastewater, the resulting biomass are subjected to scrutiny as wastewater contains very hazardous compounds and these biomasses are deemed as unsafe for consumption. Hence, the need for an alternative medium that is considered safe for humans and plants needs to be discovered and applied in order to reduce the total cost of large scale microalgae cultivation.

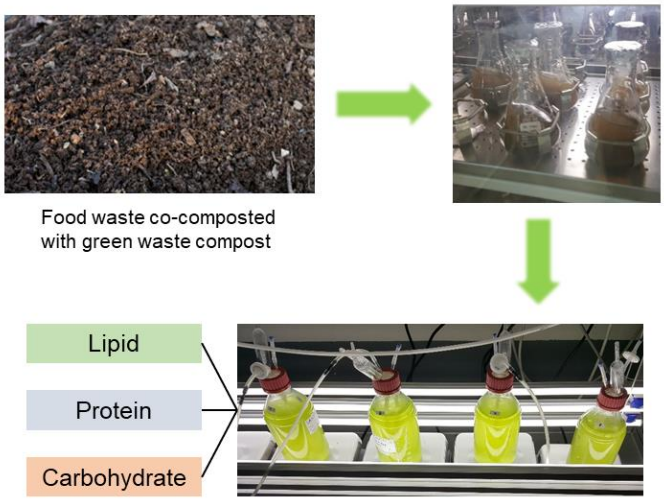
Advanced bioprocessing techniques such as liquid biphasic systems, utilize two immiscible phases to rapidly extract biomolecules. The multiphase bioseparation process has proven to extract high yield and selectivity of the desired bioproducts. However, it is essential to further integrate the process with various pretreatment methods in order to enhance the yield and separation efficiency of the process. Many of these techniques have been done enzyme extractions from many food sources, but less information is found on its application for biomolecules extraction from microalgae. The exploitation of an integrated liquid biphasic system for the recovery of proteins and pigments from various microalgae will contribute towards the development of more efficient bioprocessing methods for nutritional supplements production.

Last but not least, the handling of waste sources can be hazardous depending on the properties of the waste. Food waste compost possess the

difficulty of having low density and it is in powdery form. The handling, management and storage of food waste compost is tedious due to its properties of being bulky and dusty, as well as its lower nutrient content compared to chemical fertilizer. Therefore, the incorporation of an additive derived from waste sources, to aid in the densification of these compost materials into pellets will be greatly needed to ease the management of compost. These research gaps are investigated in detail and addressed in the following chapters (Chapters 3 to 6).

CHAPTER 3

FOOD WASTE COMPOST AS AN ORGANIC NUTRIENT SOURCE FOR THE CULTIVATION OF *CHLORELLA VULGARIS*



This Chapter covers the utilization of organic medium derived from food waste and green waste compost as an alternative nutrient source to inorganic medium. The potential replacement of a portion of inorganic mediums currently used for microalgae cultivation with organic medium has been studied. The effects of organic medium on the overall biomass production and microalgae biochemical contents were evaluated and the most suitable replacement rate of inorganic mediums were identified. This Chapter consist of a thesis-version of the manuscript published in the Bioresource Technology journal (Chew K.W. et al., 2018, *Bioresource Technology*, 267: 356-362).

Food waste compost as an organic nutrient source for the cultivation of *Chlorella vulgaris*

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Authors' Contributions

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

3.1 Abstract

The wastage of food products made for human consumption is continuously increasing every year and the need to transform these food waste into value-added products such as compost are substantial. The present study investigates the prospective of substituting inorganic medium with organic food waste compost medium as a nutrient supplement for the cultivation of *Chlorella vulgaris* FSP-E. Various percentages of compost mixtures (15 - 100%) were replaced in the inorganic medium to compare the algal growth and biochemical composition of the microalgae. The use of 25% compost mixture combination was found to yield higher biomass concentration (11.1%) and better lipid (10.1%) and protein (2.0%) content compared with microalgae cultivation in fully inorganic medium. These results exhibited the potential of combining the inorganic medium with organic food waste compost medium as an effective way to reduce the cultivation cost of microalgae and to increase the biochemical content in the cultivated microalgae.

Keywords: *Chlorella*; cultivation; food waste compost; microalgae; organic medium

3.2 Introduction

Over one billion tonnes of food wastes are produced every year and these food wastes either require treatments or are disposed in landfills. The transformation of food waste into value-added products through biological means such as composting is an environmental friendly method for effective organic waste management (Cerda et al., 2018). The end product of composting is a stabilized organic matter rich in humic substances which contains essential plant nutrients to be used as organic fertilizer (Zhou et al., 2016). Compared with inorganic fertilizer, the production of organic fertilizer can achieve a saved emissions of 4 – 81 kg CO_2 per tonne of food waste and 4 – 67 kg CO_2 per tonne of garden waste (Boldrin et al., 2009). The nitrogen and phosphorus content in compost produced from food waste can be used in agriculture for food production. Moreover, food waste contains high content of organic components such as carbohydrates, proteins, lipids and organic acids which makes it a reasonable feedstock for cultivating microorganisms (Zeng et al., 2018). The recycling of nutrients from food waste could potentially replace the non-renewable feedstock in various microorganisms' cultivation, which will lead to the alleviation of the global energy crisis. This will contribute to better nutrient use efficiencies and a reduction on the pollution that would otherwise be caused by inorganic fertilization.

Microalgae has been cultivated and applied extensively due to its promising properties as a biomass feedstock. Microalgae has high growth rates, less competition with terrestrial crops and a wide distribution. The production of biofuel from microalgae was found to be much greater than food crops and non-food crops feedstock, in addition to the shorter cultivation time need for

microalgae and its renewability (Chia et al., 2018b). Apart from that, its untapped potential to produce multiple valuable products in addition to biofuels has aroused the interest of various industries (Chew et al., 2017; Yen et al., 2013). The expanding interest and commercial development of products with functional compounds puts focus on microalgae metabolites which can be used in food, nutraceutical and pharmaceutical industries (Cuellar-Bermudez et al., 2015). Microalgae can produce a wide range of functional ingredients which include proteins, polysaccharide, natural pigments, vitamins, essential amino acids and other bioactive molecules. The functional protein biomolecules from microalgae are especially of interest for their health-promoting effect an biological properties like antioxidants, anticancer, anticoagulant, antitumor, antihypertensive and immune-modulatory activities (Samarakoon & Jeon, 2012).

The commercialization and scaling up of microalgae production for biomolecules extraction requires a large quantity of freshwater and high-cost nutrients. This creates the need to find a sustainable low-cost alternative medium which can provide similar nutrients needed for microalgae cultivation (Chia et al., 2018a). Wastewaters have been used to cultivate microalgae since their growth can purify the wastewater before discharging them back into water bodies (Wang et al., 2016). Nevertheless, the utilization of wastewater poses a few uncertainties such as the possibility of contamination, presence of hazardous chemicals and varying nutrient contents which will affect the growth of microalgae. Another potential source of nutrient medium is the use of food waste medium for microalgae cultivation. Food waste hydrolysates have successfully been used for the cultivation of microalgae and showed good

growth of microalgae (Lau et al., 2014; Pleissner et al., 2013). A coupled system of dark fermentation and microalgae cultivation was also performed using substrates of food waste and provided a sustainable route for efficient energy recovery (Ren et al., 2018). The major pollutant in food waste is mainly ammonium and phosphate, and the concentration of these pollutants can be reduced and utilized effectively to grow microalgae, bringing about a minimization of the contamination level in water (Cerda et al., 2018; Zhang et al., 2018). The integration of food waste compost medium and inorganic medium could potentially substitute the major nutrients such as nitrate and phosphate needed for microalgae cultivation. This will lead to a more sustainable industrial microalgae growth process for subsequent biorefinery.

From the literature review study in Section 2.2.4, it is identified that food waste can be used as an alternative culture medium due to its rich nutrient content, in addition to it being a waste source. Hence, the aim of this study was to investigate the potential of using food waste compost as a nutrient source for the cultivation of *Chlorella vulgaris* FSP-E. The feasibility of replacing inorganic fertilizers with organic fertilizers for microalgae cultivation were explored by using different concentrations of food waste compost mixture and to determine the algal biomass accumulation from each combination. The optimum concentration mixture was identified and the biochemical compositions in microalgae, such as lipid, protein and carbohydrates contents were extracted from the microalgae biomass to evaluate the productivity of the biomass for fuel, food and feed productions.

3.3 Materials and Methods

3.3.1 Microalgae strain and medium compositions

A high protein-yielding microalgae strain *C. vulgaris* FSP-E used in this study was isolated from a freshwater area located in southern Taiwan. The microalgae was cultivated in a modified version of BG-11 medium containing 1.5 g $NaNO_3$, 0.03 g K_2HPO_4, 0.075 g $MgSO_4.7H_2O$, 0.006 g citric acid anhydrous, 0.02 g Na_2CO_3, 0.036 g $CaCl_2 \cdot 2H_2O$, 0.006 g Ammonium iron (III) citrate, 0.001 g EDTA.2Na, 0.00286 g H_3BO_3, 0.00181 g $MnCl_2.4H_2O$, 0.000222 g $ZnSO_4.7H_2O$, 0.00039 g $Na_2MoO_4.2H_2O$, 0.000079 g $CuSO_4.5H_2O$, and 0.000049 g $Co(NO_3)_2 \square 6H_2O$ in 1 L of deionized water. The pre-culturing was done in an indoor photobioreactor (PBR) which consist of a 250 ml glass vessel containing 200 ml of medium, aerated with compressed air and equipped with external LED light sources on both sides of the vessel. The PBR was operated at room temperature with agitation. The light intensity on the vessel wall of the PBR was adjusted to 200 μmol/m^2/s using a LI-250 light meter with a LI-190SA pyranometer sensor (LI-COR, Inc., Lincoln, USA). Air was filtered (0.45 μm) and mixed with CO_2 to give a CO_2 concentration of 2.0%. The culture broth was aerated continuously at a rate of 200 ml/min (0.2 vvm, volume gas per volume media per minute). Similar PBR operating conditions were used for the subsequent batch cultures but in 1 L of medium. All cultures were performed in duplicate and average values were reported.

3.3.2 Microalgae cultivation with food waste compost

Food waste co-composted with green waste compost was provided by the Campus Services of University of Nottingham Malaysia Campus. The

compost was generated using the aerated windrow composting method and produced using food waste obtained from the cafeteria in the campus. The compost solutions were prepared by immersing 10 g of compost in 200 ml of water into individual 250 ml Erlenmeyer flasks. The flasks were placed in a shaker incubator and rotated at 150 rpm in room temperature for 24 h. The compost mixture was filtered through glass microfiber filters (934H, Whatman, USA) to remove large particles and indigenous bacteria. The organic compost mixture rates applied for microalgae cultivation was 15% organic (85C), 25% organic (75C), 35% organic (65C), 50% organic (50C), 75% organic (25C), 100% organic (100F), 100% organic with 75:25 compost solution to water dilution (75F), using 100% inorganic (100C) as control. The characteristics of the food waste compost and resulting organic compost medium are shown in Table 3.1. Sufficient compost mixture volumes were generated to fulfil the respective rates.

Table 3.1: Characteristics of the food waste compost and organic compost medium.

Parameter	**Unit**	**Concentration**
Food waste compost (Ultimate analysis)		
C	%	30.84 ± 0.45
H	%	4.10 ± 0.04
N	%	2.68 ± 0.01
S	%	0.84 ± 0.15
Organic compost medium		
Total NO_3	mg/L	360 ± 4
Total P	mg/L	208 ± 12
pH	-	7.2 – 7.4
Colour (dark brown)	λ_{max} (nm)	284 - 293

3.3.3 Determination of microalgae cell concentration

The microalgae biomass concentration was determined by measuring the optical density at 680 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu). The dry cell weight of the microalgae biomass was obtained by filtering 5 ml aliquots of culture using cellulose acetate membrane filter (0.45 µm pore size, 25 mm in diameter). Each loaded filter was dried at 105 °C until the weight was constant. The dry weight of the blank filter was subtracted from that of the loaded filter to obtain the microalgae dry cell weight (DCW). The OD_{680} values were converted to biomass concentration via calibration between OD_{680} and the DCW (1 OD_{680} = 0.23 g/L DCW). To eliminate the influence of the medium colour, a blank control of each medium combinations was prepared and quantified. The microalgae culture broth for each mixture combination was centrifuged at 6000 rpm for 15 min and the clear supernatant was removed. The pellet was lyophilized for 24 h. The resulting dry microalgae biomass was used for further characterization studies.

The biomass productivity (P_b) and specific growth rate (µ) of the microalgae in the exponential growth were calculated using the following equations:

$$P_b \text{ (mg/L/d)} = (N_2 - N_1)/(t_2 - t_1) \quad (1)$$

$$\mu \text{ (d}^{-1}\text{)} = \ln (N_2/N_1)/(t_2 - t_1) \quad (2)$$

where N_1 and N_2 are dry cell weight (g/L) at time t_1 and t_2, respectively.

3.3.4 Quantification of nitrate and phosphorus content in medium

Nitrate content was determined using the spectrophotometric method (Collos et al., 1999). The microalgae samples were collected from the

photobioreactor and centrifuged at 6000 rpm for 5 min. After centrifugation, the samples were diluted with deionized water and measured at the optical density of 220 nm using a UV-Vis spectrophotometer. Pure sodium nitrate ($NaNO_3$) at different concentrations was used for generating the standard curve. Phosphorus content in the medium was determined using atomic absorption spectroscopy (AAS, PerkinElmer AAnalyst 400) (Hoft et al., 1979). 30 ml of each mixture combination mediums were heated with occasional stirring to ensure the liberation of phosphorus. The spectrophotometer was set to isolate light at the phosphorus resonance line of 213.6 nm. Dibasic ammonium phosphate of known concentrations was used to establish the standard correlation between absorbance and phosphorus concentration. The total phosphorus concentration removal (%) was calculated by using the ratio of the difference in concentration over the initial concentration.

3.3.5 Microalgae lipid extraction

The lipid content from the microalgae biomass was extracted using the method by Bligh and Dyer (Bligh & Dyer, 1959) with some modification. 50 mg of dried microalgae biomass was suspended in 7.6 ml of chloroform/methanol/water mixture of ratio 1:2:0.8 (w/v). The mixture was sonicated at the frequency of 35 kHz for 5 min using an ultrasonic water bath (Elma, Germany). An additional 2 ml of chloroform and distilled water each were added, and the resulting mixture was sonicated again for 5 min. After sonication, the mixture was vortexed for 30 s and centrifuged at 3000 rpm for 5 min. The bottom layer (chloroform layer) was transferred into a pre-weighed vial and placed in a hot air oven at 80 °C for 30 min. The vials were then

transferred into a desiccator at room temperature and left to cool before being weighed. The total lipid contents were taken as the final weighed value of lipid in the vial and were expressed as the wt% of the dry cell weight.

3.3.6 Cell disruption and protein extraction

To extract the protein from microalgae biomass, the microalgae was subjected to cell disruption using ultrasonication as conducted in the research by Phong et al. (2018) (Phong et al., 2018b). 0.1 g of dry microalgal biomass was homogenized in 10 ml of water with 0.5 N KOH. The mixture was vortexed for 60 s and then subjected to ultrasonication at the frequency of 35 kHz for 20 min. After the ultrasonication, the mixture was centrifuged at 7000 rpm for 5 min. The pellet was discarded and the supernatant was collected for protein analysis. The extracted proteins form the microalgae were tested using the Bradford method (Bradford, 1976). 0.25 ml of the sample containing protein was mixed with 2.5 ml of Bradford reagent and measured using UV-Vis spectrophotometer at a wavelength of 595 nm. Bovine serum albumin (BSA) was used to establish the standard correlation between absorbance (OD_{595}) and protein concentrations.

3.3.7 Determination of carbohydrate contents

Carbohydrate compounds from microalgae biomass were extracted using a modified method by Pleissner et al. (2013) (Pleissner et al., 2013). To 50 – 100 mg of lyophilized biomass, 0.5 ml of 18 M H_2SO_4 was added. After 30 min of reaction time, 4.5 ml of demineralized water was added and the mixture was autoclaved at 121 °C for 30 min, followed by centrifugation for 10

min. The clear supernatant achieved was used for carbohydrate quantification using the phenol-sulphuric method (Dubois et al., 1951). To 10 µl of supernatant obtained, 1 ml of 18 M H_2SO_4 and 0.2 ml 4% (w/v) phenol were added and after 10 min the absorbance at 490 nm was measured using the UV-Vis spectrophotometer. Starch samples of known concentrations were used to establish the standard correlation between absorbance (OD_{490}) and carbohydrate concentrations.

3.3.8 Statistical analysis

The data of total lipid, protein and carbohydrate content was subjected to one-way ANOVA with two tailed t-test (with significance $p \leq 0.05$) data analysis using Microsoft Excel 2016. The data presented was the average of three replicates.

3.4 Results and Discussion

3.4.1 Effects of nutrient concentrations on biomass yield

The significant contributors to sustain microalgae growths are nutrient supply, light and carbon dioxide. Among the nutrient required, the supply of nitrogen is particularly important as nitrogen is the key compound to produce bulk quantities of microalgae biomass (Hsieh & Wu, 2009). Microalgae are able to utilize various forms of nitrogen such as nitrate, ammonium and organic nitrogen. The cell growth rate and nitrogen consumption are proportional as the microalgae cells actively consume nitrogen to promote growth. Nutrients derived from food waste compost were diluted into different concentrations and combined into various mixtures along with inorganic fertilizer to study the

potential of *C. vulgaris* FSP-E to absorb the nutrients for growth. Figure 3.1 shows the cultivation of *C. vulgaris* using food waste compost as the nutrients source. From the results, growing microalgae using 65 – 85% compost solutions showed a higher biomass production rate compared with the control, which is BG-11 medium. The biomass concentration of 1.72 g/L, 1.70 g/L and 1.65 g/L was achieved for 85C, 75C, and 65C mixture respectively, while 100C obtained a biomass concentration of 1.53 g/L at the end of 15 days. The 50C mixture yielded a final biomass concentration which is comparable to the control at 1.57 g/L. For 25C mixture the biomass concentration was 1.37 g/L and for the 100F and 75F compost mixture, the biomass concentration was very low, only at 0.37 g/L and 0.63 g/L, respectively.

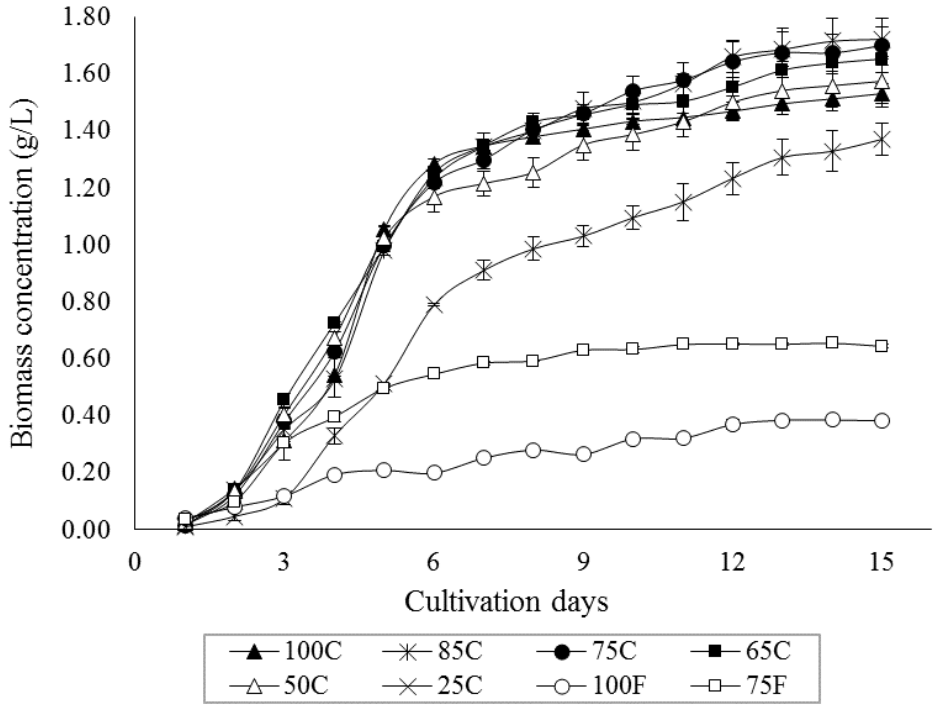


Figure 3.1: Growth curve of *C. vulgaris* cultivated in BG11 and various food waste compost medium combinations.

For all the dilutions of compost mixture up to 50% including the control, the microalgae grew rapidly during the first 6 days and continued to elevate slowly until the 15th day. The growth of 50C was slower initially but then increased slightly higher from day 9 onwards and eventually surpassed the 100C biomass concentration at the 12th day. The growth of microalgae in 100C after 15 days was found to be slightly less compared with using the combination of compost mixtures, indicating that the addition of food waste compost solution could enhance the biomass production (Table 3.2). Food waste has been discovered to contain many trace elements which can benefit the growth of microalgae (Zhang et al., 2011b). These elements are supplied in the food waste compost and can be supplemented to microalgae culture medium. Furthermore, the composting of food waste and green waste involves the decomposition of organic matter. This makes the compost medium a source of organic carbon which has been reported to greatly stimulate the microalgae growth (Zhang et al., 2018). Hence, the combining of organic compost medium with inorganic fertilizer shows improvements in the total biomass production as additional nutrients that support the growth of microalgae can be found in the compost medium.

Table 3.2: Dry cell weight concentration (DCW), average biomass productivity (P_b) and specific growth rate (μ) of microalgae cultivated in various combinations of BG11 and compost medium.

Medium	DCW (g/L)	P_b (mg/L/d)	μ (d^{-1})
100C	1.53 ± 0.07	107.63 ± 10.05	0.300 ± 0.037
85C	1.72 ± 0.08	121.93 ± 8.62	0.333 ± 0.042
75C	1.70 ± 0.08	120.31 ± 7.84	0.338 ± 0.043
65C	1.65 ± 0.07	117.16 ± 7.95	0.347 ± 0.048
50C	1.57 ± 0.07	110.48 ± 7.75	0.292 ± 0.035
25C	1.37 ± 0.06	97.23 ± 5.44	0.357 ± 0.035
100F	0.37 ± 0.01	23.42 ± 2.13	0.157 ± 0.016
75F	0.63 ± 0.03	42.86 ± 4.23	0.208 ± 0.028

For 25C, the microalgae growth was a bit slower at the initial stage, but the growth elevated around day 3 and then surges high but the biomass at day 15 remains lower compared with 100C. The colour of the compost was dark brown, and a higher rate of mixture, up to 75% compost solution for 25C resulted in a solution that was very dark brown in colour. The dark colour of compost shaded the light penetration to the microalgae, inhibiting its growth, since light is considered as one of the essential factors in microalgae growth (Ji et al., 2014). For 100F and 75F, the growth was very low and 75F obtained twice as much biomass compared with 100F. This usage of pure compost solution contains a lot of organic particles which makes the medium highly dark brown in colour. The thick colour of the medium for 100F has caused minimal light penetration into the photobioreactor, resulting in low biomass productivity. For 75F, the medium contained much lower nitrogen content compared with the others, this may cause the growth of microalgae to be limited by the presence

of nitrogen, causing nitrogen starvation which inhibits the growth of microalgae.

