SURFACTANT-AIDED DISPERSED AIR FLOTATION AS A HARVESTING AND PRE-EXTRACTION TREATMENT FOR *CHLORELLA SACCHAROPHILA*

by

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DEDICATION

I dedicate this thesis to my parents, Tayser and Manal Alhattab, my brothers (Mohammed, Ahmed, and Mahmoud), my husband (Ismail Alghalayini), my niece (Minu), and my friends (Farah Hamodat and Halah Shahin). Without their support, patience and love, the completion of this work would not have been possible.

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ABSTRACT

Microalgae are a promising feedstock for the production of biodiesel and other value added products, however, this biotechnology is hampered by high processing costs. An approach for reducing these expenses is to combine two or more processes in one technique. Thus, this thesis aimed to investigate an effective means for microalgae harvest, pre-treatment and product extraction, suitable for large scale. From comparative analyses, surfactant-aided dispersed air flotation was identified as a promising microalgae harvesting technique, with lower energy requirements compared to other techniques, and with potential to also contribute to cell wall disruption. Supercritical CO_2 (SC-CO_2) lipid extraction was determined most suitable as it is considered an environmentally friendly approach which requires only limited post processing of the extract.

Experimental investigation of these techniques on *Chlorella saccharophila* showed that surfactant-aided dispersed air flotation with surfactant CTAB resulted in a recovery and enrichment ratio of 94 % and 13, respectively, with an improvement in total suspended solids (TSS) from 0.02 to 0.30% (*w/w*). Although, a secondary centrifugation step was necessary to further concentrate the biomass to a TSS of 3.6% (*w/w*), the use of dispersed air flotation prior to secondary treatment resulted in a 14 fold reduction in working volume, which is significant as it utilizes up to 500 times less energy compared to centrifugation. SC-CO_2 lipid extraction from harvested biomass resulted in maximum fatty acid methyl ester (FAME) yields of 20.4% (dcw) using a reaction time of 90 min, 3500 psi, 73°C and 89% moisture (*w/w*). However, good FAME yields (16.5% dcw) could also be obtained when the run time was reduced to 30 min and the biomass was used without drying. These conditions are more industrially favorable, as they would eliminate drying expenses and potentially reduce the operational costs by processing three times the biomass within 90 min. The presence of surfactant CTAB improved the recovery of FAME in *C. saccharophila* using SC-CO_2 extraction, and a hold time of 24 h after harvest further improved yields. This demonstrates how CTAB can simultaneously work to improve microalgae harvest and product recovery, potentially improving the process economics by process reduction.

LIST OF ABBREVIATIONS USED

ANOVA	Analysis of the variance
ASE	Assisted solvent extraction
BD-FAME	Biodiesel dominant fatty acid methyl ester
°C	Degrees Celsius
CAD	Canadian Dollar
CO	Carbon monoxide
CO_2	Carbon dioxide
CTAB	Cetyl trimethylammonium bromide
DAH	Dodecyl amine hydrochloride
DCW	Dry cell weight
DN2	N-dodecylpropane-1,3-diamine hydrochloride
DPC	Dodecyl pyridinium chloride
FA	Fatty acids
FAME	Fatty acid methyl ester
FFA	Free fatty acids
GC-FID	Gas chromatography with a flame ionization detector
GHG	Greenhouse gases
HPH	High pressure homogenization
M	Biomass moisture content
P	Reaction Pressure
PUFA	Polyunsaturated fatty acids
PSI	Pounds per square inch
R^2	Regression coefficient
RSM	Response Surface Methodology
SDS	Sodium dodecyl sulfate
SLPM	Standard litre per minute

SS	Suspended solids
SC-CO_2	Supercritical CO_2
T	Reaction temperature
T-FAME	Total fatty acid methyl ester
TAG	Triacylglycerols
Ti	Reaction time
TSS	Total suspended solids
USD	United States dollar
v/v	Volume/volume
w/w	Weight/weight

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CHAPTER 1 INTRODUCTION

The search for sustainable energy sources is becoming the most important challenge facing humans today (Huang, Chen, Wei, Zhang, & Chen, 2010). Currently, petroleum products supply 80 % of the world's energy demand as a fuel source for transportation, and other energy demanding sectors (Sathish, Smith, & Sims, 2014). The utilization of these products releases CO_2 gas into the atmosphere, which has increased in concentration from 350 ppm in 1988 (Gonzalez & Lucky, 2013) to 403 ppm in 2016 (Dlugokencky & Tans, 2016). CO_2 makes up 63 % of greenhouse gases (GHGs) emitted to the atmosphere. GHG emissions result in adverse environmental impacts such as degradation in air quality and global warming, which impact human mortality (Demirbas & Demirbas, 2011). Furthermore, fossil fuel formation requires millions of years, and the diminishing crude oil reserves, increasing fuel prices, and environmental concerns associated with fossil fuel usage make them unsustainable energy sources (Chisti, 2007; Lardon, Helias, Sialve, Steyer, & Bernard, 2009; Demirbas & Demirbas, 2011). It is for these reasons that renewable energy sources are regarded as an important energy resource in many countries today. However, they only account for 11% of the total energy utilization (IEO, 2013). There are numerous renewable energy alternatives to fossil fuels, of which hydrogen, natural gas, syngas and biofuels have the most potential to emerge in the near future. However, biofuels are the most environment-friendly energy source, and with growing concern over global warming and environment preservation (Stephenson, Dennis, & Scott, 2008), it is this category of renewable sources that is most preferred for replacing current fossil fuels (Stephenson et al., 2008). Biofuels are of interest because they are biodegradable, renewable, and the quality of their exhaust gas emissions are acceptable (Bhatti, Hanif, Qasim, & Ata-ur-Rehman, 2008).

Microalgae are regarded as the best candidate for the production of biodiesel since these microorganisms do not compete with edible crops and can produce 20,000 - 80,000 L of oil per acre per year, which is 7-31 times greater than that produced from the leading terrestrial crop (Demirbas & Demirbas, 2011). Microalgae-derived biodiesel is a green alternative to currently utilized energy sources as it can reduce CO, CO_2 and hydrocarbon

emissions (Lee, Yoo, Jun, Ahn, & Oh, 2010; Valente, da Silva, Pasa, Belchior, & Sodré, 2010). Microalgae utilization for energy production is ideal because: (a) they have a high growth rate, and (b) they can produce neutral lipids with a low degree of unsaturation that are suitable for conversion to biodiesel and replacement for conventional diesel (Li, Miao, Li, & Zhong, 2011). Furthermore, microalgae can be used to generate alternate value added products such as pigments and fatty acids (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) (Yen, Yang, Chen, Jesisca, & Chang, 2015). Currently, only a few microalgae species are used commercially for pigment production (Yen et al., 2015). These include astaxanthin from *Haematococcus pluvialis* (Thana et al., 2008), β-carotene from *Dunaliella salina* (Solana, Rizza, & Bertucco, 2014) and lutein from *Scenedesmus* sp. (Yen, Sun, & Ma, 2011). Such high value added products (>$10,000/t USD) for human nutrition can withstand the higher processing costs associated with microalgae, however low valued commodities such as biodiesel cannot withstand these high costs (Benemann, 2013). The baseline economics of microalgae lipid production (achieving a 10 % return) was analyzed by Davis et al. (2011), and it was determined that the cost per gallon is $8.52 and $18.10 USD using open pond and photobioreactors, respectively. At present, the cost of diesel fuel per gallon in Canada is $3.75 USD (Natural Resources Canada, 2018). This emphasizes that a reduction in downstream processing costs is necessary for competing with fuel prices.

The processing of microalgae biomass begins with its cultivation, followed by its collection/harvesting, pre-treatment, oil extraction and transesterification into biodiesel. The harvesting/dewatering step accounts for 20-30% of the total costs associated with microalgae production and processing (Uduman, Qi, Danquah, Forde, & Hoadley, 2010; Barros, Gonçalves, Simões, & Pires, 2015). It is vital that biodiesel production processes are economically viable and that the costs associated with lipid recovery are reduced while maximizing product recovery from the biomass, since these lipids also require transesterification for conversion to biodiesel (Halim, Gladman, Danquah, & Webley, 2011).

Therefore, the overall aims of this project are to investigate an effective harvesting, pre-treatment and oil extraction technique for *Chlorella saccharophila* to be used for large scale production. Firstly, a comparative analysis on harvesting, pre-treatment and oil

extraction techniques is performed, with the aim of selecting the best techniques for large scale processing operations. This is followed by experimental studies on *Chlorella saccharophila* to evaluate the effectiveness and optimize process parameters for the selected techniques.

The specific objectives are to:

i) Perform comparative analyses in order to determine the most economically viable, environmentally friendly and efficient methods for industrial production of biodiesel

ii) Investigate and optimize the use of surfactant aided dispersed air flotation for harvesting *Chlorella saccharophila*, by maximizing both recovery and enrichment of the biomass

iii) Investigate and optimize lipid extraction from *Chlorella saccharophila* biomass harvested with surfactant aided dispersed air flotation using supercritical CO_2 technology, and to determine the effect of extraction parameters on lipid yields and composition

iv) Investigate the use of surfactant CTAB in a combined harvesting and pre-extraction treatment for *Chlorella saccharophila* microalgae with SC-CO_2 extraction, as a potential large scale process for biodiesel.

The goals of the thesis are addressed in the following chapters, where most chapters have been recently published as journal articles or currently submitted for publication. The content of these chapters consists of background knowledge from the literature, assessments performed to provide the research direction of the thesis, and details of the experimental work performed, research results, thesis conclusions and future recommendations.

To begin, a literature review is presented in **Chapter 2** to highlight the various harvesting, pre-treatment and oil extraction techniques employed for microalgae, which are the areas that have been described as a major bottle-neck to the large scale production of microalgae derived biodiesel. The factors affecting each technique and energy consumption are also reviewed with an advantages and disadvantages section, to serve as

a summary for the various methods. This information serves as the knowledge base necessary for performing comparative analyses on the various techniques described in later chapters.

Chapters 3 and **5** provide the methodology used for the comparative analyses for microalgae harvesting and oil extraction techniques, respectively, as well as the results of the analysis. In **Chapter 4,** an overview is given of the effect of various disruption techniques on product recovery and influence of the cell wall composition of various microalgal species. This chapter goes beyond the literature review presented in **Chapter 2**, in that it attempts to relate the effectiveness of the various disruption techniques to the structural composition of the cell wall. The results of **Chapters 3-5** were then used to determine the harvesting, pre-extraction treatment and oil extraction techniques for experimental investigation using *Chlorella saccharophila* in **Chapters 6-8**.

Chapter 6 explores surfactant aided dispersed air flotation as an effective harvesting technique for *Chlorella saccharophila.*. This technique was selected based on the results of both **Chapters 3** and **4**. Evaluation of the technique was based on the measurement and optimization of both enrichment ratio and recovery parameters, simultaneously. The optimized parameters obtained in this section were used for harvesting the biomass in the later experimental work on pretreatment and extraction of lipids.

Chapter 7 investigates the extraction of lipids from harvested biomass by supercritical CO_2, which was the extraction technique chosen from the comparative analysis performed in **Chapter 5**. The effects of varying operational parameters on supercritical CO_2 extraction were determined and the process was optimized using response surface methodology. Evaluation was based on fatty acid methyl ester (FAME) yields determined using gas chromatography equipped with a flame ionization detector. In **Chapter 8**, the use of a surfactant as a simultaneous pretreatment during the harvesting of microalgae on the FAME yield and composition using supercritical CO_2 extraction is investigated. Here, the biomass harvested via surfactant aided dispersed air flotation, both with no hold time and 24 h hold time, are compared with biomass recovered without surfactant (using centrifugation only).

Chapter 9 concludes the thesis, with a summary of the findings from this work. The novelty of the study is highlighted as well as the contributions to science and recommendations for future work.

CHAPTER 2 LITERATURE REVIEW

Parts of this chapter have been published in the Journal of *Fundamentals of Renewable Energy and Applications* and *Separation Science and Technology*, and are being reproduced here with permission from the publisher (Appendix A). The following are the full citations of the articles used.

Alhattab, M., & Brooks, M. S. (2017). Dispersed air flotation and foam fractionation for the recovery of microalgae in the production of biodiesel. *Separation Science and Technology*, 52(12), 2002-2016.

Alhattab, M., Ghaly, A. & Hammoud A. (2015). Microalgae harvesting methods for industrial use: critical review and comparative analysis. *Journal of Fundamentals of Renewable Energy and Applications*, 5(2):154-179.

Alhattab, M., & Ghaly, A. (2015). Microalgae oil extraction pre-treatment methods: critical review and comparative analysis. *Journal of Fundamentals of Renewable Energy and Applications*, *5*, 172.

2.1 Introduction

The purpose of this chapter is to provide the reader with background information pertaining to the topics of the thesis. This section is divided into three main parts: microalgae harvesting, pre-treatment and oil extraction. It highlights the various techniques that have been used with microalgae biomass in the literature. In addition, it discusses the principles of operation for each technique and highlights the factors affecting the efficiency, energy consumption for operation and the associated advantages and disadvantages.

2.2 Microalgae Harvesting

Microalgae cultures exist in dilute liquid suspensions containing 0.1-2.0 g of dried biomass per litre, and therefore need to be concentrated for further processing into value added products (Grima, Belarbi, Acién Fernández, Robles Medina, & Chisti, 2003; Danquah, Gladman, Moheimani, & Forde, 2009), since the cost of the extraction, and purification processes decrease with increased biomass concentration (Grima et al., 2003; Danquah et al., 2009; Uduman et al., 2010). Different solid-liquid separation methods have been employed to dewater/concentrate microalgae culture to values of 10-450 g biomass/L.

These include: sedimentation, filtration, centrifugation, flocculation, electrophoresis, and flotation techniques (Alhattab, Ghaly, & Hammoud, 2015). However, many of these techniques suffer from high operational costs, deeming them unsuitable for use in the production of a relatively low value end product such as biofuel (Coward, Lee, & Caldwell, 2013).

2.2.1 Sedimentation

In this technique, the solids and liquids are separated from one another by gravitational forces (Salim, Bosma, Vermue, & Wijffels, 2011). Different materials are separated from one another based on the density of the material and/or particle size. A larger difference in density would result in faster sedimentation rates while a smaller differences in densities and/or smaller particle size would require a longer time to settle out by gravitational forces (Milledge & Heaven, 2013).

2.2.1.1 Type of Settling Tanks

Lamella separator and sedimentation tanks are used to separate solids from liquid. Lamella separators offer a greater settling area than conventional thickeners as a result of plate orientation (Mohn, 1980). Lamella tanks work by inserting the microalgae biomass through the inlet. The liquid floats to the surface (effluent) and the biomass is caught onto the slanted plates. With time, the biomass settles down to the bottom of the tank and can be collected through the harvest outflow. Sedimentation tanks are cylindrical with a funnel shaped bottom so that the settled microalgae are concentrated near the outlet. The outlet is placed at the bottom of the tank so that the collection of the settled microalgae can easily be recovered (Mohn, 1980). The tank is equipped with a pump that carries the microalgae biomass from the cultivation tank into the sedimentation tank through the inlet. These tanks work by allowing the denser solids to settle to the bottom of the tank, leaving the clear water at the surface. Once the settling process is complete, the algae can be retrieved from the tank through the outlet (Mohn, 1980).

2.2.1.2 Factors Affecting Sedimentation

The factors influencing the settlement rates of microalgae include: density and particle size, temperature, aging of the cells, light intensity and time (Waite, Thompson, & Harrison, 1992; Knuckey, Brown, Robert, & Frampton, 2006; Harith et al., 2009).

Density and particle size: The density of marine microalgae varies from 1030 to 1100 kg/m^3 and the density of freshwater microalgae varies from 1040 to 1140 kg/m^3 (Van Ierland & Peperzak, 1984; Edzwald, 1993). Granados et al. (2012) reported that the densities of fresh water (1000 kg/m^3) and salt water (1025 kg/m^3) are similar to that of microalgae and as a result the rate of settlement of algae is low. Murphy and Allen (2011) stated that it is a challenge to remove microalgae biomass from the liquids because of the identical densities of the cells and media.

In the study by Cole and Buchak (2006) they indicated that the rate of settlement is dependent on the type of microalgae present, and found the green microalgae to have an average settling rate of 0.1 m/d. This was in agreement with the finding of Milledge and Heaven (2013) that the freshwater species *Chlorella* had a settlement rate of 0.1 m/d. Yang et al. (2000) also reported an algae settling rate in the range of 0.1-0.3 m/d for microalgae. Choi et al. (2006) noted that the sedimentation rate of large and small sized algae were 2.6 cm/h and less than 1.0 cm/h, respectively. However, Peperzak et al. (2003) investigated the sedimentation rate of 24 different microalgae species (ranging in size from 10-1000 μm) and found that it varied from 0.4 to 2.2 m/d, but there no correlation between the size of the cells and the sinking rates.

Temperature**:** The effect of temperature on the rate of sedimentation was investigated by a number of authors. It is thought that cold waters result in slower settling rates as a result of increased viscosity (Davis and Downs, 1997). The work of Knuckey et al. (2006) indicated that the temperature of 4°C settled a wide array of microalgae species after 24 h when the pH was adjusted in the range of 8-8.5. Harith et al. (2009) tested the effect of varying temperature (4-27°C) and the presence and absence of light on sedimentation rates of *Chaetoceros calcitrans* at a pH of 8. They found that the highest efficiency was obtained at day 8 at a temperature of 27°C in the dark. Generally it can be said the warmer waters

tend to improve settling rates, however, caution should be taken as sedimentation at high temperatures will deteriorate the cells (Greenwell et al. 2010).

Cell age: The cell age also plays a role in the settling process of microalgae cells. The study performed by Danquah et al. (2009) noted that the settling rate for microalgae harvested during the high growth phase (4-10 days) was lower than that harvested during low growth phase (10-12 days). Similarly, Choi et al. (2006) reported that the settling rate of algae significantly increased in the stationary growth phase of microalgae. Manheim and Nelson (2013) noted that in the exponential growth phase (day 6) there was little to no settling in *Scenedesmus sp*. observed over 2 h period, but the greatest removal efficiency was noted in the stationary phase (day 15). Peperzak et al. (2003) reported that the settling rate at 15 and 20 weeks for *Phaeocystis globosa* and *Eucampia zodiacus* were 0.5 and 1.0 m/day and 0.7 and 1.0 m/day, respectively. This phenomenon maybe attributed to the changes in cell (i.e. surface charge) and medium over time (i.e. pH).

Light: The investigation of the impact of light on settling rates was undertaken by Danquah et al. (2009) and others. Danquah et al. (2009) noted that the absence of light increased the settling rate during high growth and low growth phases. In the supernatant obtained during the high growth phase, the biomass concentration was 0.57 g/L in the presence of light and 0.39 g/L in the absence of light, while the supernatant obtained during the low growth phase contained 0.28 g/L in the presence of light and 0.17 g/L in the absence of light. Further, Schlenk et al. (2007) noted that the concentration of microalgae in the light and dark conditions were 1075 cells/mL and 775 cells/mL, respectively. On the other hand, the study by Harith et al. (2009) reported that the settling rates observed in the presence and absence of light in *Chaetoceros calcitrans* were similar. This is likely due to the dependence of light by photoautotrophic microalgae, therefore they will migrate to those areas.

Time: The concentration of microalgae by sedimentation requires long settling times that are greater than 24 h. The study by Park et al. (2011), noted long retention times of 1-2 days for algae recovery in large-scale settling tanks. Harith et al. (2009) reported that increasing the settling time to 15 days increased the settling efficacy to 94%. Griffiths et al. (2012) found that the percentage of biomass recovery after 24 h of settling for *S.*

platensis, C. fusiformis, T. suecica, Nannochloropsis and *Scenedesmus* were 95, 96, 80, 59 and 86%, respectively. Furthermore, Wang et al. (2014) noted that the biomass recovery for the species *S. dimorphus* and *C. vulgaris* after 2 h of gravitational settling was 80 and 55%, respectively.