The average specific growth rates (μ) during the 15 days culture are shown in Table 3.2. Higher μ was found for 85C (11% increase), 75C (13%), 65C (16%) and 25C (19%) compared with the control BG11 medium, while 75F and 100F showed a decrease in μ of about 31% and 48%, respectively. It is likely that the addition of compost mixture has provided additional micronutrients which enhanced the quantity of biomass produced. Since the compost solution contains more phosphorus content compared with inorganic medium but less nitrogen content, the higher the percentage of compost mixture will result in a lower N:P ratio. Lower N:P supply ratio has been found to increase the biomass production as the microalgae would be supplied with sufficient N and P sources (Mayers et al., 2014). Microalgae growth can be promoted with the supplement of large amount of nutrient sources, where higher biomass yield can be obtained. However, the addition of too high percentage of compost medium may not be suitable for microalgae biomass production as the impurities contained in the medium could impede the growth and biochemical content of the microalgae. The addition of up to 50% compost medium was found to be promising for biomass productivity. Further studies on the biochemical content were carried out to identify the potential of substituting the inorganic medium with food waste compost medium.

3.4.2 Nutrient removal efficiencies

Microalgae will consume nutrients and simultaneously remove organic matters in the medium during the cultivation period. Nitrogen and phosphorus

are the most essential nutrients for microalgae growth as these nutrients are consumed by microalgae for the generation of cells to produce biomass (Huy et al., 2018). The composition of food waste consists of about 60% carbohydrate, 20% proteins and 10% lipids, which makes it very valuable for the recovery of nutrients to be utilized in various applications for the production of value-added products (Lin et al., 2013). Food waste composted with green waste can produce an organic amendment that is rich in nutrients from both the food source and plant materials. The compost not only contains the major nutrients to help boost plant growth, but also the micronutrients needed for plants to grow well. The ability of microalgae to assimilate nitrogen and phosphorus effectively opens up the potential of these nutrients assimilation from organic sources like compost which contain nitrogen and phosphorus (Delgadillo-Mirquez et al., 2016). The potential of integration of food waste compost mixture with inorganic fertilizer to enhance the microalgae growth rate and bioproducts recovery then becomes a worthwhile study.

The nitrate concentration results obtained for this study showed that for the compost mixture of up to 50%, the nitrate concentration decreased in a comparable manner, with a portion of nitrate remaining after 15 days of cultivation (Figure 3.2). For compost mixtures up to 35%, the nitrate consumption rate was about 46 – 50% while for 50C, the nitrate consumption rate was the highest at 62%. For 25C, the nitrate reduction was slightly slower although the supply of nitrate was more than sufficient for microalgae growth. For 100F and 75F, the nitrate reduction was very low with only about 8 – 12% consumption rate. The replacement of a fraction of inorganic medium with compost solution does not hinder the consumption of nitrate and neither does it

cause nitrogen starvation as the quantity of nitrate available is more than sufficient. However, the higher percentages of compost mixture used will induce a darker colour solution which may lower the light penetration and availability for microalgae growth. This would explain the low growth rate and nitrate consumption for compost medium of 75% and above. The solution became very dark and cloudy, inhibiting the penetration of light which is essential for microalgae growth (Sforza et al., 2012). This indicates that substituting a portion of inorganic fertilizer medium with compost solution could yield comparable and even higher amount of biomass as the nitrate supply is sufficient.

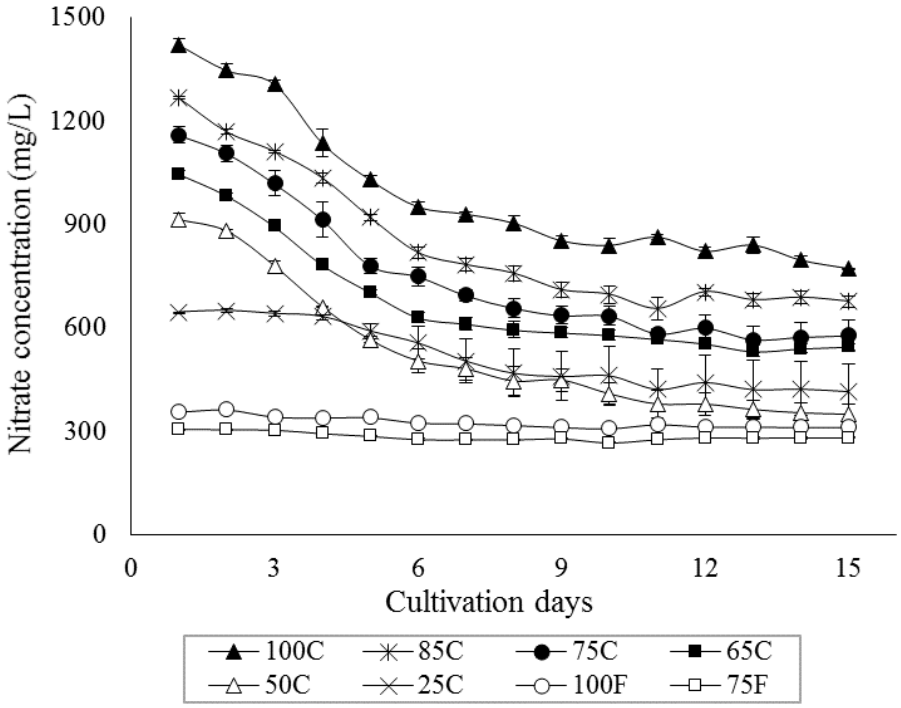


Figure 3.2: Nitrate content during growth of *C. vulgaris* cultivated in BG11 and various food waste compost medium combinations.

As seen in Figure 3.3, the removal of phosphorus from the medium culture increased with increasing compost mixture concentration up to 50%, then dropped sharply when the compost mixture concentration is 75% and above. 50C showed the highest removal at 54.3%, followed by 65C and 75C at 44.5% and 40.2% removal respectively. The 100C showed a removal of 33.1% and the mediums containing only compost mixture, 100F and 75F only had a phosphorus removal of 17.6% and 19.8% respectively. Cultivation mediums like wastewater usually contains high concentration of phosphorus which is higher than needed for growth. The amount of phosphorus present in the food wastes are also significantly high and hence the compost medium may contain more than sufficient phosphorus content for growth (Idowu et al., 2017). The phosphorus content in the compost medium was higher than the inorganic medium, so by the addition of compost mixture into the medium, the overall phosphorus content would be higher. Microalgae are able to assimilate phosphorus in excess, where the phosphorus was stored within the cells and these reserves can be sufficient for prolonged growth in the absence of available phosphorus (Delgadillo-Mirquez et al., 2016). This has led to a higher consumption rate of phosphorus in mediums containing more compost solution. Lau et al. (2014) observed negligible amounts of phosphate consumption for *C. vulgaris* cultivation using food waste hydrolysate while Pleissner et al. (2013) attained about 30% phosphorus removal for *C. pyrenoidosa* cultivated using batch culture with food waste as nutrient source. In this study, the average phosphorus removal was approximately 33%. For 50C, the phosphorus removal rate was the highest, which corresponded to it having the highest nitrate consumption as well. However, for 25C, 100F and 75F, the phosphorus removal

rate was much lower, this is caused by the slower growth of microalgae due to the insufficient penetration of light.

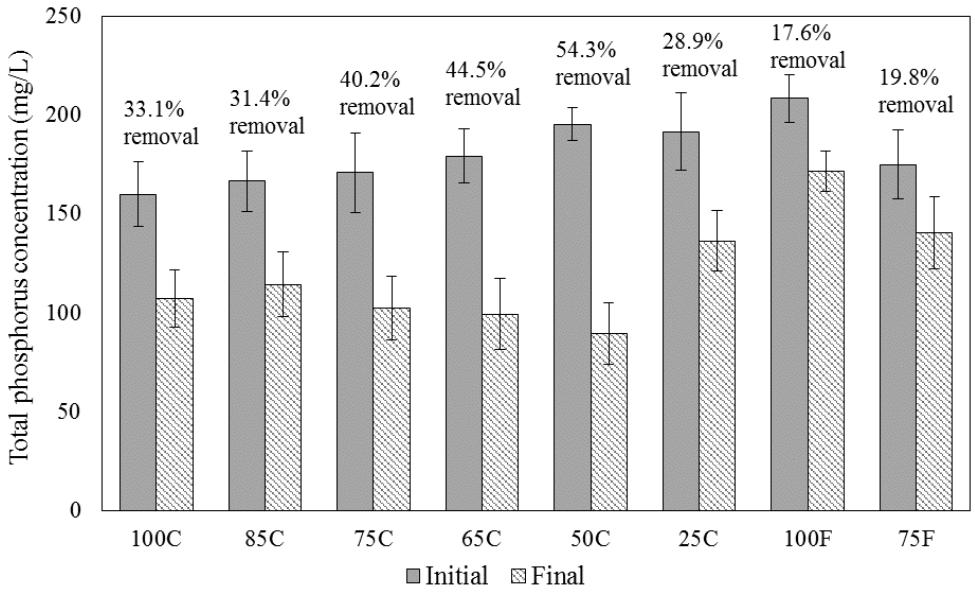


Figure 3.3: Initial and final phosphorus concentrations and removal rates from the cultivation medium consisting of BG11 and organic food waste compost medium.

3.4.3 Biochemical composition

3.4.3.1 Lipid content

The total lipid contents in the microalgae biomass from various medium combination are shown in Figure 3.4. The lipid content for 75C was the highest at 219.7 mg/g, followed by 85C and 65C at 215.0 and 208.3 mg/g. The lipid content for 100C (199.5 mg/g) is comparable to that of 50C (197.8 mg/g) and decreased more significantly for 25% compost mixtures and above. The lipid content for 100F was the lowest at 85.8 mg/g. For the different combinations of mediums used, it was found that the lipid content of the microalgae biomass was not significantly affected ($p > 0.05$) by the medium type. Only 100F showed

a significantly reduction ($p < 0.05$) in lipid content by more than half compared with the control. The lipid content for up to 35% compost mixture were higher compared with the control. This is likely due to the organic carbon present in the compost mixture which could promote carbon assimilation that will accelerate lipid accumulation (Lin & Wu, 2015). As the compost mixture contains organic substances compared with none in the inorganic fertilizer, the addition of compost mixtures can promote carbon assimilation and increase the production of various compositions in microalgae.

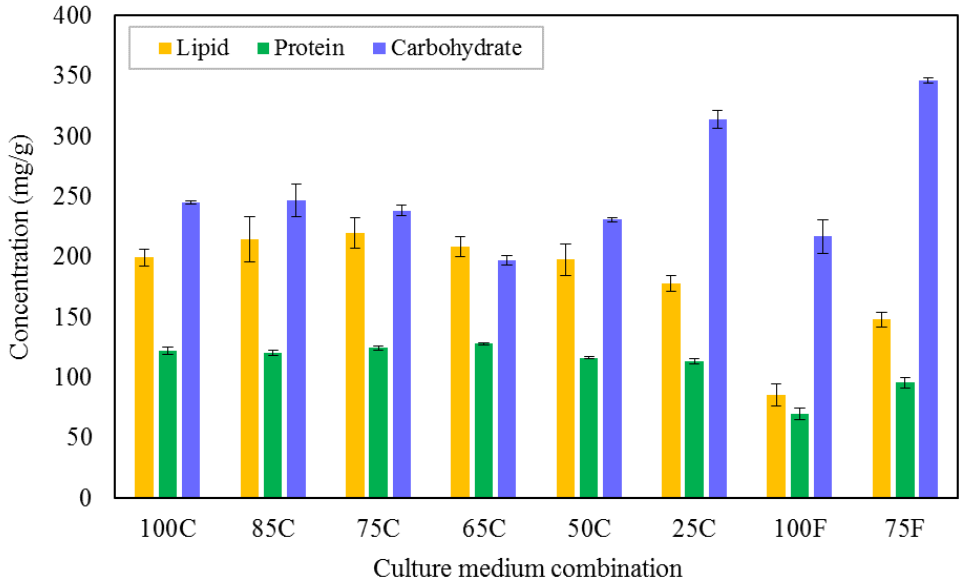


Figure 3.4: Biochemical composition of *C. vulgaris* in BG11 medium and various food waste compost medium combinations. Values shown are the mean of triplicate reading ± SD.

Studies have discovered that certain microalgae species including *Chlorella* being cultivated under stress conditions such as nitrogen or phosphate limitation can trigger lipid synthesis. It is known that the metabolic pathway of carbon assimilation diverts from protein synthesis to lipid or carbohydrate production as carbon and energy storage when under N or P limiting conditions (Kim et al., 2014). The 25C, 100F and 75F had a much larger compost mixture percentage, though the lipid content was much lower in general. Zeng et al. (Zeng et al., 2018) achieved a lipid content of about 16.8 – 25.5% from the mixed culture of *C. vulgaris* and yeast using food waste hydrolysate. It was also reported that the lipid content in pure *C. vulgaris* culture decreased from 44.75 to 16.9% with higher concentration of food waste hydrolysate. This was due to the joint effect of both photosynthesis and oxidative glucose metabolism, where the existing of sugar would inhibit the expression and activities of photosynthesis enzymes. Hence, causing a decrease in the biomass and lipid content.

The decrease in lipid content could also be due to the light intensity factor since the nitrogen and phosphorus content in the mediums used in this study was sufficient for microalgae growth. High light intensity will favour lipid production as it can lead to the storage of excess photoassimilates, where excess light energy are converted to chemical energy to overcome the photooxidative damage to the cell (Mandotra et al., 2016). All the cultivation processes for each mixture combinations were operated in the same condition with similar light intensity, duration and agitation, but the medium colour for mixtures containing compost solution appears to be darker with increasing compost mixture percentage. For 75% compost solution and above, the medium colour appears

to be very dark brown and thick. This dark coloured solution may have inhibited the optimum light penetration to the microalgae within the photobioreactor, causing the slow growth and low lipid content. The use of effluent from kitchen waste as a nutrient source was found to increase the lipid productivities by up to 37.5% compared with inorganic medium usage, yielding a lipid productivity of about 160.5 – 232.95 mg/L (Zhang et al., 2018). The increase in lipid content for 85C, 75C and 65C could be due to the presence of micronturients which enhanced the biochemical composition in microalgae. This indicates that the integration of inorganic medium and organic medium can be done but only to a certain limit in order to obtain the highest lipid production from the biomass.

3.4.3.2 Protein content

The protein content extracted from the microalgae biomass in various medium combinations are shown in Figure 3.4. The highest protein content was found in 65C at 128.4 mg/g. This was followed by 75C at 124.5 mg/g and then 100C at 122.3 mg/g. The protein content for 100F and 75F were much lower compared with the other medium combination, yielding 70.3 and 95.9 mg/g, respectively. The protein content were comparable for the control and compost rates up to 35%. The mixtures 50C and 25C yielded slightly lower protein content compared with the control though the reduction were less than 10%. The statistical analysis showed that the protein content in microalgae biomass was significantly affected ($p < 0.05$) by the combination of mediums used for all medium combinations except 75C, which value is quite comparable to the control. The nitrogen concentration in the medium can influence the protein content, whereby having nitrogen in abundance can enhance the protein

synthesis in microalgae (Martınez et al., 2000). A study by Zhang et al. (Zhang et al., 2018) reported that the protein content in microalgae cultivated using effluents from kitchen waste was lower than that in BG11 medium, as this was mainly because of the N concentration in the BG11 medium being higher that in the diluted effluent. The lower N concentration in 50C and 25C might have affected its protein content slightly. Pleissner et al. (Pleissner et al., 2013) observed that the protein composition of growing *Chlorella* cells on food waste hydrolysate was around 130.6 mg/g, which was doubled compared with the conventional medium. In this study, the improvement in protein content was found to be highest in 65C with a 5% increment only, this is likely due to the sufficient nitrogen supply throughout the dilution rates used.

The integration of compost mixture with inorganic fertilizer showed sufficient nitrogen supply for microalgae growth, whereby the growth was limited only by the light penetration into the medium. Hence, the production of protein was seen to be comparable for the 100C, 85C, 75C and 65C. The slightly higher protein content in 65C and 75C could be due to the presence of other nutrients apart from nitrate which was found in compost solutions and these nutrients have enhanced the biochemical compositions in the microalgae biomass. For 50C and 25C, the slight drop in protein content could have aroused from the lower initial nitrate content for both mediums and the slower growth for 25C. The 100F and 75F mediums did not consumed much nitrate and this has led to the low amount of protein content in the biomass. The results show that substituting up to 35% of inorganic medium with organic compost mediums could produce microalgae of comparable or higher protein content.

3.4.3.3 Carbohydrate content

The production of carbohydrate from microalgae biomass is also considered a value-added process as carbohydrates can be used as a feedstock for energy and biofuels production such as bioethanol, biohydrogen and biogas production. The carbohydrate production ability varies among the microalgae species and can be altered by using different cultivation conditions. Figure 3.4 presents the carbohydrate content obtained from biomass cultivated in different medium combinations. The highest carbohydrate content was found in 75F (346.5 mg/g) and this is followed by 25C (313.7 mg/g). The carbohydrate content found in 100C was comparable to that of 85C and 75C, ranging from 238.4 mg/g to 245.2 mg/g. 100F yielded a higher carbohydrate content compared with the control, while 65C showed a 20% reduction in carbohydrate content. The combination of mediums used showed significantly lower carbohydrate content for 65C and 50C, and was significantly higher for 25C and 75F, yielding and increase of 28 – 41% in carbohydrate content compared with the control ($p < 0.05$). Pleissner et al. (Pleissner et al., 2013) reported a reduction in carbohydrate content (21%) in *C. pyrenoidosa* when food waste hydrolysate (295.5 mg/g) was used in place of conventional BG11 medium (378.8 mg/g). In our study, the carbohydrate content was reduced slightly as well (7%) when compost medium combinations were used.

The high composition of carbohydrate in medium containing 75% or more of organic compost solution is associated with the presence of organic carbon which will promote the carbon assimilation in microalgae, thereby accelerating the TAG and carbohydrate accumulation in the biomass (Zhang et al., 2014). A study by Lau et al. (Lau et al., 2014) states that *C. vulgaris* biomass

that consisted of around 200 - 400 mg/g was produced with use of food waste hydrolysate, where the changes in carbohydrate content can be correlated to the carbon, nitrogen and phosphorus sources. The depletion of nitrogen sources which are essential for growth may affect the biomass by favouring the synthesis and accumulation of carbohydrates and lipids. However, the results from the lipids content do not show the corresponding trend of increases in lipid content in medium containing more than 75%, since the nitrogen content in the medium was sufficient for growth up to 50% compost mixture. For 75F, the carbohydrate content was very much higher and this is likely due to the weaker cell wall of 75F biomass. Autoclave process which was used for the extraction of carbohydrate can disrupt the microalgae cell to release the biomolecules (Rakesh et al., 2015). The nutrient deficient condition for 75F may have produced biomass which have weaker cell walls and are more susceptible to cell disruption through the autoclave process, resulting in more carbohydrate content released.

3.4.4 Evaluation of biochemical content in microalgae

The lipid, protein and carbohydrate contents from microalgae biomass cultivated using inorganic medium with combinations of food waste compost were compared with the controlled fully inorganic medium. In Table 3.3, the three analysed biochemical composition obtained from *C. vulgaris* using different methods as stated in the methodology are shown. For lipid, additions of up to 35% compost medium yielded higher lipid content compared to 100C. For proteins, 15% to 25% compost medium yielded higher protein content compared to 100C, and for carbohydrates, the distributions of the carbohydrate

content are suggestively uneven. The microalgae cultivated in higher concentrations of compost medium showed much higher carbohydrate content and this is likely due to the weaker development of the cells which break more easily to release the carbohydrates during the processing step. Hence, more focus has been placed on the lipid and protein content derived from the microalgae biomass.

The best compost medium combination was within the range of 25% and 35% which showed a 10.1% increase in lipid with 2% increase in protein and 4.4% increase in lipid with 5% increase in protein, respectively. The increase in biochemical content does not show a very significant improvement compared to using the controlled inorganic medium. But as food waste compost medium is considered a waste and therefore a raw material that is liken to zero cost material, the reduction of inorganic medium usage would already result in a decrease in raw materials costs, coupled with the slight improvement in biochemical content, it is worth to substitute a portion of inorganic medium with food waste compost medium. One of the main challenges in this study is the dark coloured solution obtained from food waste compost medium which inhibits the growth of microalgae. This medium substitution approach would be more promising if the organic mediums can be decoloured using bacteria or advanced oxidation processes. This constitutes to a future work in using food waste medium with additional treatment process to produce a nutrient-enriched medium made of waste sources that will improve the growth and quality of microalgae biomass produced.

Table 3.3: Lipid, protein and carbohydrate content (mg/g) in *C. vulgaris* cultivated in BG11 medium and various food waste compost medium combinations.

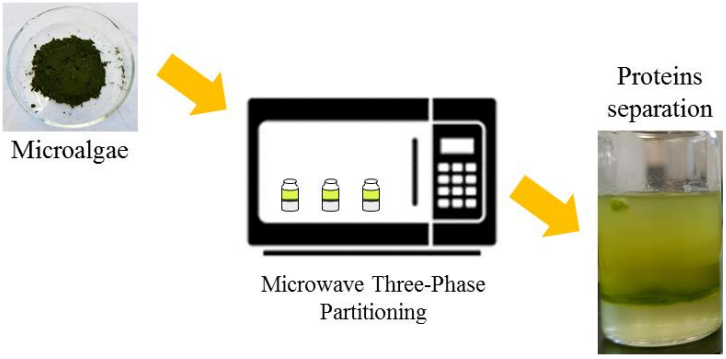
Medium	Lipid (mg/g)	Protein (mg/g)	Carbohydrate (mg/g)
100C	199.5 ± 3.5	122.3 ± 3.0	245.2 ± 0.6
85C	215.0 ± 8.7	120.6 ± 2.2	246.9 ± 5.5
75C	219.7 ± 5.6	124.5 ± 2.0	238.4 ± 1.9
65C	208.3 ± 4.0	128.4 ± 0.9	197.2 ± 2.0
50C	197.8 ± 6.7	116.5 ± 0.8	231.2 ± 0.8
25C	178.2 ± 3.6	113.5 ± 2.1	313.7 ± 2.3
100F	85.8 ± 10.5	70.3 ± 4.8	216.8 ± 6.3
75F	148.2 ± 4.1	95.9 ± 4.4	346.5 ± 0.7

3.5 Conclusion

The integration of organic medium with inorganic medium for the cultivation of microalgae offers many environmental and economic benefits. Substituting inorganic medium with 25% compost mixture yielded higher microalgae biomass as well as greater lipid and protein content. The use of organic medium can help to save microalgae cultivation cost and increase the production of biochemical compounds. Apart from that, organic compost as a substitution to inorganic fertilizer will bring environmental advantages as these compost sources are widely available and can help in food waste management. Utilization of organic medium in microalgae cultivation system to replace fossil-based fertilizers should be implemented to create a more sustainable future. The next chapter (Chapter 4) explains the use of multiphase partitioning technique for proteins extraction from the microalgae biomass.