2.2.1.3 Advantages and Disadvantages

Although sedimentation tanks are effective in concentrating microalgae suspensions to 1.5% total suspended solids (TSS), they are not widely use in the industry. The costs associated with gravitational settling are low, but the reliability of this method without the use of flocculating agents is also low (Shelef, Sukenik, & Green, 1984; Uduman et al., 2010). The settling time required is much longer than other processes (Taher, Al-Zuhair, Al-Marzouqi, Haik, & Farid, 2011) and energy is required for pumping the slurry (Uduman et al., 2010). Gonzalez-Fernandez and Ballesteros (2013) stated that this method is time consuming and the composition of the cells can change. Mata et al. (2010) stated that the cell concentrations obtained by sedimentation are low. Ras et al. (2011) indicated that harvesting microalgae biomass by sedimentation alone is not the most efficient method since the cell recovery rates of 60-65% are low.

2.2.2 Filtration

This type of algae harvesting method uses a medium that is permeable so that it can retain the algae biomass while allowing the liquid to pass through. This technique requires a pressure difference across the filter which can be driven by vacuum, pressure or gravity. The membrane filters can be classified based on the size of the pores into macro filtration (greater than 10 µm), micro-filtration (0.1-10 µm), ultrafiltration (0.02-0.20 µm) and reverse osmosis (less than 0.001 µm) (Brennan & Owende, 2010). The pressure required to force the fluid across the membrane decreases as the pore size of the membrane is increased. Filtration techniques can concentrate microalgae cells in the suspension up to 5-18% and the operating costs vary from $10 to $20/gal. The harvesting efficiency using filtration methods ranges from 20% to 90% (Green, 2008). The main types of filtration processes include vacuum and pressure (Uduman et al., 2010).

2.2.2.1 Vacuum Filtration

Vacuum filtration separates solids from liquid media by capturing the solid particles onto a filter while pulling the liquid through by suction from the filter. Microalgae range in size from 2 to 30 µm indicating that a micro-filtration membrane is suitable for vacuum filtration (Grima et al., 2003; Brennan & Owende, 2010). Milledge and Heaven (2013) stated that the macro-filtration membranes can be used for large microalgae cells or if the algae cells are flocculated together. Uduman et al. (2010) reported that the vacuum filtration harvesting technique is most suited for large microalgae cells (greater than 10 µm). Stucki et al. (2009) separated *Spirulina platensis* species using vacuum filtration equipped with regenerated cellulose membrane with a pore size of 0.45µm.

There are five different filter membranes that can be used in vacuum filtration. They are vacuum drum filter, suction filter, filter thickener, belt filter and starch precoated drum filter. Mohn (1980) noted that the suction filter, starch precoated drum filter and belt filter were suitable for concentrating the *Coelastrum* microalgae species to a range of 5-37%. The author found the drum filters were not effective harvesting techniques as a result of clogging. Filter thickeners were not recommended as a result of low solid contents (3-7%) and high energy requirements (Mohn, 1980). Ferrentino et al. (2006) noted that vacuum filtration equipped with a Buchner funnel and cellulose fiber filters were effective in the recovery of microalgae from solution.

Successful recovery of microalgae cells has been noted using filtration equipped with diatomaceous earth as a filter aid to avoid the clogging of the filter (Grima et al., 2003). Gudin and Chaumont (1991) reported that precoated drum filter with filter aid (diatomaceous earth) is effective in harvesting the microalgae *Chlamydomonas reinhardtii*. Grima et al. (2003) evaluated filters made of cellulose fibers and sand filters and obtained unsatisfactory results, but also found that diatomaceous earth filter aid effectively recovered the micro sized *Dunaliella* species. Similarly, Uduman et al. (2010) noted an exceptional recovery of *Dunaliella* species using diatomaceous earth aided filter, and Brennan and Owende (2010) stated that the use of diatomaceous earth as a filter aid can effectively remove microalgae cells from medium.

2.2.2.2 Pressure Filtration

Pressure filtration is a technique used for separating particles form a liquid suspension into a compacted form. It works by separating the liquid from the particles (that are collected onto the filter) by means of pressure (Velamakanni & Lange, 1991). The flow of fluids through the filter is created by raising the pressure above atmospheric to create a pressure differential across the filter (Rushton, 1996). This process is operated in batches that are most often fed from and discharged to a continuous process. A surge tank is required upstream to the filter and one is required for the collection of the filtrate (Raghvan and Sanga, 2003).

Pressure filtration harvesting can be achieved by plate-and-frame filter presses or by using a pressure vessel that is equipped with filters (Show, Lee, & Chang, 2013). The plate-and-frame filter press works by forcing the liquid in the microalgae suspension through the filter using high pressure. A series of rectangular plates that are mounted in a vertical position, face to face, make up the press system. A fitted filter cloth is applied to each of the plates and they are held together with one another by force under pressure (Show et al., 2013). The fluid that contains the algae is pumped into the gaps between the plates and the pressure is applied in order to force the liquid through the plate outlets and filter cloths. After separation, the dewatered microalgae cake is recovered (Show et al., 2013).

Cross Flow Filtration

Cross flow filtration is an effective means for harvesting large volumes of microalgae cells. In this technique, the sample flows tangentially across a membrane. The particles larger in size than the membrane pores are retained and referred to as the retentate. Smaller particles pass through the membrane with the liquid solution and are referred to as the permeate (Uduman et al., 2010).

Ultrafiltration or microporous membranes are the type of filter membranes used in this technique. These membranes are available with a wide range of pore sizes and molecular weight retentions (Petrusevski, Bolier, Van Breemen, & Alaerts, 1995). Polymer membranes have a long operating life when used at suitable cross flow velocity conditions and low transmembrane pressures. The study of Petrusevski et al. (1995) used a cross

filtration with a membrane pore size of 0.45 μm and achieved a biomass recovery efficiency of 70-89%. The study of Rossignol et al. (1999) found that polymer membranes were effective in recovering the marine microalgae species *Haslea ostraria* and *Skeletonema costatum*, but the performance depended on the hydrodynamic conditions, properties of the microalgae and the concentration of the microalgae cells.

In the study of Uduman et al. (2010), they reported that the initial flux for microfiltration membranes were much higher than those of ultrafiltration, but they clogged more easily. In addition, Zhang et al. (2010) also used a cross-flow ultrafiltration membrane with a cross-flow velocity of 0.17 m/s and noted an increase in algae concentration from 0.104% to 92.5% at the membrane surface with a harvesting efficiency value of 46.01 $g/m^2/h$. Rossi et al. (2005) tested 14 various inorganic membranes and noted that the ultrafiltration membrane ATZ-50 kDa illustrated the best performance and concentrated the *Arthrospira platens* species by a factor of 20. The study of Rossi et al. (2004) investigated the use of crossflow filtration technique equipped with an organic ultrafiltration membrane (polyacrylonitrile, 40kDa), and it was found to concentrate *Arthrospira platens* by a factor of 10. The species *Skeletonema costatum* was also effectively concentrated using cross-flow ultrafiltration with a flux of 30 l/h for 12 h by Rossignol et al. (1999). The study of Rose et al. (1992) also effectively concentrated the species *Dunaliella salina* using cross-flow ultrafiltration with flux rates of 30-40 l/h. Walsh et al. (1986) concentrated the species *Thalassiosira pseudonana* to 2.3 L from 2840 L which was composed of 2.33×10^{12} cell/L using microfiltration membrane system. Ahmed et al. (2012) noted that the resistance of the cross-flow microfiltration decreased as the cross-flow velocity increased from 0.13 to 4 m/s while harvesting *Chlorella* sp. species.

2.2.2.3 Energy Consumption

Filtration techniques can be considered energy efficient since a minimal amount of energy is required upon assessment of the output product and the amount of initial feedstock added (Danquah et al., 2009). The energy consumption for the application of the various types of filtration on microalgae, have been evaluated and noted in a number of research articles and summarized below.

The use of vacuum filtration, as reported by Shelef et al. (1984), utilizes an energy consumption in the range of 0.1-5.9 kWh/m^3 which is dependent on the type of filter used, and a pressure drop of 70-80 kPa is required for operation. Mohn (1980) noted that vacuum filtration consumed 5.9 kWh/m^3 of energy in order to concentrate the suspended solids (SS) in solution to 18-27%, and further noted that dewatering *Coelastrum proboscideum* using suction filtration (8% SS), belt filter (9.5% SS) and filter thickener (5-7% SS) required 0.1, 0.45 and 1.6 kWh/m^3 of energy, respectively. The work of Umesh (1984) reported that harvesting the microalgae strain *Spirulina fusiformis* under vacuum filtration with a coarse pores medium was low in cost ($83.3/ m^2) and capable of harvesting 23 kg/m^2 kwh. Milledge and Heaven (2013) reported a power consumption of 0.25 kWh/m^3 for microalgae harvest using vacuum belt filter. Finally, Mohn (1988) further reported an energy consumption value of 3 kWh/m^3 for microalgae harvesting, using vacuum drum filtration.

Similarly, the energy utilization by pressure filtration has also been assessed and it was noted by Grima et al. (2003) that the amount of energy required to harvest 22-27% (w/v) of C. *proboscideum* species is 0.88 kWh/m^3. In contrast Mohn (1980) found that recovery of *C. proboscideum* using a cylindrical sieve (7.5% SS) and filter basket (5% SS) only required 0.3 kWh/m^3 and 0.2 kWh/m^3, respectively. However, as mentioned above, pressure filtration of smaller species such as *Dunaliella* and *Chlorella*, was found ineffective (Harun, Singh, Forde, & Danquah, 2010), which is in agreement with the study of Mohn (1980), that found that pressure filtration was not suitable for the species *Scenedesmus, Dunaliella* and *Chlorella*, but was satisfactory for other larger microalgae species such as *C. proboscideum* and *Spirulina platensis*.

The energy consumption of cross flow filtration appears to be greater than those reported for vacuum and pressure filtration. In the study of Rossignol et al. (1999), they noted that the energy consumption using cross-flow filtration technique can range from 3 kWh/m^3 to 10 kWh/m^3 depending on the feed characteristics, the system design and the pressure used. This range is greater than that noted by a more recent study (Danquah et al., 2009) of 0.38-2.06 kWh/m^3 for concentrating suspended solids in the range of 2.5-8.9 %. However, Crittenden et al. (2012) reported that the energy consumption for a cross-flow filtration technique was 5 kWh/m^3.

2.2.2.4 Advantages and Disadvantages

Harvesting microalgae using filtration techniques is more efficient than sedimentation, but drawbacks of this method include membrane replacement and/or periodical washing of the membrane to avoid clogging of the pores (Gonzalez-Fernandez & Ballesteros, 2013). The effectiveness of the filtration process is dependent on the membrane size and the microalgae cell size (Uduman et al. 2010). Filtration has been noted most suitable for larger microalgae cells, but inadequate for recovery of smaller microalgal species (Uduman et al. 2010). Other drawbacks are associated with large energy requirements and costs associated with periodic replacement of membrane as a result of clogging (Grima et al., 2003; Rossi et al., 2004; Mata et al., 2010; Amaro, Guedes, & Malcata, 2011; Pittman, Dean, & Osundeko, 2011). Grima et al. (2003) recovered microalgae biomass using filtration method and concluded that this harvesting method is not economically viable for large scale operation. The coupling of flocculation to aid in filtration processes would lower the energy requirements, but additional costs for the flocculant would be encountered (Milledge & Heaven, 2013).

Some of the advantages of using pressure filtration are: the cakes collected (composed of the particles in the liquid suspension) have low moisture content, the soluble recovery from the cake is high, re-circulating the filtrate for 1-2 min will clean the filter, high degree of clarity in solutions can be achieved and alloy and synthetic materials can be used to construct the filters and the internal parts (Rushton, 1996; Spellman, 2008). The disadvantages of using this technique include: the difficulty in washing the filter medium which increases when the solid is sticky, the internals are difficult to clean in food-grade applications and the difficulty in viewing the condition of the filter due to vessel encapsulation (Rushton, 1996; Spellman, 2008).

Cross flow microalgae filtration, however, has been noted superior to other conventional harvesting methods such as sedimentation, flocculation and centrifugation because it results in the complete removal of debris and microalgae cells (Uduman et al., 2010). The structure and properties of the recovered microalgae are preserved using this filtration technique (Petrusevski et al., 1995). The equipment is considered to be cheap because the costs are only associated with pumping and replacement of membranes

(Rossignol et al., 1999). Nonetheless, large scale recovery of algae cells can be limited due to fouling and frequent replacement of the membrane (Uduman et al., 2010), as is also the case for other types of filtration.

2.2.3 Centrifugation

This type of removal mechanism is widely used in beverage, food and pharmaceutical industries. Centrifugation is a process in which a centrifugal force is used to enhance the separation of solids. Spinning the suspension creates the pressure differential necessary for particle separation from the liquid suspension. Thus, the efficiency of the recovery process is dependent on the centrifugal force (Grima et al., 2003). Two types of centrifugations are generally employed for microalgae harvest, which are disc stack and decanter types (Uduman et al., 2010).

2.2.3.1 Disc Stack Centrifuge

Disc stack centrifugation is the most common industrial centrifuge used today in commercial plants producing high value added products. It consists of a shallow cylindrical bowl that has numerous stacks of metal cones (discs) which are closely spaced together (Grima et al., 2003). Separation of the materials is based on density. The mixture is placed on the centre of a stack of discs and the lighter phase of the mixture remains on the inside towards the centre while the denser phase is displaced outwards to the underside of the discs. This technique separates materials of different densities by layering them (Mannweiler & Hoare, 1992). It is most suited for separating materials with particle sizes in the range of 3-30 μm and for concentrations of suspensions that have SS content ranging from 2 to 25% (Milledge & Heaven, 2013).

Heasman et al. (2000) evaluated the cell recovery efficiency of nine different microalgae species using a disk stack centrifuge and noted a recovery efficiency greater than 95% at a force of 13,000g. They also noted that the recovery efficiency declined with a decrease in the gravitational force to 60% and 40% at gravitational forces of 6,000g and 1,300g, respectively. The work of Sim et al. (1988) found a 90 % microalgae removal efficiency using a disc stake centrifuge. Vasudevan et al. (2012) achieved an 18% microalgae concentration using a disc centrifuge. Mackay (1996) effectively harvested cells using a disc centrifuge operating at a force of 4,000-15,000g for a biomass suspension

containing 0.2-20% v/v algae cells. Chojnacka et al. (2005) harvested the *Spirulina* sp. using a disc type centrifuge operating at 6,000 rpm for 5 min.

2.2.3.2 Decanter Centrifuge

Decanter types are based on the concept of using a special settling tank in which the solids in suspension are forced to fall down due to the gravitational forces (Smith and Charter, 2009). The decanter centrifuge operates continuously by pumping the cultivated microalgae biomass into the centrifuge bowl whereby the suspended particles in solution are forced to the bottom of the bowl. The liquid left after the particles have been extracted is passed through the overflow pipe (Rees, Leenheer, & Ranville, 1991).

The work of Grima et al. (2003), employed this technique on microalgae biomass and noted that concentration of biomass using a decanter centrifuge is better than other harvesting methods. That of Dassey and Theegala (2013), achieved a harvesting efficiency of 28.5% for microalgae that was at a flow rate of 18 L/min using continuous flow decanter centrifuge. Smith and Charter (2009) noted that the clarity of the liquid produced after separation was not as great as that achieved using disc-stack centrifugation. It was stated by Mackay (1996), that the decanter centrifuge operating using a force of 4,000-10,000g is effective for slurries with a biomass content of 5-80%. The study of Vasudevan et al. (2012), reported that a 12% microalgae concentration was achieved using a decanter centrifuge. Further, they stated that microalgae biomass needs to undergo an initial thickening step such as dissolved air flotation in order to concentrate microalgae suspensions (0.02-0.05 weight %) to 2-3% before using decanter centrifugation.

2.2.3.3 Energy Consumption

The energy consumption reported in the literature for the disc stack centrifuge varied from 0.53 kWh/m^3 to 5.5 kWh/m^3. It was noted by Alfa Laval (2010), that a disc type centrifuge for dewatering microalgae achieved a 16% solids concentration with a power consumption of 0.53 kWh/m^3. While, Mohn (1980) noted a 12% SS concentrate for the microalgae species *Scenedesmus* using a disc-stack centrifuge with an energy consumption of 1 kWh/m^3. Goh (1986) harvested microalgae grown in pig waste using disc centrifugation with an energy consumption of 1.4 kWh/m^3. Finally, Sharma et al. (2013) noted that the disc stack centrifuge consumed 5.5 kWh/m^3 for *Chlorella sp*. harvesting.

In comparison, the energy consumption of decanter centrifugation reported in the literature varies from 1.3 kWh/m^3 to 8 kWh/m^3, which is greater than disc stack type. Sim et al. (1988) noted that an energy consumption of 1.3 kWh/m^3 was required for concentrating microalgae biomass from 0.04% to 4.00% using a decanter type centrifuge. The study by Grima et al. (2003) achieved a microalgae concentration of 22% (w/v) using decanter centrifuge with an energy utilization of 8 kWh/m^3, and Mohn (1988) reported a concentration of microalgae to 20 % solids required 4 kWh/m^3 for the same technique.

2.2.3.4 Advantages and Disadvantages

The advantages of using disc-stack centrifuge for harvesting microalgae is their high removal efficiency compared to other industrial centrifuges. The concentration of the feed for these units is typically in the range of 0.5-10% *w/w*. This type of centrifuge handles a high flow rate and is capable of separating fine (0.1-100 μm) particles (Tarleton & Wakeman, 2006). This device can be used to separate solid from liquid in continuous, semi-continuous and batch operation. Some of the disadvantages of this type of centrifuge include: low dry substance content in the discharge system (Sharma et al., 2013), mechanically complex, high cost and the small space between the closely stacked discs makes it harder to clean and may require chemicals for cleaning (Tarleton & Wakeman, 2006).

The dewatered biomass using the decanter centrifuge is much more concentrated than that achieved using the disc centrifuge. However, the decanter centrifuge is more suited for suspensions with higher solid particles and is unsuitable for microalgae suspensions as indicated by Mohn (1988). This type of centrifuge is most suited for separating materials with particle sizes greater than 15 μm and solid suspensions containing higher than 15% (Milledge and Heaven, 2013). It operates at inertial forces that are less than 6000*g*. The disadvantages of using this method for microalgae harvesting are: they require highly concentrated feeds (typically in the range of 4-40% *w/w*), the liquid leaving the system may not be clear due to the presence of fines, the use of decanter centrifuge for processing of finer particles may result in poor flow properties of the thickened solids and some mechanical difficulties (Tarleton & Wakeman, 2006), and are much more energy intensive than disc centrifuges (Metcalf & Eddy, Burton, Stensel, & Tchobanoglous, 2003; Milledge

& Heaven, 2013). The limitation of using this type of centrifugation is the high costs associated with the equipment required for processing large volumes. This type of centrifuge has been estimated to consume 3000 kWh/ton of dry alga biomass (Grima et al., 2003; Packer, 2009).

2.2.4 Flocculation

On the basis of energy requirement, chemical flocculation as a dewatering method seems to be the most promising for large scale utilization (Uduman et al., 2010; Wijffels, Barbosa, & Eppink, 2010; Araujo et al., 2013). These flocculation methods work by concentrating the cells (coagulation) followed by settlement to the bottom of the cultivating apparatus due to the increased density of the concentrate (Mata et al., 2010; Araujo et al., 2013).

There are various types of flocculation techniques that can be applied to induce microalgae harvesting, these include chemical flocculation, autoflocculation and bio-flocculation. All of which are reviewed in the following sections, highlighting the factors that affect each type and their advantages and disadvantages.