CHAPTER 4

ENHANCED MICROALGAL PROTEIN EXTRACTION AND PURIFICATION USING SUSTAINABLE MICROWAVE-ASSISTED MULTIPHASE PARTITIONING TECHNIQUE



This Chapter covers the utilization of a microwave-assisted multiphase partitioning technique for the extraction and purification of proteins from microalgae. Various parameters which affect the multiphase system was investigated. These parameters were optimized to obtain the best conditions for maximal protein recovery yield and separation efficiency. The surface characterization of the microalgae after the treatment was also performed. This Chapter consist of a thesis-version of the manuscript accepted in the Chemical Engineering Journal (Chew K.W. et al., 2019, *Chemical Engineering Journal*, 367, 1-8).

Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique

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Authors' Contributions

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

4.1 Abstract

A green and effective method of microwave-assisted three phase partitioning (MWTPP) was developed for the extraction and purification of proteins from *Chlorella vulgaris* microalgae species. Five types of salts were investigated and ammonium sulphate was selected as the best to form a three phase system with t-butanol as the solvent. The optimal conditions for maximum protein recovery are as follow: ammonium sulphate concentration 30%, ratio of slurry to t-butanol 1:1, microwave time 120 s, microwave duty cycle 80%, microwave power 100W and microalgae biomass concentration 0.5 %w/w. Under the optimized conditions, the protein recovery yield from microalgae was found to be 63.2% with a separation efficiency of 67.2%. Compared to the TPP process and initial MWTPP process, the recovery yield was enhanced by 2.54 and 1.49 times, respectively. The FESEM characterization of the microalgae after MWTPP process also showed the potential effect of microwaves to disrupt the cell wall and release the biomolecules for ease of recovery. MWTPP represents an efficient, economic and environmentally friendly technique for the extraction of bioactive compounds from microalgae.

Keywords: extraction; microalgae; microwave; protein; three phase partitioning

4.2 Introduction

Microalgae is an attractive third generation feedstock as it has a high areal productivity, can be cultivated on non-arable land, composes of various biomolecules and has potential as renewable alternative to fossil fuels (Chia et al., 2018b; Kim et al., 2015). The rich composition of microalgae, particularly its protein content, has made it a potential feedstock for various sectors including food, feed and pharmaceuticals (Chew et al., 2017). Microalgae can accumulate high contents of proteins under suitable cultivation conditions and these algal proteins quality are comparable to that of proteins from other crops. The ability to extract functional proteins from microalgae has gained attention and focus are being placed on the commercial developments of these products with functional compounds (Cuellar-Bermudez et al., 2015). Many researches have been performed for the investigation of the best cultivation conditions for microalgae in indoor and outdoor systems (Praveen et al., 2018; Wu et al., 2017). Nevertheless, the key challenge to obtain the functional protein biomolecules lies in the downstream processing, where an efficient and cost-effective protein recovery process needs to be developed.

Conventional separation techniques such as precipitation, column chromatography, membrane separation, crystallization and ultrafiltration require multiple steps, higher maintenance and processing cost, longer processing time and have difficulties during scale up of the technique. In contrast, three phase partitioning (TPP) which is an emerging separation process could overcome the associated limitations of the conventional processes (Chew et al., 2018c). TPP is a system that comprises of three phases as a result of two liquid mixtures consisting of a salt phase and alcohol phase, where the proteins

present in the aqueous solution would form a third intermediate phase between the two phases. T-butanol is commonly used as the organic solvent as it has been found to produce the best results for protein separation, due to its branched structure that prevents it from permeating into folded protein molecules. The three phase mixture will be formed when the two immiscible liquid layers separate, along with an intermediate layer of precipitate present that is with the desired products (Dennison & Lovrien, 1997). TPP represents a simple and quick method for extraction, purifying and concentrating proteins and has been successfully used to purify various protein biomolecules including: proteases from *Wrightia tinctoria* stem (Rajagopalan & Sukumaran, 2018), peroxidase from bitter gourd (Panadare & Rathod, 2017b), laccase from *Trametes hirsute* (Patil & Yadav, 2018), serratiopeptidase from *Serratia marcescens* (Pakhale & Bhagwat, 2016), peroxidase from orange peels (Vetal & Rathod, 2015) and many others. It is a simple, inexpensive and easily scalable technique which can be used directly with crude suspensions.

The cell disruption step of microalgae is an important part of the processing as it corresponds to a large proportion of the overall process cost and can aid in obtaining higher protein extraction efficiency. Usually, cell disruption processes are conducted prior to extraction to disrupt the microalgae cells to promote the release of valuable biomolecules (Bai et al., 2013; Concas et al., 2015). Microwave is a cell disruption approach that utilizes the thermal effects by microwave energy to heat polar solvents in contact with solid samples. The increase in temperature and pressure conditions caused by the heating will accelerate the release of functional compounds from the cells (Dahmoune et al., 2015). Microwave extraction offers an alternative green method for cell

disruption and has been considered for industrial scale applications. It has effective cell wall disruption abilities with short processing time, low energy requirements and are free of hazardous chemicals usage. Microwave has also been successfully applied along with multiphase partitioning techniques for the recovery of various biomolecules which includes mangiferin from *Mangifera indica* leaves (Kulkarni & Rathod, 2015), polyphenol and lutein from marigold (Fu et al., 2018), laccase from *Trametes hirsuta* (Patil & Yadav, 2018) and polysaccharides from *Gentiana scabra* (Cheng et al., 2017). These studies have established that microwave-assisted multiphase extraction can generate high-quality products with better desired compound recovery. This shows the potential of integrating microwave with TPP system to maximize the recovery of proteins from microalgae.

The aim of this study was to investigate the potential of integrating microwave and TPP system in a combined microwave-assisted TPP (MWTPP) for the extraction of proteins from *Chlorella vulgaris* FSP-E. In Chapter 3, food waste compost was used as an alternative medium for the cultivation of microalgae. These microalgae biomass contains valuable bioproducts that can be extracted using simple and efficient techniques like multiphase partitioning. Hence, a detailed study was made by optimizing the operating conditions for the MWTPP technique for proteins extraction from microalgae. The effects of types of salt, concentration of alcohol and salt, volume ratio of biomass to solvent, pH of the system, microwave power, microwave time, microwave duty cycle and biomass concentration were studied.

4.3 Materials and Methods

4.3.1 Materials

Ammonium sulphate, sodium sulphate, magnesium sulphate, magnesium acetate, dipotassium hydrogen phosphate, t-butanol, tris-HCl buffer and Bradford reagent were purchased from R&M chemicals (Malaysia). All chemicals and solvents used were of analytical grade.

4.3.2 Microalgae strain and medium compositions

Chlorella vulgaris FSP-E used in this study was provided by National Cheng Kung University, Taiwan. This strain was selected due to its advantageous properties such as rapid growth rate and high protein content. *C. vulgaris* FSP-E was cultivated in a laboratory scale photobioreactor (PBR) using a modified version of BG-11 medium containing (g/L): $NaNO_3$, 1.5; K_2HPO_4, 0.03; $MgSO_4.7H_2O$, 0.075; citric acid anhydrous, 0.006; Na_2CO_3, 0.02; $CaCl_2.2H_2O$, 0.036; Ammonium iron (III) citrate, 0.006; EDTA.2Na, 0.001; H_3BO_3, 0.00286; $MnCl_2.4H_2O$, 0.00181; $ZnSO_4.7H_2O$, 0.000222; $Na_2MoO_4.2H_2O$, 0.00039; $CuSO_4.5H_2O$, 0.000079; $Co(NO_3)_2.6H_2O$, 0.000049. The pre-culturing and batch culture were done in an indoor PBR which consist of a 250 ml and 1 L glass vessel respectively. The PBR was aerated with compressed air and illuminated by external LED light sources with a light intensity of 200 µmol/m^2/s on both sides of the vessel. The light intensity was measured using a LI-250 light meter with a LI-190SA pyranometer sensor (LI-COR, Inc., Lincoln, USA). The PBR was operated at room temperature with agitation speed of 300 rpm. Filtered air (using 0.45 µm air filter) was mixed with CO_2 to give a CO_2 concentration of 2.0%. The culture broth was aerated

continuously at a rate of 0.2 vvm (volume gas per working volume per minute). Sufficient microalgae batch cultures were cultivated to fulfil all experimental needs.

4.3.3 Determination of nitrate content and microalgae cell concentration

Nitrate content in the medium and microalgae cell concentration was determined using the spectrophotometric method as described in a previous research (Chew et al., 2018a). The microalgae samples were centrifuged, then diluted with deionized water and measured at the optical density of 220 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu) for nitrate content. The microalgae biomass dry cell weight was obtained by drying the microalgae loaded filter at 105°C until the weight was constant. The dry weight of the blank filter was subtracted from that of the loaded filter to obtain the microalgae dry cell weight (DCW). The microalgae biomass concentration was measured at optical density of 680 nm and converted to biomass concentration via calibration between OD_{680} and DCW. The harvesting of microalgae was performed when the biomass growth of the microalgae has reached a constant level (approximately 14-16 days). The harvested microalgae culture broth was centrifuged at 6000 rpm for 10 min and the clear supernatant was removed. The pellet was lyophilized for 24 hours. The resulting dry microalgae biomass was used for further extraction studies.

4.3.4 Protein extraction with TPP system

The TPP system was generated by initially adding 20 %w/w of ammonium sulphate salt to a 0.5 %w/w microalgae solution (5 ml) followed by

the additional of 1:1 volume ratio of mixture to t-butanol (5 ml). The aqueous mixture was stirred using a magnetic stirrer at 200 rpm for 1 h at room temperature. The mixture was then allowed to settle and separate for 30 min. Three phase formation was observed which includes an organic upper phase, intermediate precipitate and aqueous bottom phase. The top and bottom layer was separated from the system using a micropipette. The middle phase was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The top, bottom and dialyzed solution was analyzed for total protein concentration. The TPP tests were performed in triplicates.

4.3.5 Protein extraction with MWTPP system

After obtaining the protein concentration for the TPP process, the MWTPP system was performed by using a microwave oven (Samsung, Malaysia) and glass vials of 2 mm thickness and 20 ml capacity. MWTPP experiments were conducted by initially adding 20 %w/w of ammonium sulphate salt to a 0.5 %w/w microalgae solution (5 ml) followed by the additional of 1:1 volume ratio of mixture to t-butanol (5 ml). The aqueous mixture in the glass vials was exposed to microwave for an initial value of 120 s at 180 W. The formation of three layers was present and the mixture was left to settle for 30 min at room temperature. The top and bottom layer was separated using a micropipette and the middle phase was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The top, bottom and dialyzed solution was analyzed for total protein concentration. One-variable-at-a-time (OVAT) approach was used to investigate the effects of different parameters on the extraction of protein from the microalgae biomass. The operating parameters of the MWTPP system such

as types of salt, concentrations of salt, volume ratio of slurry to solvent, pH of the system, the microwave time, microwave duty cycle, microwave power and concentration of microalgae were optimized to obtain the best recovery yield and separation efficiency. All MWTPP tests were performed in triplicates. The initial settings of the MWTPP were selected based on literature study and the variables of the MWTPP are listed in Table 4.1.

Table 4.1: Operating parameters of MWTPP system for protein extraction from *Chlorella vulgaris*.

Step No.	**Operating parameter**	**Initial condition**	**Variables**	**Unit**
1	Type of salt	Ammonium sulphate	Ammonium sulphate (AS), sodium sulphate (SSE), magnesium sulphate (MS), magnesium acetate (MA), dipotassium hydrogen phosphate (DHP)	-
2	Concentration of salt solution	20	15, 20, 25, 30, 35	%w/w
3	Volume ratio of slurry to solvent	1:1	1:0.5, 1:1, 1:1.5, 1:2	-
4	Microwave time	120	60, 90, 120, 150	s
5	Microwave duty cycle	100	20, 40, 60, 80, 100	%
6	Microwave power	180	100, 180, 300	W
7	Microalgae biomass concentration	0.5	0.25, 0.5, 0.75, 1.0, 1.25	%w/w

4.3.6 Protein assay

The extracted proteins from the microalgae were tested using the Bradford method (Bradford, 1976). 0.25 ml of the sample from each phase was mixed with 2.5 ml of Bradford reagent and measured using UV-Vis spectrophotometer at a wavelength of 595 nm. Bovine serum albumin (BSA) was used to generate the standard for proteins, where a concentration range of 0 – 1 g/L was used. The absorbance readings obtained were converted into protein concentration via calibration between OD_{595} and BSA protein concentrations.

4.3.7 Determination of recovery yield and separation efficiency

The recovery yield (R, %) of proteins from the total protein content was represented by Eq (1):

$$R(\%) = \frac{Total\ proteins\ recoverd\ in\ the\ intermediate\ phase}{Total\ protein\ content\ in\ microalgae\ biomass} \quad (1)$$

Where the total protein content in microalgae biomass is calculated using Eq. (2):

$$Total\ \ protein\ \ (\%) = N_{ea} \times NTP \quad (2)$$

Where N_{ea} stands for the total nitrogen % of *Chlorella vulgaris* with a value of 4.14% obtained by elemental analysis, and NTP stands for nitrogen-to-protein conversion factor, with a constant value of 4.78 (Lourenço et al., 2004).

The separation efficiency (E, %) was calculated using Eq. (3):

$$E\ (\%) = \frac{C_I V_I}{C_T V_T \times C_I V_I \times C_B V_B} \quad (3)$$

Where C stands for concentration of proteins, V stands for volume in each phase, T, I and B stands for top, intermediate and bottom phase, respectively.

4.3.8 Cell disruption by microwave

The structural impact caused by microwave treatment on *C. vulgaris* was observed by using a Field Emission Scanning Electron Microscope (FESEM, Quanta 400F) (FEI, USA). Two set of samples consisting of samples after microwave-only treatment (using optimized microwave conditions) and samples after MWTPP (using optimized MWTPP conditions) were prepared. The resulting intermediate phase was dissolved and washed with Tris-HCl buffer. The mixture was then centrifuged and lyophilized for 24 h. Freeze-dried fresh microalgae samples, samples after microwave-only treatment and samples after MWTPP were used for the SEM characterization.

4.4 Results and Discussion

4.4.1 Effect of type of salts

Different salts like ammonium sulphate (AS), sodium sulphate (SSE), magnesium sulphate (MS), magnesium acetate (MA) and dipotassium hydrogen phosphate (DHP) were used in this MWTPP study. T-butanol was the solvent used as the upper organic phase for all the salt combinations. The recovery yield and separation efficiency of proteins in the systems containing various salts are shown in Figure 4.1. The highest R was observed in the system containing ammonium sulphate (42.3%). This was followed by systems containing SSE and MS. MA and DHP salts obtained much lower yields compared to the salts

containing sulphate, about up to 40% lower yield in protein content. The E of proteins in salts containing sulphate are also higher compared to MA and DHP, with AS having the best E of 56.3%. Sulphate ions are part of the early member of the hofmeister series and they interact well with water, thereby forming H-bonds and dehydrating proteins. The good hydration capacity and affinity for water of the sulphate salts resulted in higher protein yield compared to the other salts. The water molecules involved in hydration of the sulphate ions will promote protein stability by favouring hydrophobic interactions. The large ions gather together and the proteins are able to be segregated after being precipitated out of the water phase (Ward, 2009).

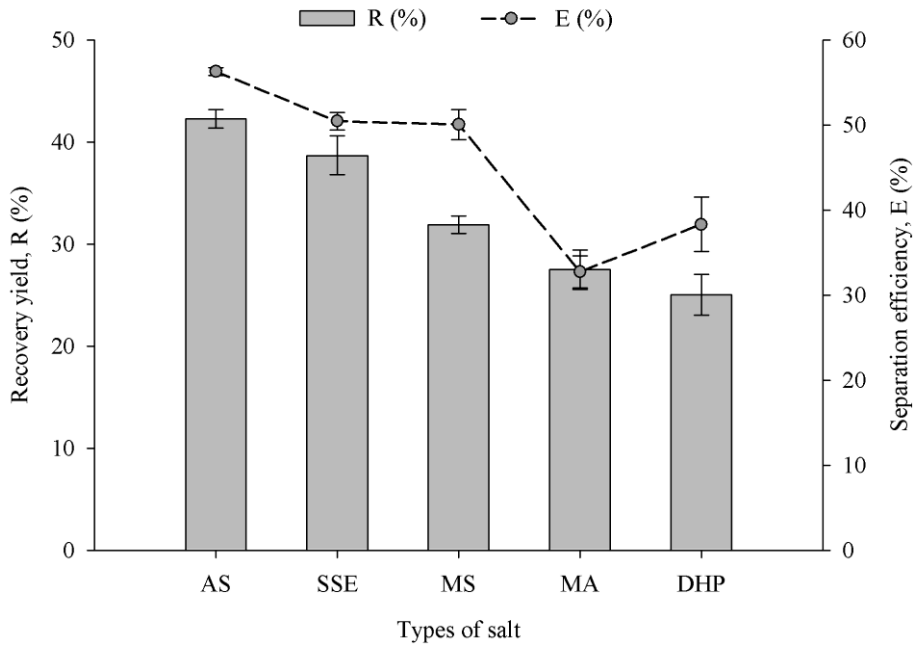


Figure 4.1: Effect of various types of salt (Ammonium sulphate, sodium sulphate, magnesium sulphate, magnesium acetate and dipotassium hydrogen phosphate) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

On the other hand, potassium salts have a more hygroscopic nature and tends to denature considerable amount of proteins which will lower the protein recovery yield (Panadare & Rathod, 2017b). AS was found to be most effective salt in protein extraction and partitioning and it has also been found to be the best phase forming salt in various other works (Avhad et al., 2014; Gagaoua et al., 2017; Pakhale & Bhagwat, 2016). Using AS to salt out proteins and concentrate them into the intermediate phase can be done according to the net charge of the proteins and concentration of salts in the system. Hence, AS was selected for further studies on the most effective concentration of salt to be used.

4.4.2 Effect of salt concentration

The concentration of salt used is a vital parameter that plays a major role in the protein interaction and precipitation. The optimization of salt concentration is necessary as the solubility of proteins and other biomolecules will vary according to the ionic strength of the solution (Chew et al., 2018c). The effect of salt concentration was investigated in the range of 15 – 35%. Figure 4.2 shows the results of different concentration of salts on the recovery of proteins from *C. Vulgaris.* The highest R and E were achieved at salt concentration of 30% with values of 48.5% and 59.6%, respectively. The recovery yield of protein increases with the higher salt concentration up to 30% but decreases at 35% salt concentration. The recovery of proteins at 30% salt concentration were found to be improved by about 15% compared to initial 20% salt concentration setting. Higher amount of salt content creates a stronger salting out effect which reduces the free water available to dissolve the protein, this would enable the AS salt to flocculate the proteins and separate them into

the intermediate phase. The effective dielectric constant of water will greatly increase with increasing salt concentration, resulting in the exclusion of t-butanol from the water due to elevating hydrophobic properties. Hence, the addition of higher salt concentration will lead to higher E.

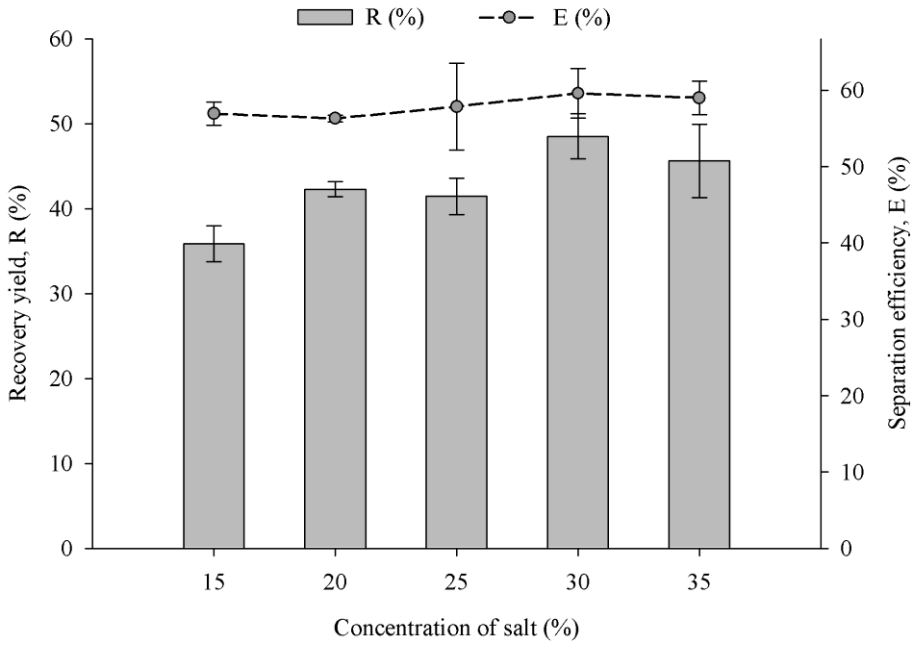


Figure 4.2: Effect of salt concentration (15, 20, 25, 30 and 35%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

However, too high concentrations of salts would lead to the irreversible denaturation of the proteins. The excessive dehydrating effect of the sulphate ions may also create an unfavorable condition for proteins in the aqueous phase and hence pushing them into the butanol phase (Kulkarni & Rathod, 2015). This is seen by the further increase of salt concentration to 35%, where lower protein recovery was obtained with a slight decrease in the E. The work by Waghmare et al. (2016) also reported that higher salt concentration lead to higher protein recovery, where the optimized salt concentration was found at 40% from the

initial setting of 20% (Waghmare et al., 2016). The salt concentration of 30% gave the best R in our experiment and was selected for subsequent experiments.

4.4.3 Effect of t-butanol ratio

T-butanol is considered as one of the best solvents to be used in TPP, as it is a non-ionic kosmotrope that has very good solubility and role as a differentiating solvent (Gagaoua et al., 2017). The mixture of t-butanol with sufficient salt solution can form the three phase layers to isolate various biomolecules such as proteins, pigments and lipids effectively. It can bind to the precipitated proteins and increase their buoyancy, causing them to float above the aqueous salt layer. The size and branched structure of this solvent avoids it from simply permeating into the folded proteins molecules, thereby reducing the potential of causing denaturation to the proteins (Dennison & Lovrien, 1997). The effect of t-butanol ratio was studied for the ratio of slurry to t-butanol of 1:0.5, 1:1, 1:1.5 and 1:2. Figure 4.3 shows the results of different ratio of slurry to t-butanol on the recovery of proteins from *C. Vulgaris.* The recovery yield of proteins was found to be highest at the ratio of 1:1, which was the initial setting. At the ratio of 1:0.5, the E was the highest (73.4%) but the R was found to be much lower at about 29% lower compared to 1:1. Further increase in the t-butanol amount to ratio of 1:1.5 and 1:2 did not yield higher protein content. At lower t-butanol concentration, there might not be sufficient solvent to synergize with the salt, while at higher concentrations, it will result in the pulling of larger quantity of water from the salt layer which will increase the salting out effect at the lower phase (Patil & Yadav, 2018). Excessive addition of t-butanol to a certain degree has been found to denature protein as well (Chew

et al., 2018c). Furthermore, more amount of t-butanol would result in a total system containing more volume and this may affect the dissipation of microwave energy in the system. Higher t-butanol content in the system would lead to a decreased absorption of microwave energy in the microalgae biomass and thereby reducing the dipole rotation and heat generation. Less microwaves absorbed by the microalgae will reduce the possibility of cell wall disruption, leading to lower protein content release during the separation process. The slurry to solvent ratio of 1:1 showed the highest yield in this experiment and was selected for the next experiment.

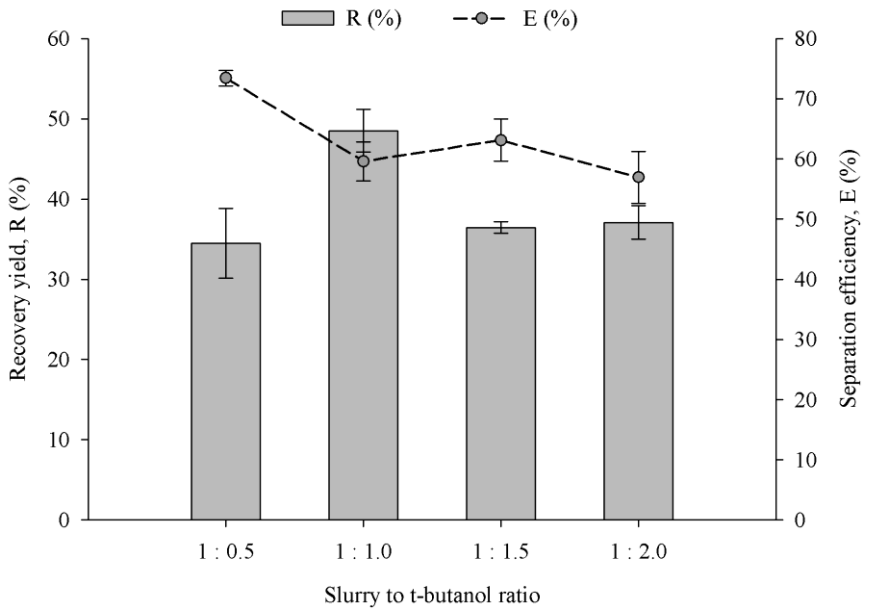


Figure 4.3: Effect of ratio of slurry to t-butanol (1:0.5, 1:1, 1:1.5 and 1:2) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

4.4.4 Effect of microwave time

The microwave irradiation time is one of the important parameters that affects the overall efficiency of the process in terms of energy consumption, yield and quality of products extracted. Microwave irradiation can cause

thermal effects on the reacting compounds, where the microwaves will create the interaction of biomass with the electric field component of an electromagnetic wave (Beneroso et al., 2017). This would result in the increase in temperature, and subsequently the evaporation of water which will exert pressure on the cell walls leading to cell disruption (Kapoore et al., 2018). However, these thermal effects will also lead to protein denaturation at high temperatures, hence, it is vital to study the suitable microwave conditions to avoid the denaturation of protein biomolecules. The effect of microwave irradiation time was studied for 60s, 90s, 120s and 150s at a power input of 180W. Figure 4.4 shows the results of different microwave duration on the recovery of proteins from *C. Vulgaris*. The increase in microwave irradiation time leads to an increase in protein yield up to 120s. At 150s, the protein yield decreases. 120s has the highest R (48.5%) and comparable E (57.7%) with 90s and 150s. Microwaves causes the polar molecules to rotate for a long time at the same frequency, thus creating a molecular friction which will release heat and increase the temperature. An increase in temperature in the three phase mixture will result in the penetration of the solvent into the matrix more efficiently and thereby facilitating the extraction process. Nevertheless, excessively long microwave time will also destroy the other active ingredients and release impurities, causing a reduction in protein yield (Fu et al., 2018). On the other hand, insufficient microwave irradiation will not alter the cell structure and membrane significantly, making the release of proteins from microalgae to be ineffective. Patil and Yadav (2018) discovered that insufficient microwave irradiation resulted in lower temperature which could only partially hydrolyze the proteins, while too much microwave irradiation elevated the temperature

and caused the denaturing of proteins (Patil & Yadav, 2018). The microwave time of 120s was considered to be the optimal time as it showed the highest R and E, and this irradiation time was selected for subsequent experiments.

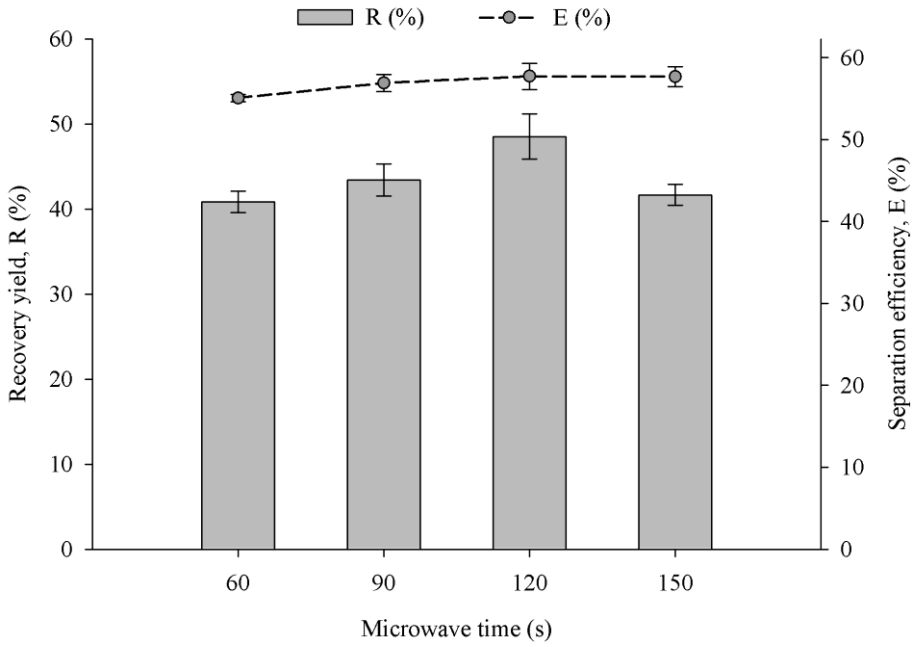


Figure 4.4: Effect of microwave irradiation time (60, 90, 120 and 150 s) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

4.4.5 Effect of microwave duty cycle

The operation of a microwave oven in continuous mode could lead to constant molecular friction, which under a long period of time, it will increase the temperature steadfastly and degrade the temperature sensitive compounds. Hence, the operation of a microwave in pulse mode, where power is supplied in intervals, can reduce the occurrence of compounds degradation. Pulse mode operation also reduces the energy consumption and will reduce the evaporation of solvents in the system. The effect of microwave duty cycle was investigated at 20, 40, 60, 80 and 100% duty cycle for a microwave time of 120s and power

of 180W. Figure 4.5 shows the results of different microwave duty cycles on the protein recovery from *C. Vulgaris*. A lower protein yield was observed for duty cycles from 20% to 60% (18s ON; 12s OFF), where 20% duty cycle had the lowest yield of 36.3%. For 80% duty cycle (24s ON, 6s OFF), the yield increased slightly (51.9%) compared to the full cycle of microwave irradiation. Slightly lower yield for 100% duty cycle compared to 80% duty cycle could be due the prolonged microwave dose, in which using the pulse mode can effective provide a temporary stop in the irradiation to avoids solvents evaporation and reduce the molecular friction which may disrupt the protein content in the solution.

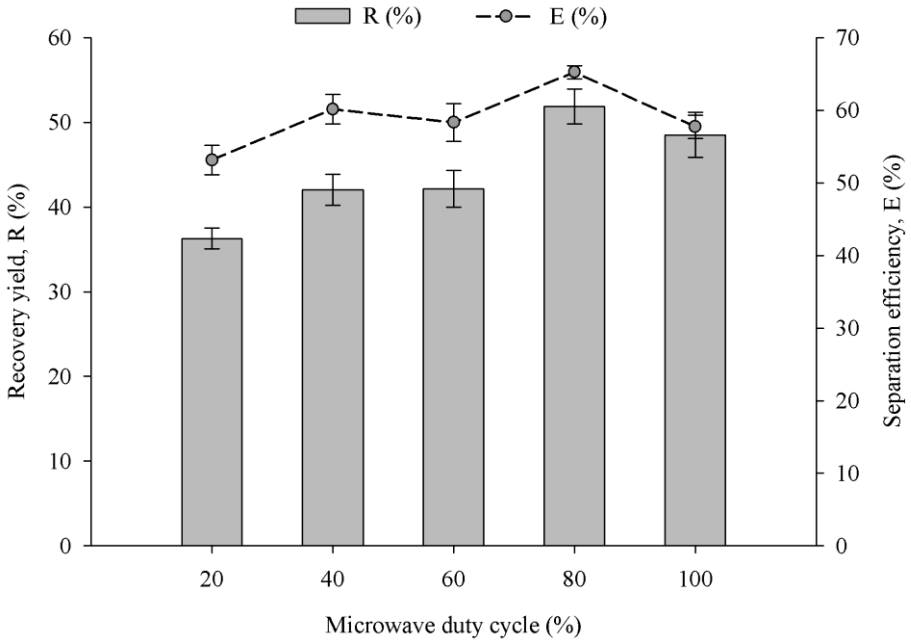


Figure 4.5: Effect of microwave duty cycle (20, 40, 60, 80 and 100%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

A related work by Kulkarni and Rathod (2015) reported that the yield of mangiferin obtained was highest at a duty cycle of 50% with a microwave time

of 5 min, as the pulse mode operation can avoid the ebullition of solvent and reduce unnecessary usage of energy (Kulkarni & Rathod, 2015). From our results, the duty cycle needed to obtained maximum R was 80%, this is higher compared to the related works as the microwave irradiation time in our study is much shorter compared to the work. Hence, a higher duty cycle would justify the total irradiation time needed to successfully disrupt the microalgae cell wall for protein release. To develop a more efficient MWTPP process, the optimum duty cycle was selected at 80% for the subsequent experiments.

4.4.6 Effect of microwave power

The microwave power input affects the yield and cost of the extraction process as power input which is too high could result in the charring of the plant materials and evaporation of highly volatile solvents. Microalgae has a good resistant to heat and many researches have been performed using microwave cell disruption on microalgae to recover biomolecules (Dai et al., 2014; Kapoore et al., 2018). However, the protein content found in microalgae or other plants are sensitive to heat and will easily denature under high temperature conditions. Higher microwave power input will also increase the energy used for the extraction process and lead to an increase in the overall operation costs. Hence, the optimization of the microwave power input is necessary to provide good stability of the protein biomolecules and reduce the cost of the process to make it economically feasible. The microwave power was examined at 100W, 180W and 300W for this experiment. Figure 4.6 shows the results of different microwave power on the protein recovery from *C. Vulgaris*. The power input of 100W shows the highest R (63.2%) and E (67.2%). Subsequent increase of

the microwave power to 180W and 300W showed lower R and E, where at 300W, the temperature of the mixture was very high after the irradiation. Hence, higher power was not tested at the resulting high temperature might cause the alcohol to boil and evaporate.

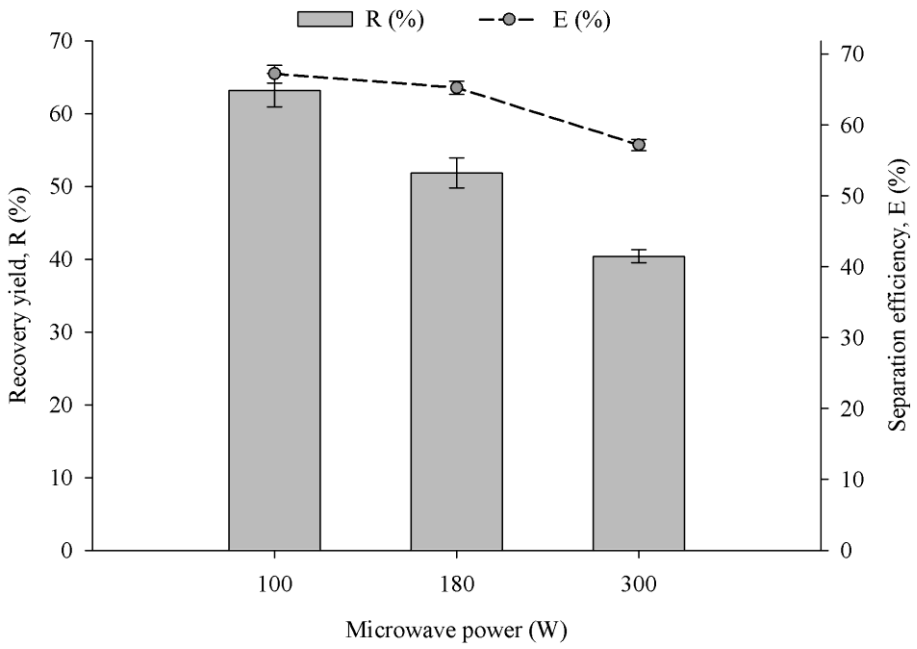


Figure 4.6: Effect of microwave power (100, 180 and 300 W) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

Power input which is too high will create an abundance of power to be dissipated in the similar mixture volume, which lead to a rapid increase in temperature that disrupts the stability of the proteins. Lower microwave power might not be sufficient to disrupt the cell wall of microalgae, Patil and Yadav (2018) reported that the microwave power range of 5 – 50W was used. It was discovered that the maximum yield was obtained at 30W, where lower power will result in insufficient dissipation of the power to enhance the protein

extraction (Patil & Yadav, 2018). For the subsequent MWTPP process, the microwave power was selected at 100W.

4.4.7 Effect of microalgae biomass

The amount of feedstock in the system could affect the system as biomass concentrations which are too high will result in a more viscous solution which may cause difficulties in the separation process (Sankaran et al., 2018a). The biomass concentration was investigated in the range of 0.25 to 1.25 %w/w. From Figure 4.7, the biomass concentration of 0.5 %w/w gives the highest R (63.2%) and moderately high E (67.2%). Higher fractions of biomass showed lower R while 0.25 %w/w showed a slightly lower R (60.0%) compared to 0.5 %w/w. The E increased as the biomass concentration increased, this is likely due to the presence of a lot more biomass in the middle phase that is increased the absorbance value resulting in higher efficiency of separation. The biomass concentrations of 0.75 to 1.25 %w/w did not perform better compared to 0.5 %w/w, this could be due to the insufficient dissipation of the microwaves to completely disrupt the cell walls of the microalgae. Larger amount of biomass would require more microwave irradiations to break down the tough cell walls of microalgae, since only a constant rate of microwaves are available, the higher concentration of biomass would mean that less microwaves are absorbed into each parts of the biomass. For 0.25 %w/w, the yield of protein is higher than 0.75 %w/w and above but not better than 0.5 %w/w. The lower biomass concentration will absorb sufficient microwaves for cell disruption but there is only limited biomass present and hence the total protein recovered is less. To optimize the power usage for generating microwaves, it is essential to place as

much biomass as possible into the system to fully utilize the microwaves generated without compromising the protein recovery. In this study the microalgae concentration of 0.5 %w/w was selected as the optimum biomass concentration for maximum protein recovery.

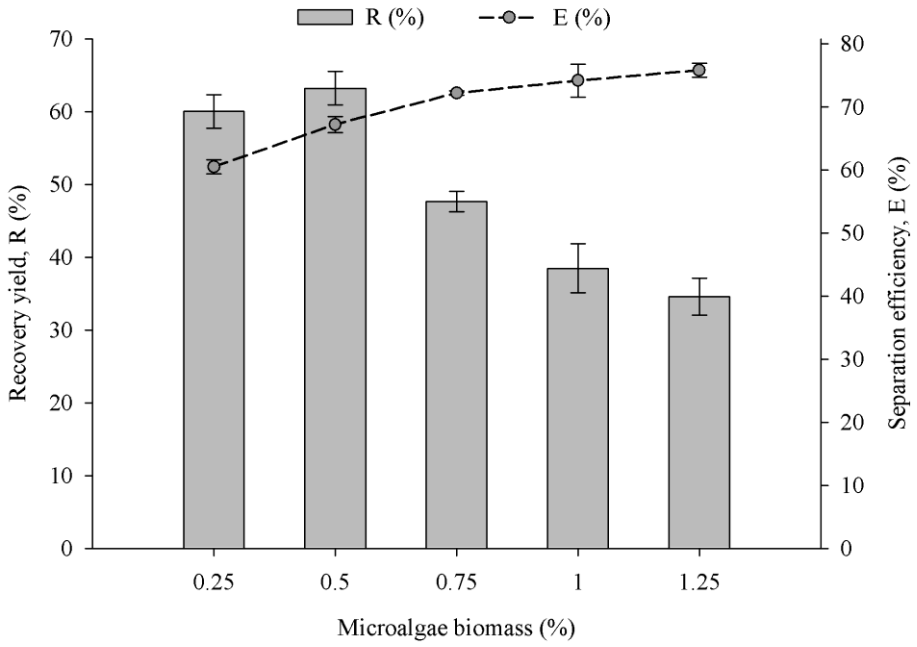


Figure 4.7: Effect of microalgae biomass concentration (0.25, 0.5, 0.75, 1 and 1.25%) on the recovery yield and separation efficiency of proteins from *C. vulgaris*.

4.4.8 Comparison of MWTPP with conventional TPP technique

The conventional TPP was conducted for protein extraction from microalgae along with a microwave-assisted TPP to enhance the protein recovery and efficiency of protein separation. Table 4.2 summarizes the comparison of R and E using microalgae as the slurry for TPP, MWTPP and the optimized MWTPP. The highest E (63.2%) for MWTPP was obtained under the optimized conditions of 30 %w/w mass fraction of ammonium sulphate as the salt phase, 1:1 ratio of slurry to solvent, where t-butanol was used as the solvent,

120s of microwave irradiation time, 80% of microwave duty cycle, microwave power of 100W and biomass concentration of 0.5%. The maximum R achieved by MWTPP was notably higher compared to conventional TPP, showing an enhancement from 46.8% to 67.2%. The extraction time for TPP was performed in 60 min while MWTPP only required a processing time of 2 min with the assistance of microwaves, excluding the fixed time for phase formation. The results from this experiment indicates that TPP combined with microwave could potentially reduce greatly the extraction time needed as well as increase the overall protein yield. This is due to the ability of microwaves to disrupt cell wall of microalgae and promote the biomolecules availability (Passos et al., 2015).

Table 4.2: Comparison of the yield of protein and separation efficiency obtained using TPP, initial MWTPP and optimized MWTPP on microalgae.

System	**Operating conditions**	**Yield of protein (%)**	**Separation efficiency (%)**
TPP	20 %w/w AS; 0.5 %w/w microalgae solution; 1:1 volume ratio; stirred for 1 h	24.9	46.8
MWTPP	20 %w/w AS; 0.5 %w/w microalgae solution; 1:1 volume ratio; 120 s microwave time; 100% duty cycle; 180 W	42.3	56.3
MWTPP (Optimized)	30 %w/w AS; 0.5 %w/w microalgae solution; 1:1 volume ratio; 120 s microwave time; 80% duty cycle; 100 W	63.2	67.2

Microwave heating has also been successfully applied to produce great potentials in energy saving, improved product quality, rapid processing, smaller processing equipment and safer implementation of harsh operating conditions (Kulkarni & Rathod, 2015; Laybourn et al., 2019; Patil & Yadav, 2018). The varying of the salt concentration, microwave duty cycle and microwave power could also significantly affect the extraction of protein, where the optimized MWTPP showed a 150% increase in protein extraction compared to the initial MWTPP settings.

In terms of energy efficiency, the estimated power usage for MWTPP, mainly generated from the microwave (1450W, 80% duty cycle, 120s) is about 144 kJ. On the other hand, the TPP which requires only a stirrer hotplate (10W, 1h) will consume about 36 kJ per experimental round. To obtain similar yield, the TPP would require about 90 kJ of power. The total power consumed by MWTPP is higher for an individual set of extraction process, however, several samples can be subjected to MWTPP simultaneously in each round of processing. This would certainly result in a much lower energy consumption to obtain similar yield compared to TPP as TPP can only be conducted for one sample at a time since stirring is needed. Besides that, in order to obtain the similar yield, the cost of raw materials would be higher for TPP as the yield is lower, meaning that more biomass and chemicals would be needed to extract the same amount of proteins compared to MWTPP. MWTPP has the advantage of faster processing and lower operating costs. Therefore, the application of microwave-assisted TPP under optimized conditions proves to be an attractive purification technique for the recovery of proteins from microalgae.

Figure 4.8 shows the FESEM images of *C. vulgaris* before treatment, after microwave-only treatment and after MWTPP process. Initially, the microalgae cells portray many round cells which are spherical-like clumped on the surface (Figure 4.8a). After microwave-only treatment (Figure 4.8b), the microalgae cells start to disrupt and shrinks, caused by the effect of microwaves irradiation. When MWTPP was applied on microalgae (Figure 4.8c), the microalgae cells were discovered to be almost completely flat and shrunk. An enlarged view of the microalgae cells without treatment and after MWTPP are shown in Figure 4.9. The SEM image shows that microalgae cells after MWTPP has ruptured due to the microwave treatment and shrinked as a result of the biomolecules within the cells being released. This indicates that MWTPP contributed to the major cell wall damage with leakage of the cell content. The findings show that microwave irradiation coupled with TPP is very effective in disrupting the microalgae cell wall for biomolecules extraction.