2.2.4.1 Chemical Flocculation

There are two categories of chemicals (Table 2.1) that can induce flocculation: inorganic and organic polymers (Oh et al., 2001). Examples of various types of flocculants used by researchers are noted in Table 2.1. The effects of these polymers on microalgae are outlined in Table 2.2. Typically, cationic, anionic and non-ionic polyelectrolytes are used to flocculate the microalgae cells (Uduman et al., 2010).

Types of Chemical Flocculation

Inorganic flocculation

Microalgae flocculation using inorganic compounds works by charge neutralization (Bernhardt, 1991). The flocculation process using these compounds works in low pH environments in order to form cationic hydrolysis products (Briley & Knappe, 2002). Under optimal pH, these flocculants form polyhydroxy complexes. A large number of chemicals (ferric sulfate, ferric chloride, aluminum chloride, aluminum sulfate) have been

Table 2.1. Chemical flocculant types used on microalgae by various studies.

Chemical Type	Flocculant	Reference
Inorganic	Ferric sulfate	Kwon et al. (2014)
	Ferric chloride	Papazi et al. (2010)
	Aluminium chloride	Grima et al. (2003)
	Aluminium sulfate	Oh et al. (2001)
Organic	Magnafloc LT 25	Knuckey et al. (2006)
	Zetag	t'Lam et al. (2014)
	Praestol	Pushparaj et al. (1993)
	Chitosan	Chen et al. (2014)

Table 2.2. Chemical flocculant effect on microalgae.

Flocculant Type	Effect on Microalgae	Reference
Inorganic	Toxic to the cells	Papazi et al. (2010)
	Alter the color of the medium	Schenk et al. (2008)
	Alter the pH of the media which may make it unsuitable for reuse	Grima et al. (2003)
Organic	High cell viability (>75%)	Harith et al. (2009)
	No inhibitory effect the cells	Pushparaj et al. (1993)
	Non-toxic	Vandamme et al. (2011)

tested with the microalgae inorganic flocculation process, the most effective of which was aluminum sulfate (Oh et al., 2001). In wastewater treatment, multi-valent metal salts such as ferric sulfate, ferric chloride and aluminum chloride have been used to remove microalgae (Uduman et al., 2010).

Papazi et al. (2010) achieved a harvesting efficacy for *Chlorella minutissma* species greater than 85% using ferric salts. The study of Kwon et al. (2014) reported a flocculation efficiency of 85.6% for *Tetraselmis* sp. using ferric sulfate (a dose of 0.7 g/L) at a pH of 4-8. Wyatt et al. (2012) attained a harvesting efficacy greater than 90% for the *Chlorella zofingiensis* species using a ferric chloride concentration of 40% (w/v) at a pH of 4.0. Similarly, Xuan (2009) noted a 90% removal efficacy for *Nannochloropsis* sp. using ferric chloride administered at 0.18 mg/l. Sukenik et al. (1988) reported a flocculation efficiency greater than 80% for marine microalgae using ferric chloride. Bintisaarani (2012) found the ferric chloride flocculation to be the most effective for harvesting *Nannochloropsis* species and reported a removal efficiency of 89% using a concentration of 0.9 M and a pH of 7.5.

The use of aluminum (alum) salts has been noted to effectively flocculate the microalgae species *Chlorella* and *Scenedesmus* (Grima et al., 2003). Papazi et al. (2010) found aluminum salts to be more effective in flocculating *Chlorella* species than ferric salts. Shelef et al. (1984) noted that alum was a superior flocculating agent compared to ferric sulfate in terms of pH, amount of flocculant and the quality of the final water slurry. The work of Kwon et al. (2014) reported a flocculation efficiency of 92.6% for *Tetraselmis* sp. using aluminum sulfate dose of 1.2 g/L at a pH of 5-6. Millamena et al. (1990) stated that alum was effective in flocculating *Chaetoceros calcitrans, Tetraselmis chui, Skeletonema costarum* and *Isochrysis goibana* species at a pH of 6.5. Furthermore, Aragon et al. (1992) used aluminum sulfate to harvest a culture made up of *Scenedesmus acutus* (80%) and *Chlorella vulgaris* (20%) using a dosage of 30-50 mg/L at a pH of 6-6.5.

<u>*Organic flocculation*</u>

Organic polymers (chitosan) or polyelectrolyte (polyelectolyte polyamine) flocculants are known as polymeric flocculants (synthetic and natural) that consist of both ionic and non-ionic species. The use of organic compounds for flocculation works by combining

both particle bridging, and charge neutralization. The charge density and polymer chain length determines the extent to which each is used. The process begins by the attachment of the polymer onto the microalgal surface through chemical or electrostatic forces (Uduman et al., 2010). The polymer is able to attach to the surface of the cells through Coulombic (charge-charge), dipole-dipole, van der Waals or hydrogen interactions (Uduman et al., 2010). Coulombic force attraction works by having unlike charges on the surface of the polymer and the microalgae attach to one another, following the notion that like charges repel one another and unlike charges attract one another. Dipole-dipole interactions occur when two polar molecules approach one another and the partially negative portion bonds to the partially positive one. Van der Waals forces are the attraction of intermolecular forces between molecules. Hydrogen bonding is a type of dipole-dipole attraction in which a hydrogen atom is bonded to a highly electronegative atom nearby (Bondi, 1964). In this manner, the polymer attaches to the surface leaving its tail out into the solvent forming loops. The loops and tails of the polymer allow it to attach to other cells to from bridges between them (Hunter, 1993).

The efficiency of this flocculation process depends on the degree to which the polymer covers the microalgae cells. If the attachment of the polymer to the cell surface is less than the optimum amount, then it may not be able to withstand shear forces as a result of agitation. On the other hand, excess coverage of the polymer onto the cell surface can cause static hindering of the bridging process (Hogg et al., 1999).

Recent studies have revealed that cationic polyelectrolytes flocculant agents are the most effective for microalgae recovery (Uduman et al., 2010; Granadoes et al. 2012). Granadoes et al. (2012) noted that inorganic flocculants were less efficient in the flocculation of *Muriellopsis* sp. species than organic agents. The study of Knuckey et al. (2006) reported that adding 0.5 mg/L of non-ionic polymer Magnafloc LT25 (anionic polyacrylamide from BASF chemical company) to a medium with a pH adjusted to 10-10.6 effectively concentrated and settled a wide range of microalgae species at rates that are 200-800 times higher than the control treatment. Harith et al. (2009) maintained a high microalgae cell viability (75%) for the *Chaetoceros* species using Magnafloc LT25 flocculant at a dosage of 1 mg/L. Further, they stated that increasing the Magnafloc LT 25

and Magnafloc LT 27 dosage did not increase the flocculation efficiency but increased the settling rates.

The work of T'Lam et al. (2014) reported a 98% flocculation efficiency for the species *P Phaeodactylum tricornutum* using Zetag flocculant at 10 ppm, but only achieved a 52% recovery for the *N. oleoabundans* species. Udom et al. (2013) found that using Zetag at a dosage of 34 mg/l flocculated microalgae and resulted in a 98% recovery efficiency. Similarly, Buelna et al. (1990) noted a 95-100% removal efficiency for *Chlorella* culture using 5 mg/L Zetag 63 at a pH of 6-9.

Pushparaj et al. (1993) flocculated *Teraselmis* and *Spirulina* with a 70% biomass recovery efficiency using praestol (a cationic organic polyacrylamide based flocculant) with no inhibitory effects on recycled and reused media. However, inhabitation of flocculation has been noted for organic cationic polymers in environments with salinity above 5 g/L (Grima et al., 2003; Knuckey et al., 2006). Sukenik et al. (1988) found that the amount of flocculant required to remove 90% of microalgae from liquid suspension increased linearly with increased salinity.

The study of Chen et al. (2014) explored the use of chitosan, and found that the general dosage range required to effectively flocculate microalgae species was 5-200 mg/L. This was also demonstrated by the various studies investigating the use of these flocculants. The work of Xu et al. (2013) noted a 99% clarification efficiency for *Chlorella sorokiniana* using chitosan at an optimal dosage of 10 mg/g dried microalgae and pH values below 7. Ahmed et al. (2011) achieved a 99% flocculation efficiency in 20 minutes for *Chlorella* sp. with a chitosan dosage of 10 ppm. Chang and Lee (2012) reported a flocculation efficiency of 99% for *Chlorella vulgaris* using chitosan at a dosage of 200 mg/L and a pH of 8.7. Sirin et al. (2012) reported a flocculation efficiency of 92% in 10 min for the *P. tricornutum* species using chitosan (20 mg/L). Morales et al. (1985) noted a 100 % flocculation efficiency for *Chlorella* sp. using chitosan at a concentration of 40 mg/L.

Factors Affecting Chemical Flocculation

Inorganic flocculation

The factors affecting inorganic flocculation include: concentration of the flocculant, pH and the surface charge of the flocculant.

Flocculant concentration**:** The concentration at which the flocculant is administered into the system has been noted to affect the efficiency of the microalgae recovery. Rakesh et al. (2014) used aluminium sulfate concentrations ranging from 50 to 300 mg/L for the recovery of *Chlorella sp*., *Chlorococcum sp*. and *Chlorella sorokiniana* and found 50 mg/L to be the most effective dose. The work of Garzon-Sanabria et al. (2012) evaluated the recovery efficiency of *N. oculata* using aluminum chloride at concentrations in the range of 50-100 mg/L and found 50 mg/L to be the most effective dose. Ferriols and Aguilar (2012) reported on the use of calcium chloride and sodium hydroxide at concentrations of 100-200 mg/L for the recovery of *Tetraselmis terrahele* and achieved the highest recovery efficiency at 200 mg/L. Wyatt et al. (2012) noted that in media with low algae concentrations, the concentration of ferric chloride required to flocculate *Chlorella zofingiensis* increases linearly with cell concentration.

pH**:** It was determined that varying the pH of the medium using inorganic flocculation can promote cell aggregation as a result of changes in surface charge, see below. Knuckey et al. (2006) noted effective flocculation (>80%) of *Chaetoceros calcitrans*, *Chlorella muelleri*, *Thalassiosira pseudonana*, *Attheya septentrionalis*, *Nitzschia closterium*, *Skeletonema* sp., *Tetraselmis suecica* and *Rhodomonas salina* by altering the pH with the addition of sodium hydroxide. Garzon- Lee et al. (1998) noted that changing sodium hydroxide concentration affected the flocculation efficiency of *Botryococcus braunii* as a result of pH change in the medium. Sanabria et al. (2012) used aluminum chloride (50-100 mg/L) to modify the pH (4-7) and achieved the highest recovery efficiency of *N. oculata* using a dosage of 50 mg/L (pH =5.3). Finally, Sanyano et al. (2013) successfully flocculated *Chlorella* sp. using ferric chloride at a pH of 8.1.