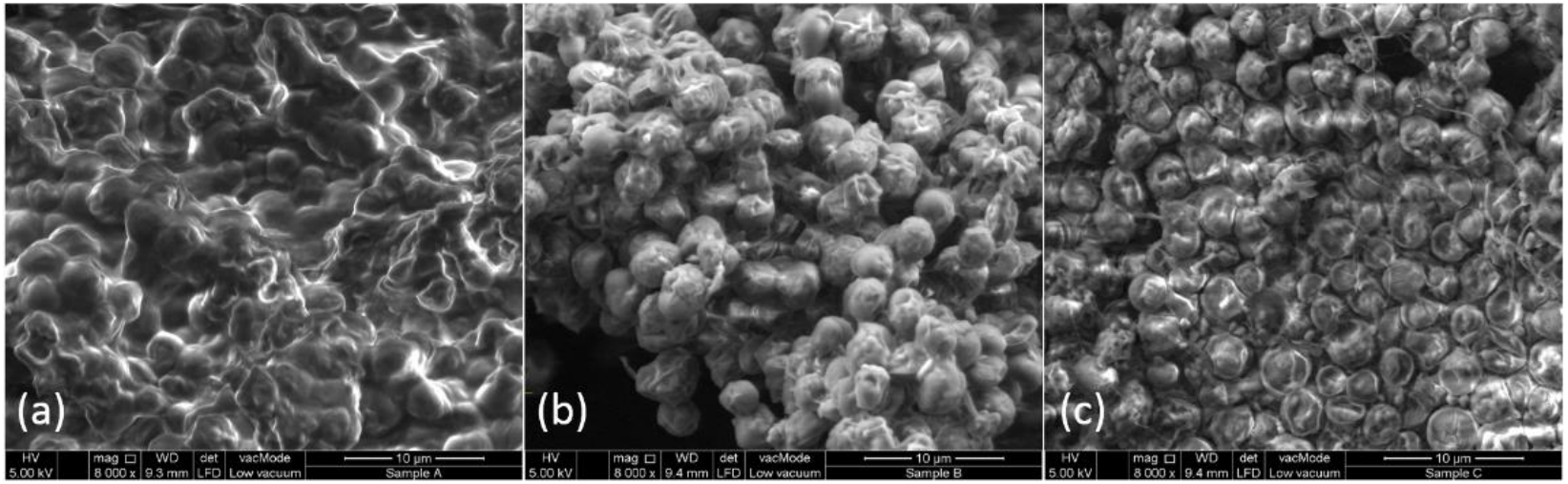


Figure 4.8: FESEM visualization of *C. vulgaris* cell disruption: (a) microalgae cells without treatment; (b) microalgae cells with microwave-only treatment (Microwave time: 120s, duty cycle: 80%, power: 100W); (c) microalgae cells with optimized MWTPP process (AS concentration: 30 %w/w, ratio of slurry to t-butanol: 1:1, microwave time: 120s, duty cycle: 80%, power: 100W).

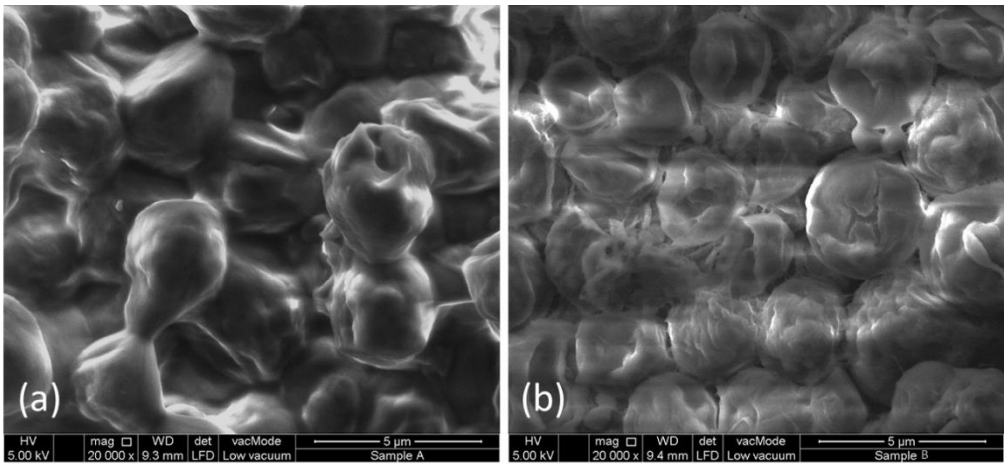


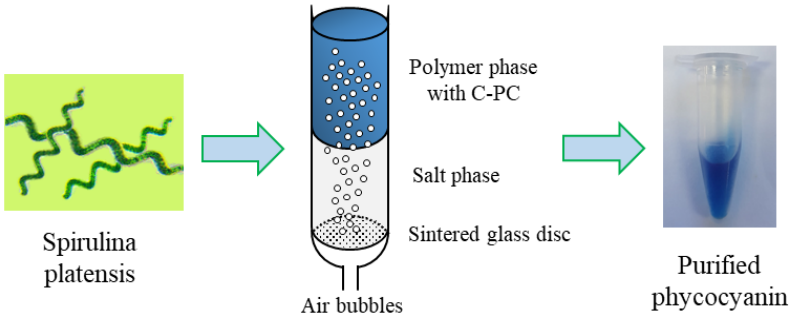
Figure 4.9: Enlarged FESEM visualization (Mag: 20000×) of *C. vulgaris* cell disruption: (a) microalgae cells without treatment; (b) microalgae cells with optimized MWTPP process.

4.5 Conclusion

The extraction of proteins from *Chlorella vulgaris* was performed using MWTPP, which is an improved version of the traditional TPP process with the incorporation of microwaves into the system. The optimized conditions for the MWTPP was found to be: Ammonium sulphate concentration (30 %w/w), slurry to solvent ratio (1:1), microwave irradiation time (120 s), duty cycle (80%), microwave power (100W) and microalgae biomass concentration (0.5 %w/w). The MWTPP increased the protein yield by about 2.54 times to 63.2% from 24.9% obtained in conventional TPP process alone. The incorporation of microwaves has contributed tremendously in the cell disruption of the microalgae, leading to the release of valuable protein and other biomolecules. Following the success of multiphase partitioning for proteins recovery from microalgae, the next chapter (Chapter 5) investigates the potential use of bubbles-assisted multiphase partitioning for pigments purification.

CHAPTER 5

LIQUID BIPHASIC FLOTATION FOR THE PURIFICATION OF C-PHYCOCYANIN FROM SPIRULINA PLATENSIS MICROALGA



Liquid Biphasic Flotation

This Chapter covers the utilization of a bubbling-assisted liquid biphasic partitioning technique for the purification of phycocyanin from microalgae. Various parameters which affect the liquid biphasic flotation system was investigated. These parameters were optimized to obtain the best conditions for maximal phycocyanin recovery yield and purification fold compared to the crude extract. The molecular weight characterization of the pigment purified after the biphasic process was also accomplished. This Chapter consist of a thesis-version of the manuscript submitted to the Bioresource Technology journal (Chew K.W. et al., 2019, *Bioresource Technology*).

Liquid biphasic flotation for the purification of C-phycocyanin from *Spirulina platensis* microalga

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Authors' Contributions

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

5.1 Abstract

Liquid biphasic flotation (LBF), an integrated process of liquid biphasic system (LBS) and solvent sublation, was used for the purification of C-phycocyanin from *Spirulina platensis* microalgae. Various experimental parameters such as type of phase forming polymer and salt, concentration of phase forming components, system pH, volume ratio, air flotation time and crude extract concentration were evaluated to maximise the C-phycocyanin recovery yield and purity. The optimal conditions for the LBF system achieving C-phycocyanin purification fold of 3.49 compared to 2.43 from the initial LBF conditions was in polyethylene glycol (PEG) 4000 and potassium phosphate combination, with 250 g/L of polymer and salt concentration each, volume ratio of 1:0.85, system pH of 7.0, air flotation duration of 7 min and phycocyanin crude extract concentration of 0.625 %w/w. The LBF has effectively enhanced the purification of C-phycocyanin in a cost effective and simple processing.

Keywords: C-phycocyanin, liquid biphasic flotation; pigment; purification; *Spirulina platensis*

5.2 Introduction

The importance of high-value natural products that can be obtained from renewable source and their nutritional quality have been growing in recent times (Chew et al., 2017). The cyanobacterium *Spirulina platensis* has been on the spotlight for its great potential in the production of food and nutritional compounds, namely, proteins, carbohydrates, vitamins, fatty acids and food colorants (Kulshreshtha et al., 2008). One of the major pigments that can be extracted from *Spirulina platensis* is C-phycocyanin (C-PC). C-PC is a natural blue pigment that is part of the phycobiliprotein group in the proteins present in *Spirulina*. C-PC is widely used in food processing and cosmetic industry and possess anti-oxidant, anti-inflammatory and anticancer activity. It can help to modulate lipid and carbohydrate metabolisms and control the occurrence of chronic diseases like obesity, cardiovascular disease and diabetes (Chew et al., 2017; Khan et al., 2005). These benefits have led to researchers putting focus on the development of an efficient process for the commercial extraction and purification of phycocyanin.

The main difficulties in phycocyanin purification involves the great number of processing steps whereby precipitation, centrifugation and dialysis are used in the initial purification, followed by ion-exchange chromatography and gel filtration chromatography in the final purification (Patel et al., 2005; Patil & Raghavarao, 2007). These long steps needed for phycocyanin purification will result in higher product yield loss, apart from posing difficulties in the scale-up process. The major portion of production costs arises from the purification steps, hence, a more efficient and economical large scale

bioseparation method is required to help elevate the costly conventional processing for phycocyanin purification.

Liquid biphasic system (LBS), also known as aqueous two-phase system (ATPS), is an economical and effective downstream processing that has been widely used in the recovery of bioproducts. This technique is favoured for its simplicity, high extraction efficiency, biocompatibility, ease of operation, rapid processing time and feasibility for scale-up (Lee et al., 2017a; Phong et al., 2018a). LBS consists of two hydrophilic but immiscible solutions, made using either polymer/salt, alcohol/salt or other combinations. The partitioning behavior of biomolecules such as proteins in LBS can be affected by the type of raw materials used, concentration of the polymer/salt, volume ratio, temperature, pH of the system, biomass concentration and many more (Asenjo & Andrews, 2012; Chia et al., 2018c). LBS has been successfully used for the downstream processing of C-PC from *Spirulina*, for example: ATP extraction with PEG and salt (Patil & Raghavarao, 2007), ATPS with ionic liquid and salt (Chang et al., 2018; Zhang et al., 2015), integrated ATPS with cell debris (Antelo et al., 2010) and vortex fluidic device-intensified ATP extraction (Luo et al., 2016). These processes have demonstrated the potential purification of C-PC using multiphase systems which are able to yield high recoveries with low cost operation and are suitable for food processes.

Liquid biphasic flotation (LBF) is an emerging bioseparation process for the extraction and purification of biomolecules (Sankaran et al., 2018b). The LBF system is an integration of the conventional LBS and solvent sublation (SS) system, where the biphasic system is supported with air bubbling to transport the biomolecules from one phase to another. The surface-active

compound of biomolecules present will adsorb onto the surface of the ascending gas bubbles and be brought from the bottom aqueous phase to the top organic phase. LBF has been applied for the purification of various biomolecules which include lipase from *Burkholderia cepacia* (Sankaran et al., 2018b; Show et al., 2013), proteins from microalgae (Sankaran et al., 2018a), betacyanin from *Hylocereus polyrhizus* (Leong et al., 2018) and many more. The LBF which contains the benefits of both LBS and SS, has shown to achieve high separation efficiency, ease of operation and environmental friendliness. This indicates that LBF has high prospects for the purification of biomolecules from microalgae.

The aim of this study was to investigate the potential of integrating LBS and SS system in a combined LBF the purification of C-PC from *Spirulina platensis*. From Chapter 4, the successful extraction of proteins shows that multiphase partitioning systems have the potential to extract and purify various biomolecules from microalgae. In this chapter, the potential technique of integrated liquid biphasic system with bubbling effect, known as liquid biphasic flotation (LBF), can be used as a rapid and efficient purification method for C-PC recovery from microalgae. The LBF study was conducted by optimizing the operating conditions and evaluating the yield and separation efficiency of C-PC. The parameters that were investigated include types of PEG, types of salts, concentration of PEG and salt, volume ratio, pH of the system, air flotation time and crude extract concentration. The molecular weights of the C-PC purified were established through SDS-PAGE tests.

5.3 Materials and Methods

5.3.1 Materials

Dipotassium hydrogen phosphate [K_2HPO_4], potassium dihydrogen phosphate [KH_2PO_4], ammonium sulphate [$(NH_4)_2SO_4$], magnesium sulphate [$MgSO_4$], sodium dihydrogen phosphate [NaH_2PO_4], Polyethylene glycol (PEG) 4000, PEG 8000, phosphate buffer and sodium chloride [NaCl] were purchased from R&M chemicals (Malaysia). PEG 2000 was purchased from Alfa Aesar (UK). Dried *Spirulina platensis* powder was purchased from Charming & Beauty Co. Ltd. (Taipei, Taiwan). All chemicals used were of analytical grade.

5.3.2 Preparation of crude phycocyanin extract

Spirulina platensis dried powder were dissolved in phosphate buffer and disrupted using an ultrasonicator (Elma, Germany) at 35 kHz (40% amplitude) for 5 min. The suspension was then centrifuged at 6500 rpm for 10 min and the supernatant was separated from the cell debris and stored at 4°C for further purification studies.

5.3.3 Liquid biphasic flotation technique

The LBF apparatus used was adapted from the work by Leong et al. 2018 (Leong et al., 2018). The LBF system consists of a glass filter funnel of 20 cm length and 2 cm diameter with a sintered glass disk (Grade G4 porosity) installed at the bottom of the funnel. Air was supplied using a pump and air bubbles were generated when the air passed through the sintered glass disk. The LBF of predetermined properties was generated by initially adding 300 g/L PEG

solution (20 ml) into 250 g/L of potassium salt with 0.5 %w/w crude extract concentration of phycocyanin solution (20 ml). The aqueous mixture was aerated with air flow of 20-30 cc/min for a duration of 5 min. The mixture was then transferred to a centrifuge tube and allowed to settle and separate into two phases for 30 min. One-variable-at-a-time (OVAT) approach was used to investigate the effects of different parameters on the purification of phycocyanin from the microalgae. The operating parameters of the LBF system such as types of PEG, type of salt, concentration of PEG, concentration of salt, volume ratio of PEG to salt, pH of the system, air flow duration and concentration of crude extract were optimized for the phycocyanin purification. The initial settings and variables of the LBF system are listed in Table 5.1.

Table 5.1: Operating parameters of LBF system for C-phycocyanin purification from *Spirulina platensis*.

Step No.	**Operating parameter**	**Initial condition**	**Variables**	**Unit**
1	Type of PEG	-	PEG 2000, PEG 4000, PEG 8000	-
2	Type of salts	K_2HPO_4	$(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, NaH_2PO_4	-
3	Concentration of PEG	300	200, 250, 300, 350, 400	g/L
4	Concentration of salt	250	200, 250, 300, 350	g/L
5	Volume ratio	1:1	1:0.7, 1:0.85; 1:1, 1:1.25, 1:1.5	-
6	pH	9	6, 7, 8, 9	-
7	Air flotation time	5	1.5, 3, 5, 7, 9	min
8	Crude extract concentration	0.5	0.25, 0.375, 0.5, 0.625, 0.75	%w/w

5.3.4 Analytical procedures

5.3.4.1 Determination of C-phycocyanin concentration

C-PC concentration was determined according to Bennett and Bogorad (1973) (Bennett & Bogorad, 1973) using Equation (1), where CPC is the C-phycocyanin concentration (mg/ml), 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:

$$CPC = \frac{OD_{615} - 0.474 \times OD_{652}}{5.34} \qquad \text{Equation (1)}$$

The purity of C-PC (PT) was calculated spectrophotometrically according to Abalde et al. 1998 (Abalde et al., 1998) using Equation (2), where OD_{620} is the optical density of the sample at 620 nm, which represents the maximum absorption of C-PC, and OD_{280} is the optical density of the sample at 280 nm, which represents the total proteins in the solution. This purity is an indication of the pureness of C-PC extract with respect to other contaminating proteins. The purification factor (PF) is calculated by using the purity of the sample divided by the purity of the crude extract (Equation (3)):

$$PT = \frac{OD_{620}}{OD_{280}} \qquad \text{Equation (2)}$$

$$PF = \frac{PT_{top}}{PT_{crude}} \qquad \text{Equation (3)}$$

The partition coefficient (K) was calculated using Equation (4), where CPC_t and CPC_b are the concentration of C-phycocyanin (mg/ml) in the top and bottom phases, respectively.

$$K = \frac{CPC_t}{CPC_b} \qquad \text{Equation (4)}$$

The volume ratio (V_r) is the ratio between the volumes of the top and bottom phases and can be calculated using Equation (5):

$$V_r = \frac{V_t}{V_b} \qquad \text{Equation (5)}$$

The recovery yield (%) of the C-PC from the sample was calculated using Equation (6):

$$RC = \frac{K \times V_r}{1 + K \times V_r} \qquad \text{Equation (6)}$$

5.3.4.2 Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Deutscher's method (Deutscher, 1990), using a 30% polyacrylamide slab gel, running in a vertical gel electrophoresis unit. Electrophoresis was run at 50 V, 12.5 mA, for 3–4 h. The gel was stained with a solution containing 0.05 %w/w Coomassie Brilliant Blue R250 and destained using deionized water.

5.4 Results and Discussion

5.4.1 Effect of PEG molecular weight

The LBF study was performed using polyethylene glycol of different molecular weights, namely PEG 2000, PEG 4000 and PEG 8000. The phase compositions were set at 300 g/L of polymer and 250 g/L of salt for a system with polymer/salt volume ratio of 1:1. The influence of PEG molecular weight on the recovery yield and purification fold of C-PC are shown in Table 5.2. PEG 4000 obtained the highest yield (RC) at 81.2% while PEG 2000 obtained the best purification fold (PF) of 2.60. The RC for PEG 2000 was the lowest while

for PF the lowest was found at PEG 8000. The increase in PEG molecular weight will lead to less hydroxyl groups present, hence, increasing the top phase hydrophobicity. By adding similar concentrations of polymer, the higher molecular weights of PEG will induce higher chain lengths of the polymer that results in less free volume in the top phase for proteins. This indicates that lower molecular weight of PEG will have more free volume and hence more proteins will be present at the top phase, where a lower concentration of PC was found at the top phase of PEG 2000. On the other hand, increasing the PEG molecular weight from 4000 to 8000 facilitated the partitioning of proteins and PC to the bottom phase as there are less space for proteins at the top phase, resulting in a lower yield and purity. Higher molecular weight of PEG also has lower solubility and creates a thicker solution that may reduce the effectiveness of the air bubbles from transporting the PC to the top phase. The purity of both PEG 2000 and PEG 4000 does not vary significantly but the yield of PEG 2000 is much lower compared to PEG 4000. In view of this, PEG 4000 was selected for further experiments.

Table 5.2: Effect of polymer molecular weight on the C-PC recovery yield and purification fold from *Spirulina platensis.*

PEG MW	**PEG phase (g/L)**	**Salt phase (g/L)**	**VR**	**RC (%)**	**PF**
2000	300	250	1.02	66.5 ± 0.83	2.60
4000	300	250	0.95	81.2 ± 0.28	2.43
6000	300	250	1.08	75.3 ± 0.13	2.19

5.4.2 Effect of types of salt

The selection of suitable salts for the purification of C-PC can affect the partitioning of proteins to the top phase. Different salts like ammonium sulphate, magnesium sulphate, dipotassium hydrogen phosphate and sodium dihydrogen phosphate were studied in the polymer/salt LBF. PEG 4000 was the polymer used as the upper phase for all the salt combinations (Figure 5.1). The highest RC was found for ammonium sulphate while the highest purity was obtained by dipotassium hydrogen phosphate. The lowest yield and purity were observed when magnesium sulphate was used as the salt in LBF. It is essential to select the salt which can provide an initial pH that is higher than the pI of C-PC to allow for better partitioning. Ammonium sulphate has been commonly used for the precipitation of proteins in protein and enzymes purification process. The high RC of ammonium sulphate could be attributed to the sulphate ions which interact well with water, hence, forming good hydration capacity and affinity for water that increases protein yield. However, much of the undesired proteins are also partitioned to the top phase, leading to lower purity of C-PC. Both ammonium sulphate and magnesium sulphate produced a system of pH 4.5-5.5, which may have caused the C-PC to be unstable. Phosphate salts, on the other hand, are able to provide a comfortable phase environment (pH 6.0-8.0) for the proteins in LBF system, resulting in better purity of C-PC and good recovery yield. Patil and Raghavarao (2007) reported that among the salts tested (phosphate, sulphate, citrate), phosphate salts showed the maximum purity as well (Patil & Raghavarao, 2007). Comparing both phosphate salts (Dipotassium hydrogen phosphate and sodium dihydrogen phosphate), dipotassium hydrogen

phosphate showed higher RC and PF, hence, the PEG/potassium phosphate system was selected for subsequent experiments.

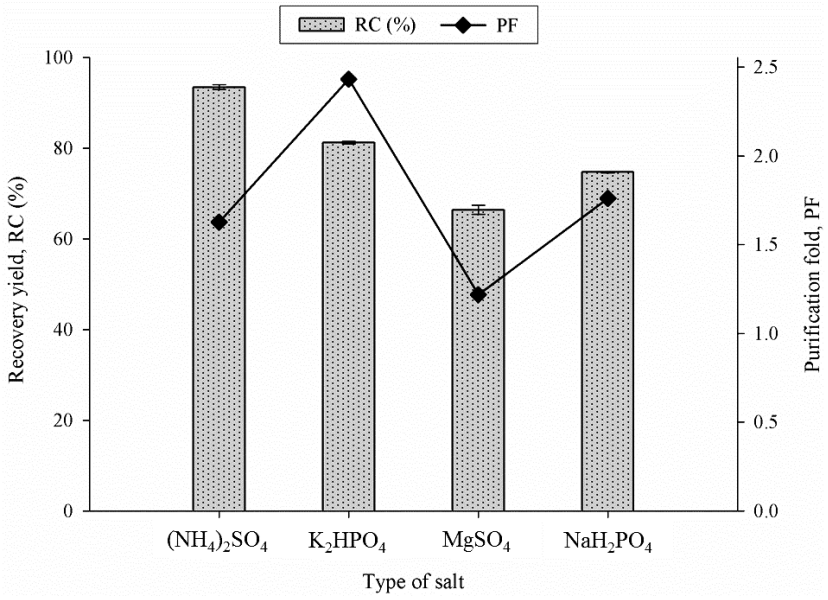


Figure 5.1: Effect of various types of salt on the C-PC recovery yield and purification fold from *Spirulina platensis*.

5.4.3 Effect of polymer and salt concentration

Changes in the polymer and salt concentration will directly influence the phase composition of the biphasic system. These changes will affect the interfacial tension, density and viscosity of the system due to the variation occurring in solute partitioning. The partitioning of C-PC was studied by adjusting the polymer concentration in the range of 200-400 g/L and the salt concentration in the range of 200-350 g/L. Figure 5.2 shows the effects of polymer concentration on the C-PC recovery yield and purification fold. Increasing the polymer concentration above the initial setting of 300 g/L showed a lower RC and PF. Lowering the polymer concentration down to 200 g/L showed an increase in both the RC and PF for 250 g/L but decreased at 200

g/L. 250 g/L of polymer concentration produced the best RC (86.3%) and PF (2.63). Increasing the polymer concentration will increase the volume of the top phase, which encourages the partitioning of contaminating proteins to the top, this would lead to a lower purity of C-PC compounds in the top phase. The higher concentration of polymer also tends to cause foaming during the bubbling process in the LBF system, where the top polymer phase will be reduced as the solution foams at the top. Foaming is less apparent when the lower concentration of polymer is used.

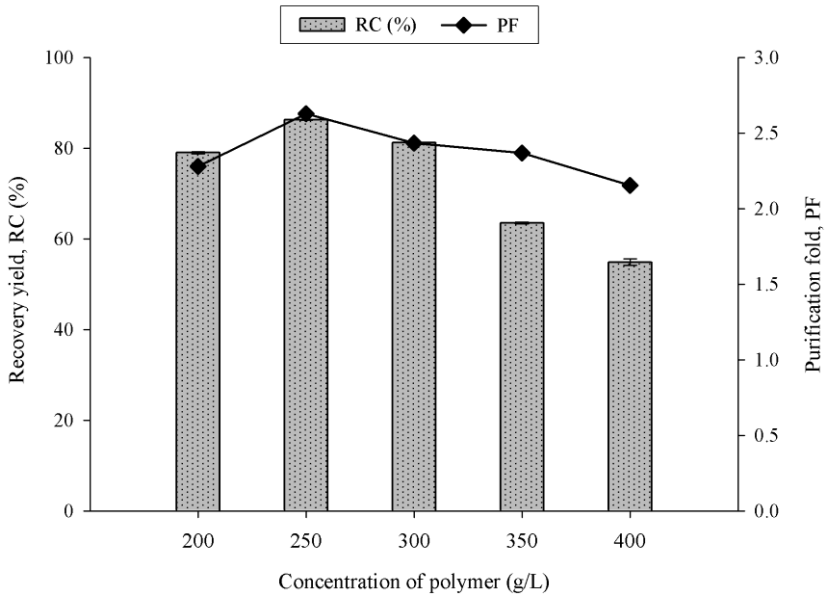


Figure 5.2: Effect of polymer concentration (g/L) on the C-PC recovery yield and purification fold from *Spirulina platensis*.

The decrease in polymer concentration will result in less free volume available for the protein and hence C-PC will be more concentrated at the top. However, too little free volume would result in the insufficient space for C-PC to be partitioned to the top. Less polymer concentration also results in lower top phase volume whereby the air bubbles flow through a longer distance of the

bottom phase, adsorbing more contaminating proteins to the top phase before dispersing at the surface. Based on the findings, polymer concentration of 250 g/L was selected for the next study.

The concentration of salt was altered while keeping the polymer concentration constant. The effects of salt concentration on the C-PC recovery yield and purification fold are shown in Figure 5.3. The increase in salt concentration up to 350 g/L led to much lower RC and lower PF, on top of that, decreasing the salt concentration to 200 g/L also led to a large decrease in RC and PF. Increase in the salt concentration will decrease the top phase volume, creating less free space for proteins and C-PC, hence, reducing the yield and purity of C-PC.

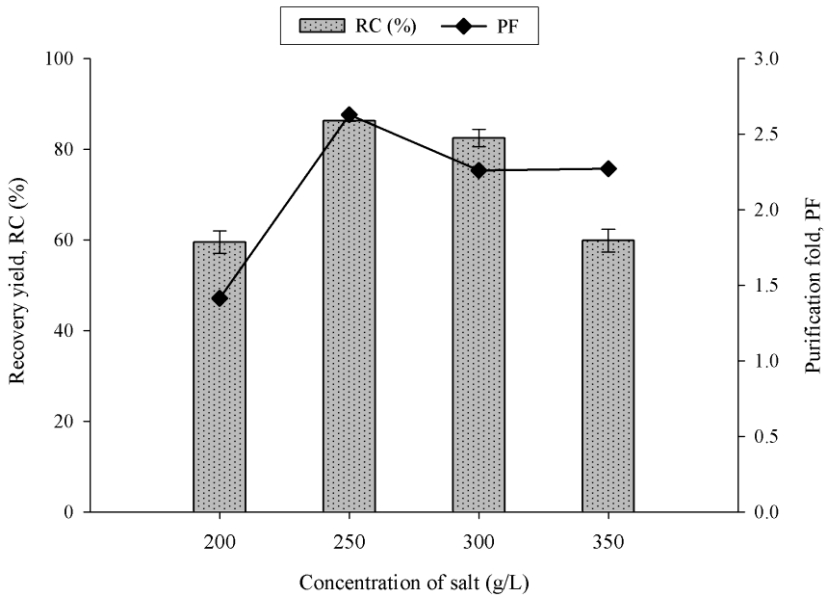


Figure 5.3: Effect of salt concentration (g/L) on the C-PC recovery yield and purification fold from *Spirulina platensis*.

The high concentrations of salt also caused slight precipitation of C-PC. The higher volume of bottom phase would contain more C-PC and this has

significantly affected the yield. At lower salt concentrations (200 g/L), the top phase volume is higher, this indirectly also increase the free volume which would be taken by C-PC and the contaminating proteins. Coupled with the gas flotation effect, more contaminating proteins would be brought to the top phase and cause a lower purity of C-PC. Chethana et al. (2015) reported that the increase in salt concentration led to the significant increase in the change in free volume, resulting in increased partitioning of C-PC and contaminating proteins to the top phase (Chethana et al., 2015). Hence, 250 g/L of salt concentration was selected to remain for the subsequent experiments.

5.4.4 Effect of volume ratio

The phase volume ratio can alter the volume in each phase in a liquid biphasic system, leading to changes in the concentration, yield and purity of biomolecules recovered. The volume ratio of bottom to top phase was adjusted from 1:0.7 to 1:1.5 to observe the concentration of the targeted protein in each phase (Figure 5.4). As the volume ratio increases, the yield of C-PC increased, while the purity decreased. The highest PF (2.91) was found at the volume ratio of 1:0.85, which showed an 11% increase from the initial setting at 1:1, although the RC dropped slightly by 3.4%. Lower RC and PF were observed in the lowest volume ratio used, and despite having the best RC for volume ratio of 1:1.5, the PF was much lower compared to the ratio of 1:0.85. The increase in volume ratio will result in a higher volume of top phase, which allows for both C-PC and the contaminating proteins to concentrate there. This would increase the yield of C-PC but reduce its purity as there will be a lot of contaminating proteins in the top phase. On the other hand, decreasing the volume ratio will

lead to lower volume for the top phase and a higher total salt concentration in the system, meaning that there will less free volume for both the C-PC and proteins. The influence of salt on the partitioning is caused by the non-uniform distribution of the salt in both phases through the difference in electric potential. This facilitates the movement of proteins to the other phase through electrostatic attraction or repulsion (Nagaraja & Iyyaswami, 2015). A lower volume in the top phase would also allow for less volume for the ascending bubbles to release the C-PC in the top phase, where the bubbles will likely bring more contaminating proteins from the bottom to the top, leading to a lower purity. The PF of the volume ratio 1:0.85 was the highest, despite its RC being slightly lower. This volume ratio was selected for the further experiments.

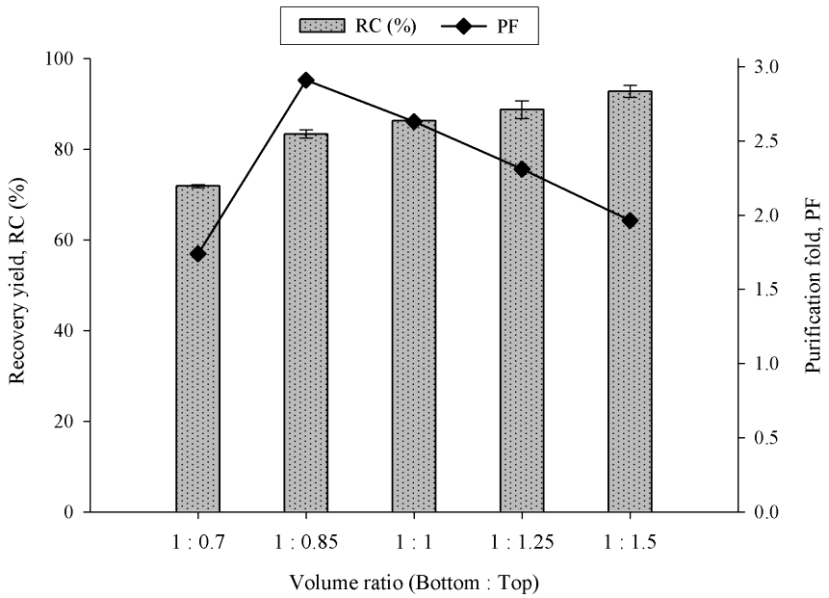


Figure 5.4: Effect of volume ratio on the C-PC recovery yield and purification fold from *Spirulina platensis*.

5.4.5 Effect of pH

The extraction pH is one of the vital parameters that produces substantial effects on the efficiency of C-PC separation. The changes in the pH value of the system can affect the net charge of C-PC, the charge distribution on the C-PC surface, zeta potential and conformational structure change (Chang et al., 2018). The pH of the system was adjusted by using various combination of potassium salts to make a final pH in the range of 6-9. The pH values range were selected based on the isoelectric point of C-PC, which is 5.8, as there needs to be a balance between the positive and negative charges of the ionic groups of a protein (Antelo et al., 2010). Figure 5.5 shows the influence of pH of the system on the RC and PF of C-PC partitioning. The highest C-PC recovery and purity was observed at pH 7, with pH 8 and 9 showing comparable purity but slightly lower yields. pH 6 showed the lower yield and purity of the C-PC. The partitioning of C-PC to the top of the system depicts that C-PC is more soluble in the PEG rich phase, where it will be more concentrated depending on the charge-charge interaction. The pH value higher than the pI of C-PC with negative charge will make the C-PC favour the top polymer phase, while the other proteins with positive charges will partition to the bottom phase. Patil and Raghavarao (2007) specified that at pH 6, the partitioning of C-PC was due to the surface properties rather than net charge as the difference of isoelectric point of C-PC and the pH was very small. While for pH of 7 and 8, the C-PC would be negatively charged and thereby attracted by the PEG-rich phase (Patil & Raghavarao, 2007). This means that C-PC would get partitioned to the top phase and the contaminating proteins would go to the opposite phase (bottom phase). Further increase in pH to 9 will lead to C-PC denaturation and conformational

changes, where C-PC is mostly found to be stable at pH of 6 to 8 (Antelo et al., 2008). Higher pH will also increase the free volume in the top phase, allowing more contaminating proteins to go to the top. The pH of 7 proved to be the best in terms of RC and PF of C-PC and hence, this pH was selected for the subsequent experiment.

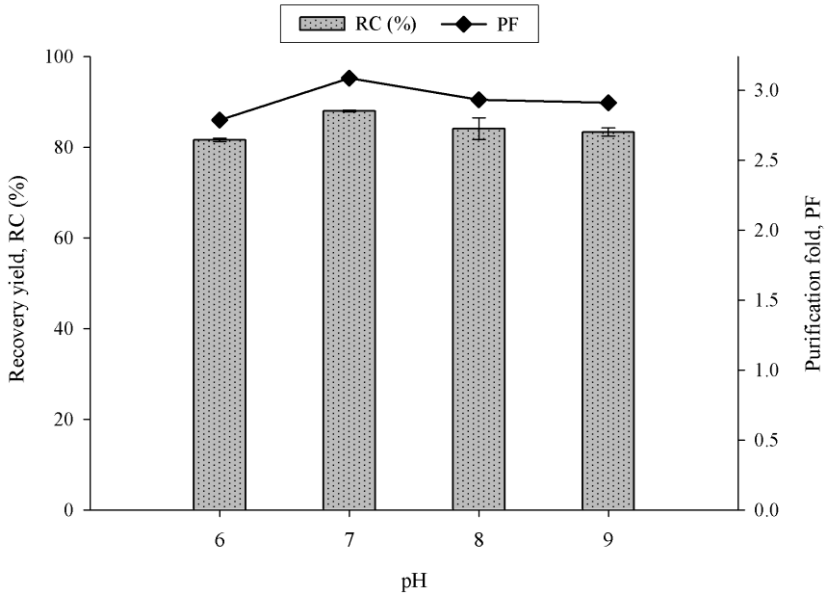


Figure 5.5: Effect of pH of the system on the C-PC recovery yield and purification fold from *Spirulina platensis*.

5.4.6 Effect of air flotation time

The air flotation time contributes significantly in the LBF system as this parameter affects the area of air-water interface per unit volume of aqueous solution in time (Sankaran et al., 2018b). Different durations of flotation time from 1.5 min to 9 min were evaluated for the LBF system on C-PC partitioning. Figure 5.6 shows the influence of air flotation time of the system on the C-PC recovery yield and PF. The yield of C-PC increases as the flotation time increased from 1.5 min to 5 min, then the yield drops slightly after 5 min of

flotation time. The purity of C-PC also observes the same trend, increasing up to 7 min and dropping significantly at 9 min. The highest RC (88.0%) was found at 5 min with PF of 3.09, while the highest PF (3.16) was found for 7 min, with a RC of 86.9%. Increasing the flotation time increases the transportation of the C-PC from the bottom phase to the top phase, however, too long a duration will also encourage the partitioning of contaminant proteins to the top. Lower flotation time are insufficient for effective C-PC partitioning to the top phase, while higher flotation time showed comparable yields but a reduction in purity of C-PC. Air flotation time of 7 min showed the highest purity and comparable C-PC yield with the initial setting of 5 min, hence 7 min was selected as the suitable flotation time for the next experiment on biomass concentration, where extension of the flotation time beyond 7 min was deemed unnecessary.

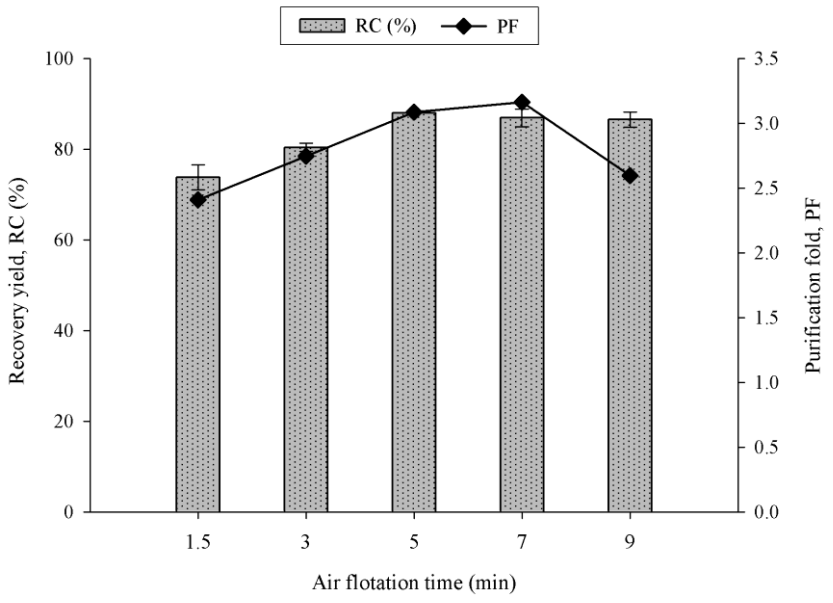


Figure 5.6: Effect of air flotation time (min) on the C-PC recovery yield and purification fold from *Spirulina platensis*.

5.4.7 Effect of crude extract concentrations

The partitioning behavior of the proteins will be affected by the amount of feedstock in the system. Crude extract concentrations which are too high will result in a more viscous solution which may cause difficulties in the separation process, while lower crude extract concentration makes the process less effective as the total product recovery would be lower (Sankaran et al., 2018a). The crude extract concentration was investigated in the range of 0.25 to 0.75 %w/w. From Figure 5.7, the concentration of 0.625 %w/w gives the highest RC (90.4%) and PF (3.49). The increase and decrease in crude extract concentration both showed increasing C-PC recovery but lower purity. The lowest yield and purity was found for concentration of 0.375 %w/w. At lower crude extract concentration, there will be more space available for both C-PC and the undesired proteins, hence the yield is higher but a drop in purity was observed. Lower crude extract concentration is also not favourable as the production output of C-PC will be lower and this makes the purification process less cost-effective. With increasing crude extract concentration, the yield of C-PC increases, and the purity of C-PC increases as well up to 0.625 %w/w, then it decreases at 0.75 %w/w. The higher concentration of C-PC with higher crude extract concentration requires more work from the air bubbles to transport the C-PC to the top phase. As both the C-PC and contaminating proteins are present in large volumes, higher proportions of the proteins will be transported to the top phase, leading to a lower purity. In this study, the crude extract concentration of 0.625 %w/w showed the best RC and PF, compared to the initial setting of 0.5 %w/w. This is likely due to the available free volume in the top phase that can accommodate additional C-PC content, resulting in a slightly

higher C-PC yield and purity in the top phase. The crude extract concentration of 0.625 %w/w was selected as the most suitable condition for the LBF study.

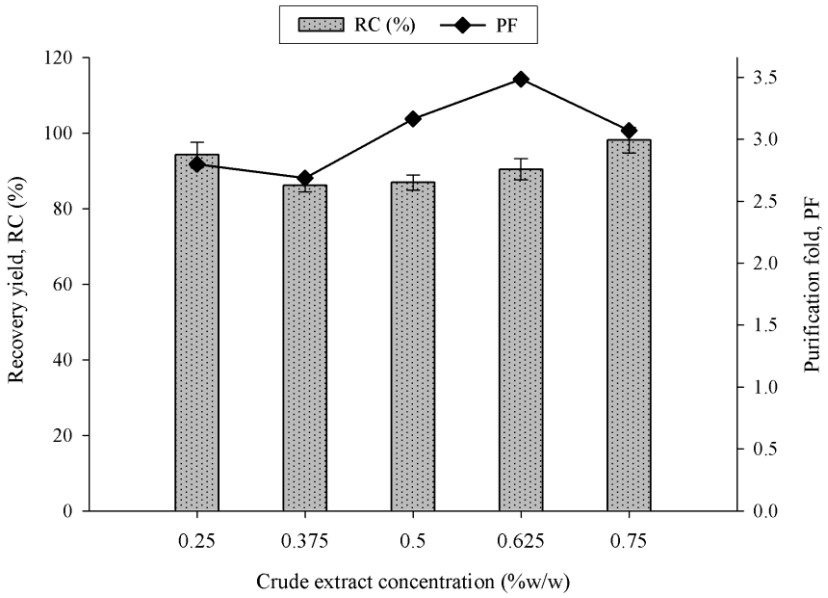


Figure 5.7: Effect of crude extract concentration (%w/w) on the C-PC recovery yield and purification fold from *Spirulina platensis*.

5.4.8 SDS-PAGE

SDS-PAGE analysis of C-PC purity from the crude extract of *Spirulina platensis* was performed to determine the molecular weights of the C-PC subunits. The purified top phase of the LBF system were subjected to gel electrophoresis as shown in Figure 5.8. Lane A indicates the molecular marker, lane B indicates the crude extract of C-PC and lane C shows the C-PC after LBF process. From the SDS-PAGE, the C-PC shows two bands in the range of 18 and 20 Da. After LBF, the majority of the contaminant proteins present in the crude extract have been partitioned to the bottom phase, resulting in less bands in the lane C compared to Lane B. This shows the potential of LBF for the effective purification of C-PC from microalgae.

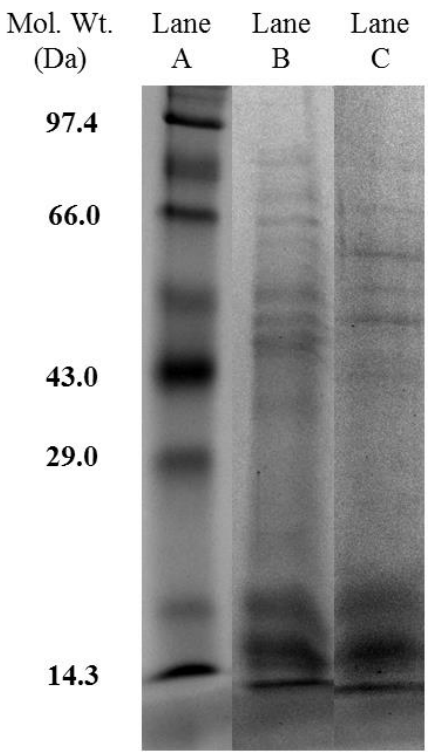


Figure 5.8: SDS-PAGE of C-phycocyanin: Lane A indicates the molecular marker; lane B indicates the crude extract of C-PC; and lane C shows the C-PC after LBF process.

5.5 Conclusion

The purification of C-phycocyanin from *Spirulina platensis* was performed using LBF, which is a combination of LBS and solvent sublation with the incorporation of bubbling effect into the system. The optimized conditions for the LBF was found to be: PEG 4000 and potassium phosphate combination, polymer and salt concentration of 250 g/L each, bottom to top volume ratio of 1:0.85, system pH of 7.0, air flotation period of 7 min and crude extract concentration of 0.625 %w/w. The maximum C-PC recovery and purification fold of the optimized LBF system was found to be 90.4% and 3.49, respectively. The next chapter (Chapter 6) studies the combination of both the food waste compost used for cultivation and dairy waste for the pelletization of compost.

CHAPTER 6

DENSIFICATION OF FOOD WASTE COMPOST: EFFECTS OF MOISTURE CONTENT AND DAIRY POWDER WASTE ADDITIVES ON PELLET QUALITY



This Chapter covers the application of dairy powder waste additives for the pelletization of food waste and green waste compost. The prospects of utilizing waste additives as a binder for pellet quality and production enhancement was investigated. The physical properties and surface characterization of the compost pellets produced were evaluated to determine the most suitable moisture content and additive addition percentages. This Chapter consist of a thesis-version of the manuscript published in the Process Safety and Environmental Protection journal (Chew K.W. et al., 2018, *Process Safety and Environmental Protection*, 116: 780-786).