Surface charge**:** Microalgae cell surfaces are negatively charged, indicating that a positively charged flocculant would be required to bind the cells to one another (Wyatt et al., 2012; Sanyano et al., 2013). Algal coagulation is induced by the attraction of the

positively charged flocculant onto the negatively charged cell surface, and the attachment of another algal cell onto the positively charged flocculant (Chang et al., 2012). The efficiency of the flocculation is dependent on the amount of flocculant available to bridge the algae to one another (Ching et al., 1994; Wyatt et al., 2012). Wyatt et al. (2012) noted that the positive nature of ferric chloride induced microalgae flocculation with a recovery efficiencies of 90% at a pH above 4.1 and below 8. Knuckey et al. (2006) flocculated microalgae with an efficiency of 80% using Fe^{3+} ions. Garzon-Sanabria et al. (2012) recovered *Nannochloris oculata* with a 90% efficiency using aluminium chloride to counteract the surface charge of the microalgae cells at a pH of 5.3. Sanyano et al. (2013) successfully flocculated *Chlorella* sp. using ferric chloride. Lee et al. (2013) achieved 100% flocculation efficiency in *Chlorella* sp. using synthesized cationic aluminum and magnesium organoclays.

Organic flocculation

The factors affecting organic flocculation include: pH, charge on polymer, dosage and salinity.

pH: Some microalgae species can flocculate together by adjusting the pH (Grima et al., 2003). Uduman et al. (2010) stated that the pH and the chemical composition of the microalgal medium impact the amount of flocculant required. They noticed less electrostatic repulsion between colloids at low pH levels resulting in increased amounts of bridging since the polymer chains are longer. They also found the dose of polymeric flocculant required to vary with microalgae concentration, because of the charge in surface area of algae. Knuckey et al. (2006) reported that the non-ionic polymer Magnafloc LT25 settled a wide range of microalgae species effectively using 0.5 mg/L in a pH adjusted media of 10-10.6. Tenney et al. (1969) noted the most effective flocculation resulted when using cationic polyelectrolytes at low pH levels. Ras et al. (2011) noted that the *Chlorella* species flocculated when the pH was increased to 11-12. Lee et al. (2009) stated that extreme pH levels can result in cell death or impairment.

Charge on polymer: The polyelectrolyte charge also plays an important role in the flocculation process of microalgae. Anionic polyelectrolytes are not effective flocculant agents on their own due to the negatively charged microalgae cell surface because like

charges repel one another and/or the length of the polymer is not sufficient enough to bridge the particles together (Tenney et al., 1969; Bernhardt et al., 1991). It is for this reason that cationic polyelectrolytes are found to be much more effective in the flocculation of microalgae. Morrissey et al. (2014) noted that the N,N-dimethylaminopropyl acrylamide polymer (positive character) resulted in recovery efficiencies of microalgae greater than 99% at a pH of 7 and increasing the pH to 13 (activating negative functionality) resulted in flocculation efficiencies of less than 12%. Chang and Lee (2012) noted that the positively charged surface of chitosan resulted in a 99% removal efficiency for *Chlorella vulgaris*.

Flocculant concentration: The amount of cationic flocculant required for effective bridging between the cells depend on the amount of negative charges present in the system, the surface charge density, the cell counts per volume, the total cell surface area and the charge density of the positively charged polyelectrolyte. The negative charge on the surface is induced by the functional groups (carboxyl groups) present on the microalga cells which have been noted to affect the isoelectric point of the cells (Bernhardt and Clasen, 1994). Uduman et al. (2010) stated that the growth phase and the metabolic conditions of the microalgal cells dictate the concentration and the reactivity of these functional groups. The study of Granados et al. (2012) showed that the species *Chlorella* and *Scenedesmus* were effectively flocculated to a concentration of 2 g/L after 15 min using polyelectrolyte dosages of 2-25 mg/g. Further, Tenney et al. (1969) stated that the cationic polyelectrolyte polyamine was effective in flocculating the microalgae cells at a dosage of 2.5 mg/l. Sukenik et al. (1988) reported that marine microalgae *Isochrysis galbana* and *Chlorella stigmatophora* require 5-10 times more flocculant dosages than those required by freshwater microalgae.

Salinity: The salinity level affects the organic flocculation of microalgae. Bilanovic and Shelef (1988) noted that the polyelectrolyte flocculant was inhibited in the marine medium due to its high salinity and observed effective flocculation at salinity levels below 5 g/l. This was attributed to the fact that high ionic strength causes the polymer to shrink in dimension, thus failing to form a bridge to link the microalgal cells. However, Schlesinger et al. (2012) reported that the addition of alkali to *Chlamydomonas* did not result in rapid flocculation in the saline medium.

Advantages and Disadvantages of Chemical Flocculation

In comparison with other methods, chemical flocculation is considered to be one of the best methods for cell harvesting because it can handle large amounts of microalgae, it can be used with a wide range of species, it is reliable and it is cost-effective (Uduman et al. 2010; Benemann et al. 1980). Beach et al. (2012) compared the chitosan flocculation, centrifugation and filtration methods for microalgae harvesting and noted that chitosan flocculation was the least energy consuming method of the three.

The costs of inorganic flocculants are much less than those of organic ones (Shammas, 2005; Papazi et al., 2010; Zhou et al. 2014). However, the higher amounts required using inorganic flocculants can result in higher costs per unit of microalgae than the more expensive organic flocculants (Mohn, 1988). Sukenik et al. (1988) reported that the optimal dosage of inorganic flocculant required to flocculate marine microalgae was 5-10 times higher than that required to flocculate freshwater microalgae. Shammas (2005) also noted that the higher cost of organic coagulants can be offset by the low dosages required compared to those of inorganic flocculants.

Microalgae harvesting techniques using chemical flocculation are not environmentally friendly because they introduce chemicals into the system which increase the dissolved solids and change color (Bukhari, 2008). Inorganic flocculants can be toxic and can also have negative effects on microalgae by modifying their growth media and changing their color which prevents the reuse and recycling of water (Grima et al., 2003; Schenk et al., 2008; Papazi et al., 2010). Both Hee-Mock et al. (2001) and Vandamme et al. (2013) stated that chemical flocculants that are toxic and carcinogenic and are not suitable for harvesting microalgae biomass that is being processed for food supplements and food additives. Therefore, selection of flocculant should be based on cost, toxicity and reusability of the media (Grima et al., 2003).

2.2.4.2 Autoflocculation

Some microalgae species can flocculate spontaneously in response to certain environmental stresses. This phenomenon is known as autoflocculation.

Environmental Conditions for Inducing Autoflocculation

There are several factors that affect the efficiency of autoflocculation, which include: pH, dissolved oxygen content, nitrogen concentration and the amount of calcium and magnesium ions in solution.

pH**:** When the pH of the medium is increased, the cells come together and settle by gravitational force. The addition of more base increases the formation of flocs which result in shorter settling times. However, it is important to note that not all species flocculate with increased pH levels (Grima et al., 2003; Wu et al., 2012).

The study of Harith et al. (2009) noted that at pH values less than 10, only slight separations between the microalgae and liquid media occurred after 4 h and increasing the pH from 8 to 10 using NaOH and KOH increased the flocculation efficiency from 13 to 82% and from 35 to 78% in 4 h, respectively. Further, Wu et al. (2012) found that a pH of 10.5 resulted in 90% flocculation efficiency for the freshwater species *Chlorella vulgaris*, *Scenedesmus* sp. and *Chlorococcum* sp. and a pH of 9.0-9.3 resulted in 90% flocculation efficiency for the marine species *Nannochloropsis* sp. and *Phaeodactylum tricornutum*. The investigation of Horiuchi et al. (2003) reported a 96% flocculation efficiency in the marine species *Dunaliella tertiolecta* when the pH was adjusted to 8.6. Millamena et al. (1990) also noted effective flocculation of microalgae when the pH was maintained above 10 in salt water.

Dissolved Oxygen**:** It is thought that increased dissolved oxygen in solution triggers autoflocculation by increasing the binding sites available on the cell surface (Schenk et al., 2008; Uduman et al., 2010). Greater binding sites result in bulk formation of the cells which increases the weight of the flocs and increases the settling rate (Liao et al., 2011). Increased photosynthetic activity by the microorganisms increases the dissolved oxygen content and the formation of dense flocs (Liao et al., 2011). Wilen and Balmer (1999) also observed that large flocs can be generated when the dissolved oxygen concentration is high in the media. Similarly, Koopman et al. (1982) noted that dissolved oxygen concentrations of 14-16 mg/l promoted flocculation in the system.

Nitrogen**:** Autoflocculation in microalgae cells may be triggered naturally as a result of environmental stress caused by nitrogen concentration (Schenk et al., 2008; Uduman et al.,

2010). Sukenik and Shelef (1984) noted that certain species of microalgae flocculate with one another as a result of nitrogen stress in the media. Becker (1994) found that microalgae cells can aggregate with one another as a result of nitrate assimilation. Assimilation of nitrate nitrogen increases the pH of the medium and promotes cell flocculation (Uusitalo, 1996; Wu et al., 2012). Nurdogen and Oswald (1995) also noted that nitrate assimilation resulted in auto-flocculation in microalgae species. Nguyen et al. (2014) noted a nitrate concentration of 840.4 mg/L was sufficient in flocculating *Chlorella vulgaris* in mBB medium.