Densification of food waste compost: Effects of moisture content and dairy powder waste additives on pellet quality

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Authors' Contributions

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

6.1 Abstract

Densification of food waste compost through pelletizing is essential to increase the bulk density, expand its storability, provide ease of transportation, as well as to enable easier handling of the compost. Compost in its natural form takes up a lot of space and has a high powder dispersion rate which makes it less safe and difficult to handle. The compression of these composts into pellet forms will reduce the managing issues associated with the natural compost feedstock. In this study, the pelletizing of food waste compost by adjusting the moisture content and dairy powder waste addition was performed to evaluate the pelletization performance. The physical and mechanical properties such as moisture content, bulk density, producibility, particle density, compressive strength and moisture resistance were evaluated for the pelletized compost products. This research indicates that the addition of dairy powder additives containing lactose is very beneficial in improving the pellet production rate as well as the properties of the pellets. The pellets with lactose addition showed better producibility, better compressive strength and higher density. The presence of more solid bridges was also observed with the addition of lactose additives which crystallize and lead to stronger bonds within the pellet.

Keywords: Compost pellet; food waste compost; lactose additive; moisture content; pelletization

6.2 Introduction

A major fraction of the total municipal solid wastes disposed consists of food waste. These food wastes are disposed in landfills or incinerated, and the increasing generation of food waste has created an unsustainable situation of this disposal method due to the environmental impacts incurred. Many countries have explored into alternative waste management methods that are more environmental friendly which have the ability to produce value-added product through waste conversion (Evangelisti et al., 2015; Thi et al., 2015; Tumwesigye et al., 2016). One such method is composting, which is a biological process that is performed by the degradation of organic matters under aerobic or anaerobic conditions. The products from composting are the compost or organic amendments, and biogas that contain mainly methane and carbon dioxide. This composting process is very efficient for the bioconversion of food waste into valuable fertilizer and is promising as an alternative for the management of food waste (Cerda et al., 2018; Lou et al., 2015). Composting provides the benefit of food waste utilization which would otherwise end up in landfills and it can process the food waste into low cost amendments which will help in balancing the nutrient content for plant growth. Composting also has the potential to sequester carbon and this could lead to the mitigation of climate change (Asses et al., 2018; Mohammadi et al., 2017).

Some of the challenges in managing compost include the handling, application and storage of compost. Compost generally have low density and can take up a lot of storage space, hence, the densification of these compost would helped in reducing the storage capacity needed. Furthermore, a hazard posed by compost handling is the dust dispersion which can potentially carry

pathogens and toxic organics and if inhaled, could cause respiratory problems. The densification of compost is required to overcome these hazards, where an example of densification process is pelletization. Pelletization is the process of intensifying the bulk density of biomass through mechanical pressure (Pampuro et al., 2017). The purposes of pelletizing compost are to produce pellets which are the densified form of compost and to obtained high quality products. Several types of pelleting machines can be found on the market such as diskpelleter or extruder type. The diskpelleter type uses a rotating disk and a die to force composts through the holes in the die. Examples of diskpelleter types include roller disk die type, roller ring die type and double die type. For the extruder type pelleter, the compost material would be compressed through a die installed at the end of the machine to produce pellets (Zafari & Kianmehr, 2014). The compost should not contain metal debris or stones as these might cause damage to the machines, creating higher production costs for repairs and maintenance works. Compost pellets produced will be durable and sufficiently strong to withstand transportation to different areas as well as to be applied efficiently by agricultural machines.

The quality of the pellets is mainly dependent on the types of raw materials used, size and power of the pelletizing machine and characteristics of the compost (Castellano et al., 2015; Lehtikangas, 2001). The addition of excess bulking agents like sawdust can also reduce the processing speed and strength of the pellets (Pampuro et al., 2017). Besides that, the pellets are costly to produce and should contain a stable and certain amount of nutrients, in order to produce high quality pellets (Hara, 2001). The benefits of compost pellets are mainly the compactness of the pellets which will contribute to the conservation

of space. Compost pellets are densified and contain more mass per volume compared to the original compost. The compression of compost into pellets reduces their volume, making them more suitable for transportation to long distances. Pellets also produce less dust and pollution to the environment, enabling them to be used around residential areas. The characteristics of the compost also plays a major role in producing good pellets. Pellets are usually uniform in size and quality, they can be applied by agricultural machineries for implanting and scattering. Their increase in strength also helps avoid the crumbling of the pellets during distribution. When compost pellets are applied to the soil, the breakdown of the pellets will be slower compared to ordinary compost, as it takes a longer time for the distortion and decomposition of the pellets. This is advantageous if the raw compost is immature as the negative effects caused by the rapid decomposition of ordinary organic matter to plants can be avoided. Nonetheless, the fertilizer efficiency of compost pellets is highly dependent on the nature of the compost used as well as the raw materials and soil conditions (Hara, 2001).

The moisture content is a significant factor as it can affect the strength and processing speed of the pellet production. As the compost would pass through the holes of the die, the frictional resistance will increase, hence, higher moisture content would be needed to enhance the fluidity of the compost, allowing it to pass through the holes smoothly. Insufficient moisture content will tend to lead to a blockage in the molding die, requiring considerable repair works such as electrical drilling to remove the blocked compost. Moreover, various additives can be added during pelletization to enhance the quality of the pellets (Mavaddati et al., 2010; Zafari & Kianmehr, 2014). A potential additive

that can be added is lactose. Lactose is a disaccharide sugar found usually in dairy products and has been classified as safe for utilization in drug delivery and pharmaceutical applications (Gutiérrez et al., 2012; Zhou & Morton, 2012). Lactose has been used as a binder for tablet production where it has been reported to form a binding layer on the particle surface area (Vromans et al., 1987). Tonnes of excess dairy products are wasted down the drain annually, where these products are derived from agriculture, post-harvest, processing losses, distribution and consumption wastage. Reutilizing these dairy products as binders for organic fertilizer enhancement is certainly a better and more environmentally friendly solution which can contribute to resource recovery as well as effective waste management (Kasmi, 2018; Mahboubi et al., 2017).

The aim of this study was to investigate the effects of moisture content and dairy powder additive addition on the densification of food waste compost. The idea of this study was derived from the remaining food waste compost solids that could be obtained from the cultivation of microalgae. In Chapter 3, food waste compost was used as an alternative nutrient source for microalgae cultivation. However, only the liquid medium from the compost was utilized and the remaining compost solids, despite being lower in nutrient content, could still be pelletized and used as an organic amendment rather than disposing them as a solid waste. Hence, this study explores the combination of food waste compost and diary powder waste for the densification process. New compost sources were used in this experiment. The major component in the dairy powder waste is lactose which has been found to contain binding properties that might help in the pelletization of the compost. The physical properties of the compost were initially determined to evaluate its suitability for pelletizing. The compost

pellet products were then characterized physically and mechanically to observe the pellet density and compression strength, which are factors that affect the pellet quality during handling and storage.

6.3 Materials and Methods

Food waste co-composted with green waste compost was provided by the Campus Services of University of Nottingham Malaysia Campus, and partly supported by Impact: The Nottingham Campaign, under the Cascade grant: Implementation of Green Campus Model. The compost was generated using the aerated windrow composting method and produced using food waste obtained from the cafeteria in the campus. The aerated windrow composting allows for large volumes of compost to be generated through aerating the compost piles periodically. Green waste was shredded using a garden wood chipper (SIMA, Malaysia) and placed as the base while food waste was added for the enrichment of nutrients in the compost production. During the incubation period of the compost, the compost piles was turned manually thrice every week to ensure good aeration. The addition of effective microorganisms was performed to aid the decomposition of the organic materials. The compost piles were provided with freshwater to regulate the moisture content, in the event of dry season. The composting process was performed for 4 months and then harvested and stored in a 500-gallon water tank for maturation, cooling and drying before grinding using a feed grinder (SIMA, Malaysia). The grinded compost was used as the compost for the experiments.

6.3.1 Physico-chemical characterization of food waste compost

The elemental composition (C, H, N, S) of the compost samples was determined by using an elemental analyzer (LECO CHN628S, USA). The moisture content (MC) of the compost was determined according to the American Society of Agricultural and Biological Engineers (ASABE) standard S358.2 (ASABE, 1988), where the compost samples were placed in an oven for drying at 103 °C for 24 h. Moisture content was taken as the percentage of the dried compost weight over the initial compost weight. The bulk density of the compost samples was determined using the ASABE standard S269.4 (ASABE, 2007). The bulk composts were poured into a cylindrical container with a diameter of 140 mm and height of 190 mm with total volume of 0.0293 m^3. The composts were poured at a height of 200 mm from the top edge of the container and the excess material on the top surface was levelled. The weight of the container with compost was weighed. The weight of the sample was taken as the total weight subtracting the weight of the empty container. The bulk density was given by the mass per unit volume (kg/m^3). Each measurement for bulk density was repeated five times and the mean values were reported. The particle size distribution of the compost was determined by using a sieving machine. A set of 4 sieves with mesh sizes 5, 2.36, 1.6 and 0.5 mm were arranged from the largest to the smallest in the sieve, with a collecting container at the bottom. 100 g of compost was placed on top of the sieves and the compost samples were sieved for 10 min. Each fraction of the sieves were collected and weighed. The sieving tests were replicated three times.

6.3.2 Preparation of compost for pelletization

A roller rotating pelletizing machine was used to make the compost pellets. The die inside the pelletizer is powered by an electric motor, where the die rotation will initiate the rotation of the rollers which will force the compost materials and compress it through the die holes to form pellets. The pellets which emerge from the die holes are sliced with cutting knives in order to obtained pellets of acceptable length (< 40mm) and the pellets are collected in a basin. The fresh pellets leaving the pelletizer are usually at high temperatures and was left to cool for 2 hours. Cooling stage is essential to improve the mechanical durability and reduce moisture content in the pellets (Protic et al.).

To determine the suitable moisture content and additive addition for the best compost pellets production, the compost samples were added with different amounts of water and additives. Based on the initial MC value obtained from the physical characterization of compost, water was added into the compost samples to make the final MC of 30%, 35% and 45% (equation (1)). The additive was added into each MC in 0%, 10% and 20% in terms of the mass of the water added. For samples with additive addition (AA), the additives were first added into the water at 50 °C and mixed thoroughly. The additive mixture was then poured and mixed with the compost samples. The compost samples mixed with water were then packed in separate bags and stored in a cooler at 4 °C for at least 72 hours. 500 g of compost samples were used for each treatment method and the compost pellets produced were dried in an oven at 55 °C for three days before storage.

$$m_w = \frac{m_i\left(MC_D - MC_i\right)}{100 - MC_D} \qquad (1)$$

Where

m_w = Mass of water to add (g)

m_i = Initial mass of samples (g)

MC_D = Desired moisture content of sample, wet basis (%)

MC_i = Initial moisture content of sample, wet basis (%)

6.3.3 Physical characterization of compost pellets

6.3.3.1 Pellet production

The formation of good pellets acts as an indication to observe the effects of moisture content and additive addition on the compost pellets production. The pellets produced by the roller rotating pelletizer were collected, cooled and dried in an oven at 55 °C for three days. The initial pellets weight was recorded and the pellets were sieved through a 2.36 mm mesh size sieve. Good and acceptable pellets were the pellets remaining after sieving and calculated as the percentage of pellets remaining with respect to the total initial pellets weight after pelletization.

6.3.3.2 Pellet density

The pellet density was determined by measuring the pellet's weight and volume. The pellets were of cylindrical shape and the height and diameter of the pellets were measured using a Vernier caliper (Mitutoyo, Japan). The weight of the pellets was determined with a digital balance with an accuracy of 0.01 g (Mettler Toledo, Switzerland). The density of the pellets were the taken as the weight over volume of the pellets and are calculated using equation (2) and (3). The average pellet density value was taken from at least 10 tests of repetition.

$$V_p = \frac{\pi}{4} d^2 H \quad (2)$$

$$\rho_p = \frac{m_p}{V_p} \quad (3)$$

Where

V_p = Volume of pellet (m^3)

d = diameter of pellet (m)

H = Height of pellet (m)

ρ_p = Density of pellet (kg/m^3)

m_p = mass of pellet (kg)

6.3.3.3 Compression test

Compressive strength represents the force needed to cause a deformation on the pellet and can be related to the pellet resistance during storage. The compressive strength of the pellets were determined using an Instron Universal Testing Machine (Instron, USA). The diameter and height of each pellet was measured before compression. The pellets were laid vertically on the plate and compressed individually in the axial direction until reaching the breakage of the pellets. A load cell of 5 kN was used with a constant displacement rate of 1 mm/min. The compressive strength (N) of the pellet was taken as the maximum force recorded during the compression of the pellet before breakage. The test were repeated five times, using pellets of equal height to produce the similar conditions.

6.3.3.4 Wettability Index (WI)

Wettability index describes the moisture resistance of the samples. The pellet was weighed and then submerged in water for 30 s. After submerging, the pellet was reweighed. WI was taken as the percentage of the difference in weight of the pellet before and after submerging from the initial weight of the pellet before submerging (equation (4)). The tests were repeated five times.

$$WI = \frac{m_f - m_i}{m_i} \qquad (4)$$

Where

m_f = final weight of pellet after submerging in water (g)

m_i = initial weight of pellet before submerging in water (g)

6.3.3.5 FESEM analysis

A Field Emission Scanning Electron Microscope (FESEM, Quanta 400F) (FEI, USA) analysis was performed to study the effect of additive addition on the surface morphology of the pellets. During the sample preparation process, the pellets of 35%MC with 0 and 20%AA was selected for viewing. A total of four samples were analysed, pellets with two different additive addition percentage (0 and 20%) and pellets in different conditions (uncompressed and compressed). The compressed pellets were subjected to compression of 20% their original height using a load of 5 kN.

6.4 Results and Discussion

6.4.1 Physico-chemical characterization of raw material

The physico-chemical characteristics of the food waste compost are presented in Table 6.1. Moisture content is a key factor that can significantly affect the operational properties of the pelletizer as well as the strength and formation of the pellets produced. The initial MC of the compost was found to be 18.44 ± 0.29% wet basis. This moisture range is not suitable for pelletizing as it is too dry and will likely cause the pelletizer to be clogged during the pelleting process. Hence, the addition of moisture was required in order to successfully pelletized the compost, where the moisture content of 30 to 40% was selected for this experiment. The bulk density of the compost was 218.0 ± 8.5 kg/m^3. An optimal range of particle size is recommended for producing pellets of acceptable quality. Tests have shown that samples with particle size larger than 1.5 mm are unable to produce good quality pellets (Zafari & Kianmehr, 2014). Approximately 70% of the particle size from the compost falls within the range of 1.6 mm and this indicates that the compost is able to produce pellets of acceptable quality. Based on the elemental analysis performed, the carbon-to-nitrogen ratio (C/N) of the compost was found to be less than 10. The low C/N ratio signifies that the compost is readily matured and can provide good mineralization in the form of accessible nitrogen for the plants to uptake. On the contrary, a high C/N ratio will promote nitrogen immobilization, which means that the accessible nitrogen would be taken up by microorganisms preventing the plants from absorbing them (Al-Bataina et al., 2016; De Neve et al., 2004).

Table 6.1: Properties of food waste compost.

Properties	**Food waste compost**
Moisture content (%wt)	18.44 ± 0.29
Bulk density (kg/m^3)	218.0 ± 8.5
Particle size distribution (%)	
< 0.5 mm	31.41 ± 0.32
< 1.6 mm	42.03 ± 0.85
< 2.36 mm	13.71 ± 0.76
< 5 mm	10.48 ± 0.88
> 5 mm	2.37 ± 0.53
Ultimate analysis (%)	
Carbon	24.16 ± 0.62
Hydrogen	3.79 ± 0.01
Nitrogen	2.54 ± 0.01
Sulfur	0.56 ± 0.02

6.4.2 Effect of MC and AA on pellet production

The moisture content is an important factor that affects the producibility and compressive strength on the pellets. Figure 6.1 shows the results for the producibility of good pellets with varying MC and AA. An increasing good pellets production rate was observed with increasing additive addition, with the highest rate of each MC being 69.0%, 88.6% and 87.1% for 30%, 35% and 40%MC, all with 20%AA, respectively. The increase in MC is likely to make the materials more flexible and easier to be pushed through the die during pelletization (Filbakk et al., 2011), hence increasing the MC from 30% to 35% showed great increase in the pellets production. However, higher MC will create pellets which are soft and break more easily, hence, further increase in the MC from 35% to 40% showed a slight decrease in good pellets production, where

more of the pellets produced were shattered and excluded through sieving. The MC range of 30 to 50% has been regarded as the suitable MC which can promote the pellet forming process and produce homogenous pellets (Pampuro et al., 2017; Yilmaz et al.; Zafari & Kianmehr, 2014). For this research, the MC range of 30 – 40% was selected for the pelletizing of the food waste compost. When the moisture content was increased further above 40%, it was found that the fresh pellets produced were moist and much softer and could be compressed by using just the hands, hence, the higher limit of 40%MC was taken for this experiment. MC of lower than 30% was found to have difficulties in producing much good pellets and has even caused the pelletizer to be blocked easily as the materials were too dry. The optimum MC for densification process is dependent on the type of material and processing conditions (Zafari & Kianmehr, 2014), so the range of 30 - 40% was selected as the basis for compost pelletization in this research. MC ranges of 15% to 60% were also used in various other pelletizing processes using compost or wood blends as feedstock (Alemi et al., 2010; Monedero et al., 2015; Zafari & Kianmehr, 2014). The MC of 35% with 20%AA showed the best producibility rate for pellets production.

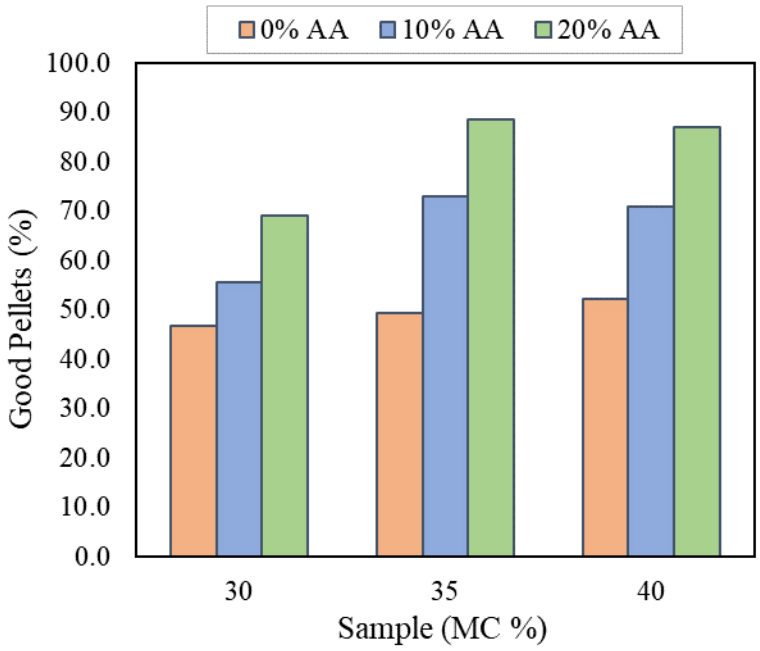


Figure 6.1: Effects of MC and additive addition on the pellet production.

The major compound in the dairy powder additive is lactose which comprises of more than 65% of the total composition, where the remaining composition constitutes of minerals found in dairy products such as iron, zinc, vitamins and others. The rate of additive addition was selected as 0 – 20% to provide additional micronutrients during the fertilization. Too much nutrient would result in over-fertilization of the soil and hence 20% was set as the limit for nutrient content addition (Gupta et al., 2008). The addition of dairy powder additive showed an increasing trend with increasing additive concentration across all MC. The increase of AA from 10 to 20% showed almost two times the increased pellet production from 0 to 10%AA. Lactose is very ideal as a binder as it shows stickiness properties which can help bind the fine particles in the compost (Zhou & Morton, 2012). Lactose will crystallize when heated and dried and the crystallization of the lactose will form compost pellets that are more firm and dense. The binding capability of lactose is also likely to produce

pellets of higher compressive strength, while requiring only a low dosage of the binder (Srivastava et al., 2013). This shows great potential in the application of dairy powder wastes which contains valuable minerals for use as an additive and binding agent in compost pellets production for plant growth.

6.4.3 Densification of compost pellets

The bulk density is important for the development of the handling and storage procedures in order to preserve the pellet integrity (Jiang et al., 2014; Pampuro et al., 2017). Figure 6.2 shows the pellet density for each of the treatments. The results from the pelletization shows the particle density of around 1116.8 – 1169.2 kg/m^3, 1076.1 – 1178.7 kg/m^3 and 1106.2 – 1154.0 kg/m^3 for 30%, 35% and 40%MC respectively. The high density of the pellet is likely attributed to the small particles size of the compost material which increases the pellet density during pelletization (Pampuro et al., 2017). The addition of the additive does affect the pellet density, though only a small increase in density was observed with the lactose binder addition. The binding effect of the lactose additive helps to bind the particles within the pellet, preventing the volumetric expansion of biomasses which is likely to occur during pelletization (Stelte et al., 2012; Zaini et al., 2017). The density of the compost pellets in this research is quite similar with the values reported by Zafari and Kianmehr (2014) for biomass pellets from compost (1038 – 1324 kg/m^3), which was produced using a hydraulic press and a single pelleter (Zafari & Kianmehr, 2014). Other studies on the pelletization of cow manure compost using single pelleter also produced pellets having density of about 1200 – 1300

kg/m^3 (Alemi et al., 2010), and the pelletization of woody biomass and agri-food wastes using flat die pelleter obtained pellet density of 950-1105 kg/m^3.

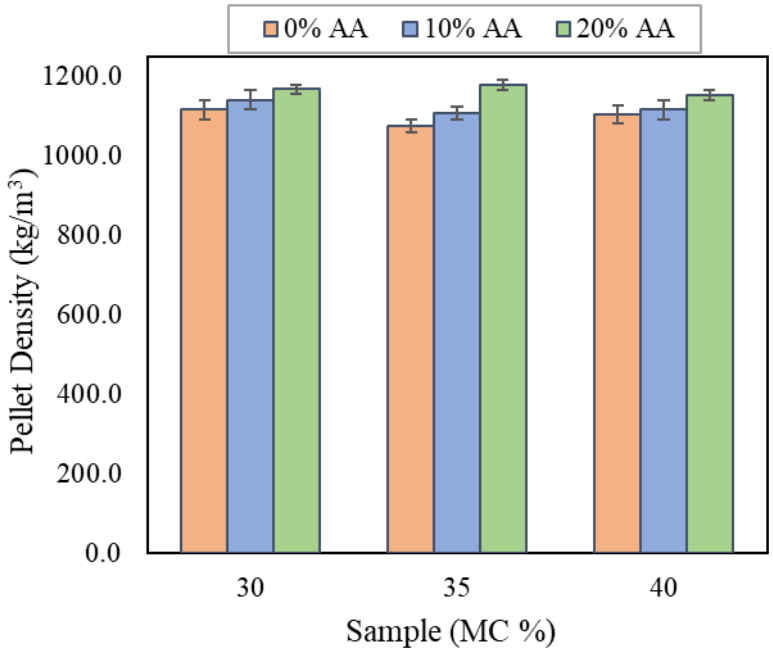


Figure 6.2: Effects of MC and additive addition on the pellet density of the compost pellets.