***Addition of Ca^{2+} and Mg^{2+}*:** Autoflocculation has also been reported to occur in the culture media spontaneously as a result of coprecipitation of calcium and magnesium salts present in the media which results in changing the pH of the medium (Shelef et al., 1984). Smith and Davis (2012) evaluated Mg^{2+}, Ca^{2+} and CO_3^{2-} ions for their effectiveness in flocculating and settling microalgae cells and found that Mg^{2+} ions with high pH levels resulted in effective flocculation and rapid sedimentation. They achieved settling rates that were 100-fold higher than those achieved with sedimentation. The reason for this phenomenon is that magnesium hydroxide flocs are positively charged while calcium carbonate flocs are negatively charged (Ayoub et al., 1986). Thus, destabilization of the negatively charged microalgae cells is greater using magnesium as opposed to calcium. However, the optimal pH for autoflocculation is strain specific (Sukenik and Shelef, 1984). Nguyen et al. (2014) reported that the species *Chlorella vulgaris* autoflocculated with an efficiency of 90% by addition of Ca^{2+} and Mg^{2+} at concentrations of 120 mg/l and 1000 mg/l, respectively. Furthermore, Vandamme et al. (2012) noted that addition of Mg^{2+} in *Chlorella vulgaris* culture induced autoflocculation.

Advantages and Disadvantages of Autoflocculation

The advantages of this harvesting technique are the simplicity and low operational costs. The process can be reverted by pH adjustment using HCl to decrease the pH back to 7.5-8 (Knuckey et al., 2006). Using pH flocculation is beneficial since pH induced flocculated cells are identical to non-flocculated microalgae cells. This means that this autoflocculation technique has low-shear force on the cells when compared to centrifugation (Knuckey, 1998). Knuckey et al. (2006) found that the chlorophyll *a* from

T. pseudonana cultures were intact using pH-induced methods, but centrifuged microalgae cells only had slight chlorophyll *a* peaks. This is important when the microalgae biomass is required for use as feed or for the extraction of certain compounds (such as chlorophyll a) from the cell. Furthermore, they noted that the harvested microalgae species using pH induced flocculation were a better diet choice for oyster feed compared to those harvested by centrifugation and much better than those harvested using ferric chloride flocculation. D'Souza et al. (2002) also reported that pH induced harvesting of *C. muelleri* for feed to tiger prawn *P. mondon* was only slightly slower in developmental rate compared to those using fresh *C. muelleri*. However, autoflocculation does not occur in all species making it an unreliable process (Schenk et al., 2008).

2.2.4.3 Bio-flocculation

The use of microorganisms for the recovery of microalgae biomass has been investigated and summarized in Table 2.3. This method works by the addition of microorganisms to the culture which adhere to the microalgae cells causing the weight to increase and resulting the cells settling to the bottom of the vessel. The supernatant containing the culture medium is decanted and washed with water in order to reduce the salinity (Hanifa et al., 2011; Gonzalez-Fernandez and Ballesteros, 2013).

Grima et al. (2003) noted the effective flocculation of *Chlorella* using bio-flocculant from bacteria species. The study of Oh et al. (2001) successfully harvested *Chlorella vulgaris* using the bio- flocculant bacterium *Paenibacillus* sp. Kim et al. (2011) noted effective flocculation of the species *Scenedesmus* sp. using the bio-flocculant *Paenibacillus polymixa.* The work of Ndikubwimana et al. (2014) consisted of harvesting the microalgae species *Desmodesmus sp.* using the bacterium *Bacillus licheniformis*, which resulted in a 98% removal efficiency. Zheng et al. (2012a) co-cultured the species *Chlorella vulgaris* with different filamentous fungi and extracted the microbial oil for transesterification into biodiesel.

Factors Affecting Bio-flocculation

The factors affecting bio-flocculation include: concentration of the bio-flocculant, pH and the selectivity of the microorganism.

Table 2.3. Bio-flocculation of microalgae by use of fungi and bacteria microorganisms.

Microorganism	**Bio-Flocculated Microalgae**	**Reference**
Bacteria		
Bacillus licheniformis	*Desmodesmus sp.*	Ndikubwimana et al. (2014)
P. stutzeri & B.cereus	*Pleurochrysis carterae*	Lee et al. (2009)
Paenibacillus sp.	*Chlorella vulgaris*	Oh et al. (2001)
Paenibacillus polymixa	*Scenedesmus* sp.	Kim et al. (2011)
Bacillus subtilis	*Chlorella vulgaris*	Zheng et al. (2012)
Fungi		
Ankistrodesmus falcatus	*Chlorella vulgaris*	Salim et al. (2011)
Scenedesmus obliquus	*Chlorella vulgaris*	Salim et al. (2011)
Tetraselmis suecica	*Nannochloropsis oleabundans*	Salim et al. (2011)
Skeletonema	*Nannochloropsis*	Schenk et al. (2008)

***Bio-flocculant concentration*:** The rate at which bio-flocculation is achieved depends on the ratio of the bio-flocculant to the non-flocculating microalgae species. Bio-flocculant concentrations that are greater than the concentration of non-flocculating microalgae increase the rate of sedimentation (Salim et al., 2011). Lee et al. (2009) found that the addition of bacteria to the non-flocculating microalgae culture increased the rate of sedimentation. The study of Salim et al. (2011) successfully harvested non flocculating microalgae by addition of bioflocculating species and noted that the addition of bioflocculating microalgae induced the microalgae sedimentation and increased the efficiency of harvesting. Oh et al. (2001) reported that the flocculation efficiency of *C. vulgaris* using the bacterium *Paenibasillus* sp. decreased with increasing dilutions of the bacterium. The work performed by Zheng et al. (2012a) found that the flocculation efficiency of *C. vulgaris* using the bio-flocculant *B. subtilis* increased with increasing concentrations of *C. vulgaris* biomass. Lee et al. (2013) noted that *C. vulgaris* flocculation efficiency increased with increasing bacteria (*Flavobacterium*, *Terrimonas* and *Sphingobacterium*) concentration in the culture.

***pH*:** The efficiency of bio-flocculation is affected by the pH of the medium. The pH alters the surface charge of the molecules in the medium which dictate the degree of attraction/repulsion. Oh et al. (2001) reported that the flocculation efficiency of *C. vulgaris* using the bacterium *Paenibasillus* sp. increased with increasing pH from 5 to 11. Ndikubwimana et al. (2014) noted that the flocculation efficiency of *Desmodesmus* sp. increased from 43 to 98% using the bacterium *Bacillus licheniformis* as the pH decreased from 7.2 to 3. Lee et al. (2013) found that pure *C. vulgaris* cultures showed no flocculation as the pH was increased from 3-11, but cultures with bacteria demonstrated increased flocculation efficiencies (from 43 to 94%) with increases in the pH over the range of 3-11. However, Zheng et al. (2012) noted that the bio-flocculation efficiency using *B. subtilis* with microalgae species *Chlorella vulgaris* and *Chlorella protothecoides* were not effected by pH.

***Species Selectivity*:** Bio-flocculants are species specific (Oh et al., 2001). This suggests that not all bio-flocculants will flocculate varying types of microalgae species. Oh et al. (2001) reported that the bio-flocculant bacterium *Paenibasillus* sp. resulted in flocculation efficiencies in the range of 91-95% for *Botryococcus braunii*, *Scenedesmus quadricauda*,

C. vulgaris and *Selenastrum capricornutum*, but an efficiency in the range of 38 to 49% was noted for *Anabaena flos-aquae* and *Microcystis aeruginosa*. Grossart et al. (2006) reported that bacteria were successful in aggregate formation of *Thalassiosira weissflogii* but had little effect on flocculation of *Navicula* sp. Oh et al. (2001) and Kim et al. (2011) noted that the flocculating bacterium *Paenibasillus* resulted in a flocculating efficiency of 83% and 95% with *Chlorella vulgaris* and *Scenedesmus* sp., respectively.

Advantages and Disadvantages of Bio-flocculation

The advantages of using bio-flocculants include their biodegradability, non-toxic nature and that the intermediates formed during degradation are not secondary pollutants (Salehizadeh and Shoaosadati, 2001). Salim et al. (2011) noted that a two step harvesting process using naturally flocculating microalgae to induce non-flocculating microalgae followed by centrifugation reduced the energy use from 13.8 MJ/kg (dcw) to less than 2 MJ/kg (dcw).

The disadvantages of this technique are that the microorganisms used to flocculate the algae are species-specific, and the recycling and recovery of these organisms can be difficult (Grima et al., 2003; Shen et al., 2009). It was stated by Oh et al. (2001) that the bio-flocculants used to dewater the microalgae cells should be tested for acute oral toxicity in order for the retrieved biomass to be used in food additives and feed supplements. Vandamme et al. (2013) indicated that the use of fungi or bacteria as flocculating agents results in microbiological contamination of the microalgae biomass, which needs to be assessed before use in feed or food products.

2.2.5 Electrophoresis

Electrolytic methods are used to eliminate costly and toxic chemicals, since microalgae behave much the same as colloid particles which allows for their separation from a water based medium by application of an electric field (Uduman et al., 2010). The main electrophoresis methods that can be used for harvesting microalgae are: electrolytic coagulation, electrolytic flocculation and electrolytic flotation.

2.2.5.1 Electrolytic Coagulation

This type of electrophoresis method requires both physical and chemical stimuli for the effective separation of microalgae biomass. The coagulation process is induced by the generation of current from an iron or aluminum electrode (Cerqueira and Marques, 2012). The amount of electrical current passing through the water medium dictates the amount of metal ions dissolved into the liquid suspension (Mollah et al., 2004). The metal ions released into the solution are metal hydroxides that contribute to the destabilization of colloid suspension and coagulate the biomass. Flocculation is achieved by linking the positively charged metal to the negatively charged microalgae cell and the movement toward the anode as a result of electrophoretic motion (Mollah et al., 2004; Bukhari, 2008). Rapid coagulation results from high current densities but the cost associated with the method is high.