The density of the pellets produced could also be affected by the pelletizing process through the variation in material flow rate through the pellet die. It is not unusual that certain portions of the compost may retain in the die holes longer, since the compost is not homogenous in nature. Composts that flow through the die holes faster will likely be less dense with slight ruptures on the surface, while composts that retained in the die holes longer will produce pellets of more dense and smoother surface (Mišljenović et al., 2016). But composts that remain in the die chamber for too long might be burnt as the temperature in the chamber increases rapidly during the operation of the rotating pelletizer. To avoid this variation in pellet production, the compost materials

were filled into the chamber at an almost similar rate to try and present an equal condition. The compost pellets in this research were successfully densified by a scale of 5.07 to 5.41 times its bulk density (218.0 ± 8.5 kg/m^3), which is very promising for saving storage capacity. The densification process is very helpful in allowing more compost volume to be transported in one go due to the reduction in storage space needed, densified composts are also easier to handle and stored.

6.4.4 Compressive strength of compost pellets

The compressive strength tests indicate the maximum crushing load that the pellets can withstand before cracking or breaking. This will evaluate the compression resistance of the pellets during storage, crushing or transportation (Kaliyan & Morey, 2009). Figure 6.3 shows the compressive strength of the pellets from different treatments. An increasing trend in the compressive strength is seen for all MC with increasing AA. The addition of 20%AA showed the highest compressive strength for all MC, with the highest strength in each MC being 270.4 N, 297.1 N and 286.0 N for 30%, 35% and 40%MC, respectively. The highest compressive strength was found for 35%MC with 20%AA although it is quite comparable to 40%MC with 20%AA. It is apparent that the strength of pellets tends to decrease with increase in MC, as the compactness of the pellet products decreased as MC increased. In contrary, the porosity and particle density of the pellet would increase with MC, creating a more porous structure which is less resistance to compressive forces (Alemi et al., 2010; Monedero et al., 2015). The addition of lactose additive caused a more solid bridge bonding force among the particles, leading to the production of

stronger pellets. The results of the additive incorporated pellets showed an increase of about 18 – 28% times the compressive strength of the pellets without additives. The changes in MC do not show much effect on the compressive strength of the pellets and the values are all above 200 N, which is higher than compost pellets formed using municipal solid wastes (44 - 113 N) (Zafari & Kianmehr, 2014) and quite similar to pellets made from composted pig solid fraction with biochar (200 – 400 N) (Pampuro et al., 2017).

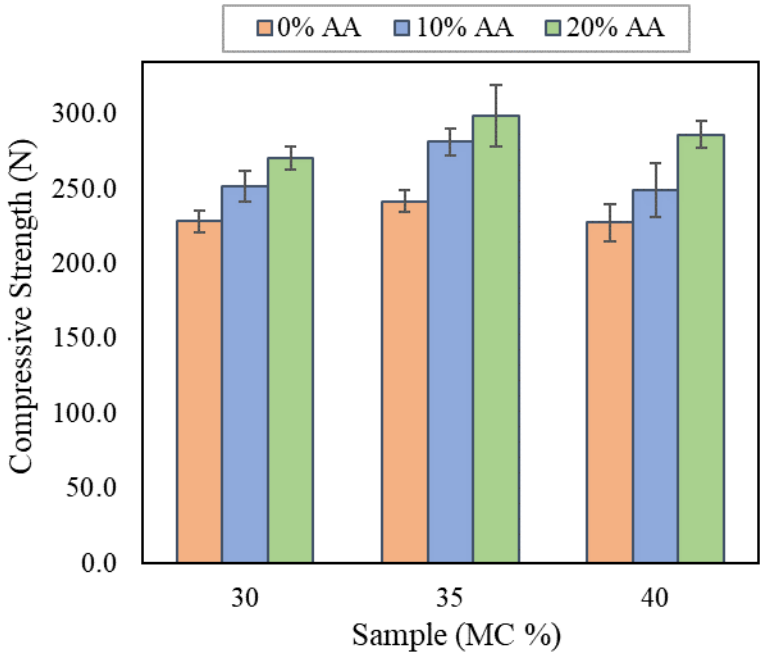


Figure 6.3: Effects of MC and additive addition on the compressive strength of the compost pellets.

A slightly higher variation in the results for each individual treatment was found as the compost materials used in each batch processing may differ in terms of composition and particle size distribution, since it is difficult to obtain homogeneity in the raw materials used for each separate pelletizing. Apart from that, the particle size of the compost material also plays a major role in the pellet

strength. Smaller particle size has been found to correspond to better pellet strength as there will be greater surface area for moisture addition and hence enhance the binding effect of additives (Zafari & Kianmehr, 2014; Zaini et al., 2017). The particle size of the compost used in this experiment consisted of more than 70% of particle below 1.5 mm. The remaining portion consisted of mainly leaves and branches which were not shredded into finer portions. It is possible that if the food waste compost were grinded into much finer particles or if only the sieved portion of the compost within the suitable range of particles size (<1.5 mm) was used for pelletizing, the pellets produced will very likely have a higher density as well as pellet strength.

6.4.5 Wettability Index of compost pellets

The wettability index (WI) of the pellets need to be taken into consideration as high moisture content are the primary causes of the pellet degradation. Depending on the biomass used for pellets production, the pellets will tend to absorb moisture if they are placed in wet or humid conditions. Prolonged absorption of moisture will weaken the strength of the pellets and promote fungal growth, leading to the decomposition of the pellets during storage (Kambo & Dutta, 2014). The wettability index values for the compost pellets are between 0.181 and 0.197 (Figure 6.4). This indicates that the pellets can absorb up to about 20%MC if contacted with humid conditions. Slightly lower WI was observed for 35% and 40%MC as these samples already contain more moisture compared to the 30%MC samples. White wood pellets made from virgin softwood could absorb up to 20.3% moisture (Graham et al., 2016) while pellets made from compost could absorb up to 18% moisture (Zajonc et

al., 2014). The strength of the pellets was found to reduce significantly after the pellets were immersed in water. When water is absorbed into the pellets, the pellets will crumble more easily as they are made of a major portion of green waste which is likened to soil that will soften when moistured. The WI results shows that the compost pellets can sustain an adequate period without deteriorating and should be stored in closed warehouses or roofed conditions before being utilized.

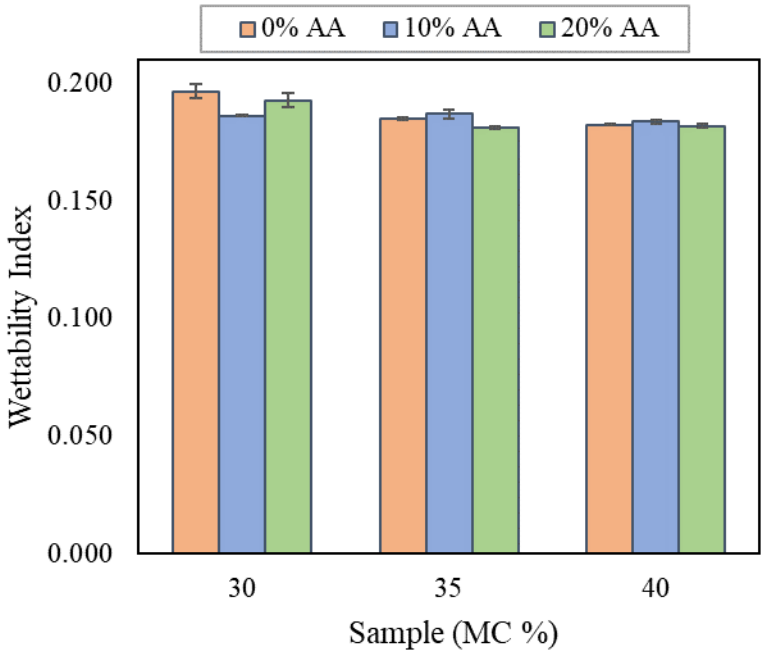


Figure 6.4: Effects of MC and additive addition on the Wettability Index of the compost pellets.

6.4.6 FESEM analysis of binding mechanism

The FESEM images were taken for 35%MC as this moisture content rate yielded the highest good pellets producibility and showed the best quality among the other two MC. Figure 6.5 shows the FESEM images of the surface of the pellet samples for with and without additive samples as well as

uncompressed and compressed samples. From Figure 6.5 (a) and (b), it can be seen that there are a lot of clusters in the pellet containing lactose additives compared to the ordinary pellet.

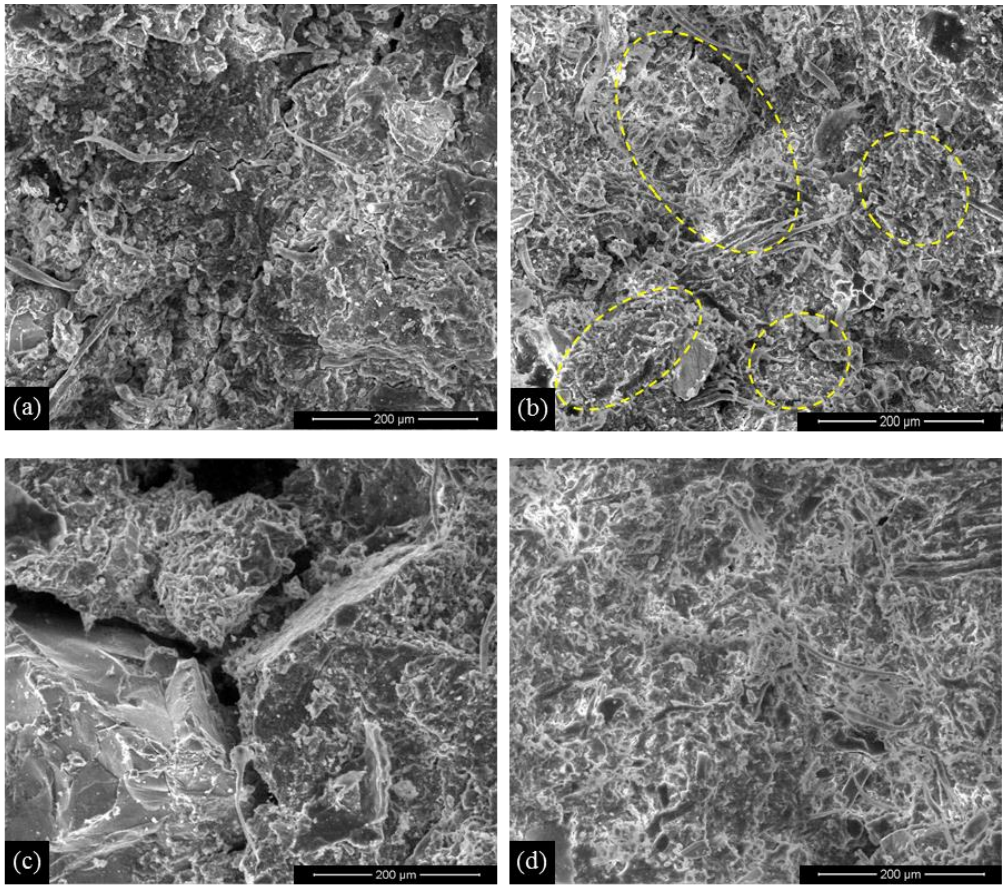


Figure 6.5: FESEM images on the compost pellet (35%MC) surface. **(a)** Uncompressed pellet with 0%AA; **(b)** Uncompressed pellet with 20%AA, yellow dotted lines represent some of the clusters; **(c)** Compressed pellet with 0%AA; and **(d)** Compressed pellet with 20%AA.

The lactose particles are well dispersed during the mixing before pelletizing and at high temperatures, the lactose particles crystallize and bind to the pellet, creating a more packed and sturdy structure. The application of high temperatures during pelletization may lead to the development of solid bridges through the interaction of the molecules (Kaliyan & Morey, 2009). These solid

bridges are formed due to the crystallization of certain materials, hardening of binders or the solidification of melted components, and are usually formed after the cooling or drying of the densified products (Zaini et al., 2017). The contents of the biomass pellets which is likely to contain lignin, together with the addition of lactose additives, has created more solid bridges and hence improve the physical quality of the pellets.

The compressed pellets for 35%MC without additive and with 20%AA are shown in Figure 6.5 (c) and (d) respectively. Larger cracks were seen in the ordinary pellet without lactose additive as there is a lack of good binding between the particles. The sticky property of lactose and its crystallization on the pellet will help in developing the inter-particle adhesion, which can lead to the formation of a better interconnected structure (Figure 6.5 (d)), enabling the pellet to withstand higher loads. The lactose additives which is slightly viscous may also adhere to the surface of the solid particles and generate strong bonds that are likened to solid bridges, these binders will harden after cooling and form the solid bridges (Kaliyan & Morey, 2009). Therefore, the addition of lactose additive exhibits great potential in the formation of dense and strong pellets.

6.5 Conclusion

The densification of food waste compost was successfully performed with the incorporation of suitable amount of moisture (30 – 40%) and dairy powder waste additives (0 – 20%). The appropriate moisture content range and feedstock materials used for pelletizing is significant to produce pellets of acceptable quality and quantity. The use of the roller rotating pelletizer was able to produce pellets at a fast rate with reasonable portions of good pellets (46 – 88%). The addition of dairy powder waste was also found to obtain higher good pellets production rate as dairy powder waste contains lactose additives which can act as a good binding agent. The effect of lactose additives addition on the physical characteristics of the compost pellets were evaluated and improved pellet properties was observed with the additive addition. The pellets produced with additive addition showed higher particle density, better compressive strength and adequate moisture resistance. The best combination of pellets production from green and food waste was found to be 35% moisture content and 20% additive addition for this experiment. These results shows the possibility of utilizing dairy powder wastes for enhancing pellet production rate through the stickiness and crystallization ability of lactose in dairy powder. Furthermore, the application of this dairy powder waste can be extended for use in pelletizing various other biomass feedstock using different types of pelleters.

CHAPTER 7

CONCLUSIONS AND FUTURE WORKS

This thesis project has demonstrated the sustainable cultivation of microalgae and its subsequent downstream processing using green and environmentally friendly techniques. This chapter summarises the research findings and achievements done throughout the study. This section also presents the recommendations for certain aspects that can be studied in the future.

7.1 Research Achievements

This thesis has accomplished the novel approaches of managing waste and resource recovery through microalgae cultivation and biorefinery. The identification of food waste compost as a potential alternative nutrient source for microalgae cultivation and the development of multiphase partitioning techniques for the biorefinery of microalgae has been successfully performed. The comprehensive description of each research achievement is stated as followed:

1) Food waste compost as an organic nutrient source for the cultivation of chlorella vulgaris
    - The prospective of substituting inorganic medium with organic food waste compost medium as a nutrient supplement for the cultivation of Chlorella vulgaris FSP-E was investigated. Various percentages of compost mixtures (15 - 100%) were replaced in the inorganic medium to compare the algal growth and biochemical composition of the

microalgae. It was discovered that the use of 25% compost mixture combination was found to yield higher biomass concentration (11.1%) and better lipid (10.1%) and protein (2.0%) content compared with microalgae cultivation in fully inorganic medium. These results exhibited the potential of utilizing organic medium in microalgae cultivation system to replace fossil-based fertilizers for a more sustainable future.

2) Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique
   - The development of a green and effective method of microwave-assisted three phase partitioning (MWTPP) for the extraction proteins from microalgae was investigated. The optimized conditions for the MWTPP was found to be: Ammonium sulphate concentration (30 %w/w), slurry to solvent ratio (1:1), microwave irradiation time (120 s), duty cycle (80%), microwave power (100W) and microalgae biomass solution (0.5 %w/w). The MWTPP successfully enhanced the protein yield by about 2.54 times to 63.2% from 24.9% obtained in conventional TPP process alone. The incorporation of microwave in TPP has contributed tremendously in the microalgae cell disruption for the release of valuable protein and other biomolecules, making MWTPP a rapid and efficient downstream processing for biomolecules extraction.

3) LBF phycocyanin

- Liquid biphasic flotation (LBF) was developed and used for the purification of C-phycocyanin from *Spirulina platensis* microalgae. The optimal conditions for the LBF system achieving C-phycocyanin purification fold of 3.49 compared to 2.43 from the initial LBF conditions was in polyethylene glycol (PEG) 4000 and potassium phosphate combination, polymer and salt concentration of 250 g/L each, volume ratio of 1:0.85, system pH of 7.0, air flotation time of 7 min and phycocyanin crude extract concentration of 0.625 %w/w. The bubbling effect in LBF has successfully improved the recovery yield and purity of C-phycocyanin and is considered a cost effective and simple downstream processing for valuable biomolecules.

4) Densification of food waste compost: effects of moisture content and dairy powder waste additives on pellet quality

- The densification of food waste compost through pelletizing to enable easier handling of compost was investigated. The densification process was performed with the incorporation of suitable amount of moisture (30 – 40%) and dairy powder waste additives (0 – 20%). The best combination of pellets production from green and food waste was found to be 35% moisture content and 20% additive addition, where the pelletizing has successfully reduced the managing issues associated with the natural compost, through increasing its bulk density, improving its storability and making it easier for transportation. This has indicated that the addition of dairy powder

additives containing lactose is very beneficial in improving the pellet production rate as well as the properties of the pellets.

7.2 Future Works

The research gaps proposed have been addressed in this thesis. Nevertheless, there are opportunities to further improve and extend these approaches to explore the remaining and consecutive research gaps.

1) Decolourisation of organic medium from food waste compost
    - The utilization of food waste compost as the medium for microalgae cultivation generates a nutrient-enriched organic medium that is dark coloured. The benefits of utilizing this organic medium for microalgae growth is then brought to a disadvantage by the inhibition of light penetration into the microalgae culture. As microalgae requires light for growth, the dark coloured solution makes it difficult to enhance the microalgae growth, despite the organic nutrient having more micronutrients compared to the inorganic medium. It is proposed as a future work to decolourise the organic medium through environmental friendly options such as co-cultivating the microalgae medium with bacteria or yeast. Certain species of bacteria and yeast have been found to successfully decolorise solutions and will not consume too much nutrients. There is also potential to utilize this organic culture medium combination for large scale microalgae biomass production.

2) Pilot study of large scale study of multiphase partitioning system

- The effective extraction and purification of proteins and pigments from microalgae have shown the potential of multiphase partitioning systems as a rapid, cost effective and green downstream process for biomolecules recovery. High recovery yield, separation efficiency and purification fold were obtained through the utilization of microwave-assisted three phase partitioning and liquid biphasic flotation. The feasibility of adapting these techniques for commercial applications can be further tested by developing a larger scale system that fits for the multiphase partitioning systems. The design and implementation of an extraction column that can accommodate the conditions needed for optimum extraction is proposed as a future work. This design can serve to examine the scale-up challenges and possibility of applying these novel multiphase techniques for marketable productions.

3) Application of compost pellets for plants growth
    - The densification of compost pellets have been successfully performed and the pellets produced have shown good physical and mechanical properties for long term storage and handling. In addition to this, the benefits of densifying the compost pellets can be further demonstrated through applying the compost pellets for crops and plants growth. The densified pellets are expected to contain more nutrients that the compost in its original powdery form. Hence, a lower amount can be used and its efficiency can be compared with the non-densified compost. The densified compost also contains added nutrients from the dairy waste that is used to improve the pellet

quality. It is proposed as a future work to test out the compost pellets for crops growth to shows its potential to supply organic fertilizers to farmers in densified pellet form.

Apart from that, there is possibility of integrating the proposed works in a complete industrial study cycle for commercialization purposes. This study cycle will start with the cultivation of microalgae biomass using sustainable organic medium, where the total raw material costs can be reduced. The microalgae biomass containing rich nutrient compounds will be harvested and next, the extraction and purification of valuable compounds from microalgae can be done using novel and effective bioprocessing techniques. These rapid, efficient and high recovery technique can produce large quantities of nutrients and bioproducts for cosmeceutical industries. This study will then be followed by the utilization of the food waste compost solid wastes from the cultivation process with dairy waste additive addition for compost pellets production. The compost pellets produced can be used for enhancing crops growth and are considered environmentally friendly as an organic amendments to soil. This study cycle has good feasibility to be applied in an industrial scale as it is cost-effective and sustainable in terms of raw materials usage and processing operation.

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APPENDIX



Figure A1: Photobioreactor with condensation column and air filter used for the cultivation of *Chlorella vulgaris* microalgae.

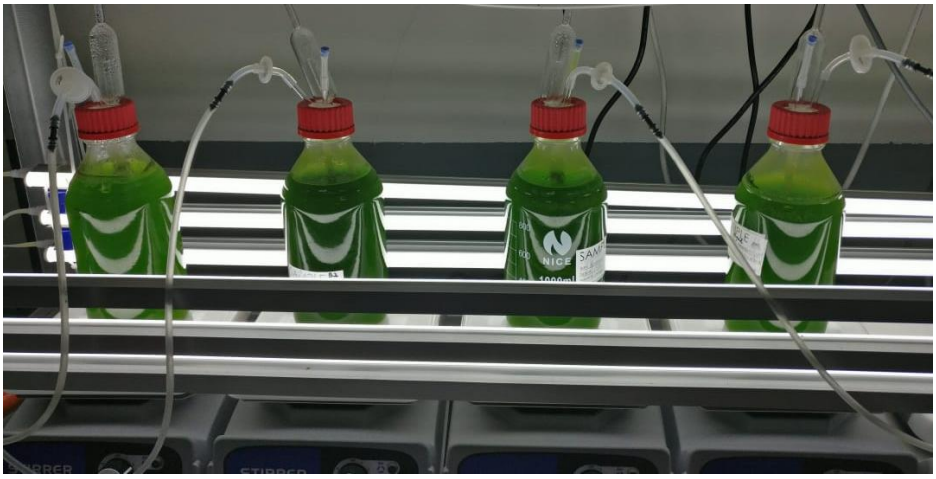


Figure A2: Multiple photobioreactor set-up used for the cultivation of *Chlorella vulgaris* microalgae to obtain biomass for experimental needs.



Figure A3: Roller rotating pelletizer used for the pelletization of food waste with green waste compost.