The work of Uduman et al. (2011), used an aluminium electrode set at 5 V and achieved electrocoagulation efficiencies of 93.3% and 87.3% in 600 s for the species *Tetraselmis* sp. and *Chloroccum* sp., respectively. In the study of Azarian et al. (2007), they recovered 99.5% of TSS, in 15 minutes by electrocoagulation using a power source of 550 W with aluminium anode. They also noted that a power supply of 100 W required 30 minutes to achieve similar results. Furthermore, Ghernaout et al. (2008) achieved a 99% removal of *Escherichia coli* in 20 min by electrocoagulation using aluminium electrode with a power supply of 12 W.

2.2.5.2 Electrolytic Flocculation

Electrolytic flocculation works by movement of negatively charged cells toward the anode (Uduman et al., 2010). At the anode, the cells lose their charge, forming flocs that can be lifted to the surface by adhering to the bubbles formed by the electrolysis of the water (Poelman et al., 1997).

Poelman et al. (1997) tested the effectiveness of electrolytic flocculation in a 100 L vessel equipped with vertical electrodes and noted a removal efficiency of 80-95% in 35 min. They also noted that the rate of microalgae removal decreased with decreasing voltages and less energy was consumed when the total surface area of the electrodes was decreased and/or the distance between the electrodes was decreased.

The work of Xu et al. (2010) noted a recovery efficiency of 93.6% for *Botryococcus branii* after 30 minutes using electrolytic flocculation technique with a power supply of 6 W. Lee et al (2013) noted recoveries of 85% and 95% after 60 minutes for marine microalgae using electrolytic flocculation with a power supply of 5 V and 5.2 V, respectively. Zenouzi et al. (2013) reported a 97.4% removal efficiency of *Dunaliella salina* after 3 min using electrolytic flocculation.

2.2.5.3 Electrolytic Flotation

This technique is similar to electrolytic coagulation in that active metal anodes are used to flocculate the microalgae cells. The difference between the two techniques is that the cathode in electrolytic flotation is made from an inactive metal (steel) that is electrochemically nondepositing (Santos et al., 2013). The inactive metal forms hydrogen bubbles from the electrolysis of the water. The particulates in the suspension attach to the gaseous bubbles and are lifted to the surface of the vessel (Alfafara et al., 2002; Azarion et al., 2007).

The use of electrolytic flotation was investigated by Alfafara et al. (2002) on microalgae cells using a polyvalent aluminium anode and titanium alloy cathode, and it was determined that that increasing the electrical power decreased the electrolysis time and increased the rate of chlorophyll *a* removal. They also noted that the amount of chlorophyll measured was related to the concentration of microalgae removed by electrolysis. The usage of high electrical power is limited by the increase in heat and the increase in pH.

De Carvalho Neto et al. (2014) reported a chlorophyll *a* removal efficiency of 99% using an electroflotation method running for 140 min with a power supply of 60 W. Similarly, Ghernaout et al. (2014) noted a microalgae removal efficiency of approximately 100% after 140 minutes in Ghrib Dam water using electroflotation method. Shelef et al. (1984) found that electroflotation technique resulted in TSS in the range of 3-5%. Despite the efficiency of the technique, Brennan and Owende (2010) stated that there is little proof of the economic feasibility of this recovery method.

2.2.5.4 Energy Consumption

Dewatering microalgae by electrophoresis techniques requires less energy (0.2-2.1 Wh/g) compared to other harvesting methods (0.18-35.62 Wh/g). It was reported by Vandamme et al. (2011) that the amount of energy required to flocculate the freshwater microalgae *C. vulgaris* and the marine *P. tricornutum* species was 2.1 Wh/g and 0.2 Wh/g (dcw), respectively. Gonzalez-Fernandez and Ballesteros (2013) noted that the marine microalgae required less energy for harvesting, because the marine medium allows for a higher conductivity that favors the electrocoagulation process. The work performed by Kim et al. (2012) on the electrical energy consumption of polarity exchange using two types of electrodes was found to range from 1.19 to 1.23 Wh/g for achieving a 99% harvesting recovery of microalgae after 15 min. Bektas et al. (2004) noted that dewatering of microalgae using electrocoagulation by 0.8-1.5 Wh/g of microalgae culture whereas cross flow filtration, pressure filters, vacuum filters, flocculation using polymers and centrifugation have been reported to consume 3.47, 0.18, 1.19, 1.67 and 35.62 Wh/g, respectively (Grima et al., 2003; Danquah et al., 2009).

2.2.5.5 Advantages and Disadvantages

The advantages of the electrophoresis harvesting technique include: versatility, energy efficiency, safety, selectivity, environmental compatibility and cost effectiveness (Mollah et al., 2004). Minimum energy is consumed when using optimum potential difference (0.331 kWh/m^3) by controlling the electrode surface area and distance between the electrodes. There are no added costs associated with flocculant products (Uduman et al., 2010). The costs associated with dewatering microalgae via electrolytic methods were significantly less than other harvesting methods such as sedimentation with flocculants, centrifugation and flotation with flocculants (Poelman et al., 1997). This indicates that although higher electrical energy is consumed using electrolytic methods, the cost to harvest is much lower than other harvesting techniques.

Some of the drawbacks associated with this harvesting technique include: cathode fouling and change in cell composition (Poelman et al., 1997). The current intensity decreases by 5-10% upon reuse of the cathode due to internal resistance (Vandamme et al.,

2011) and changes in cell composition can be induced using high current densities (Gonzalez-Fernandez and Ballesteros, 2013).

2.2.6 Flotation

Flotation is a widely used technique for the separation of dilute solid particles from liquid suspensions (Pan, Jung, & Yoon, 2012). Flotation separation works on the basis of varying surface activity (Matis & Lazaridis, 2002), and consists of three major phases which are the water, particles in suspension and the air bubbles (Zouboulis, Matis, & Stalidis, 1992). In the earlier stages of collision, which are a result of bubble deformation, a wetting film or thin liquid film, is formed between the particle and the bubble (Pan et al., 2012). The destabilization of the wetting film results in the linkage of the hydrophobic particle to the bubble (Sutherland & Wark, 1955; Klassen & Mokrousov, 1963). The flotation process scheme is depicted in Figure 2.1 (Krofta & Wang, 2000).

The primary factor influencing the rise rate of the bubble-particle complex in an unaerated system, is the density of the particles. A reduction in the density of the agglomerate, would increase the rise rate of the bubble-particle complex, through the attachment of an air bubble onto the particles in solution carrying it up to the surface (Krofta & Wang, 2000). The use of flotation to separate particles from liquid suspension follows the same principles as those of sedimentation, but occur in the "reverse field of force" (Shammas & Bennett, 2010). The air flotation separation process can be described using Stoke's Law, which is used in determining the rate of bubble-particle rise (Krofta & Wang, 2000; Wang, Fahey & Wu, 2005).

$$V_t = \frac{gD^2(\rho_a - \rho_o)}{18\mu} \tag{1}$$

Where V_t is the terminal rise velocity of the bubble-particle complex, cm/s; g is the gravitational constant, 980 cm/s^2; D is the agglomerate diameter, cm; ρ_a is the density of the agglomerate, g/cm^3; ρ_o is density of the aqueous phase, g/cm^3; and μ is the viscosity of the aqueous phase, cp.

The flotation processes are grouped by the method that is used for the bubble formation, which consist of the following types: electrolytic flotation (see section 2.2.5), fluidic oscillation, dissolved air flotation, dispersed air flotation.

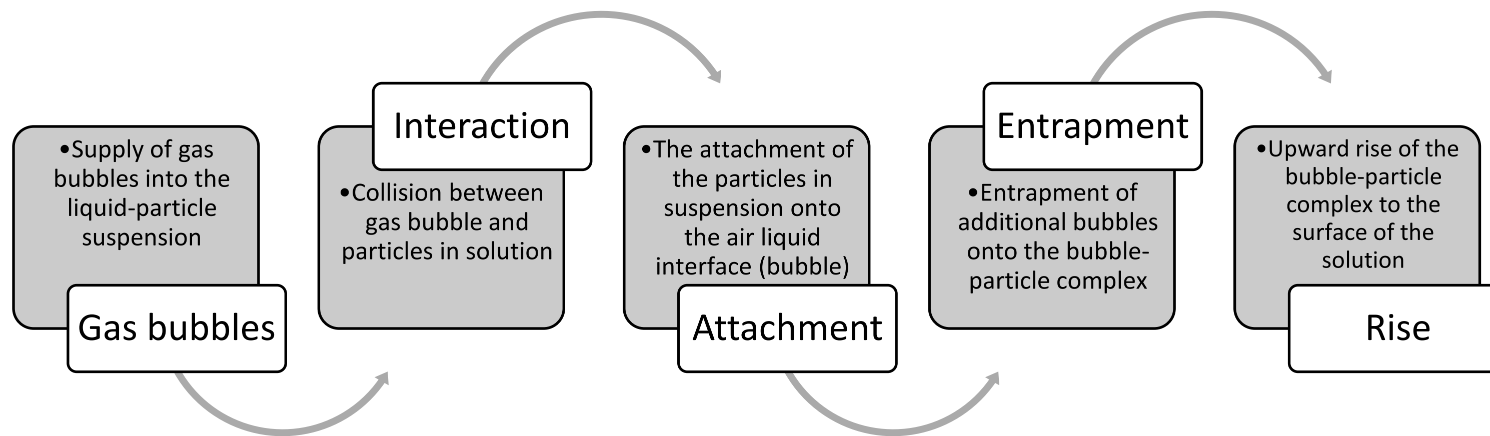


Figure 2.1. Flotation process scheme (after Krofta & Wang (2000)).

2.2.6.1 Fluidic Oscillation

The recovery of microalgae can be achieved by micro-bubble generation through fluidic oscillation (Hanotu et al., 2012). This method works by converting a continuous air supply into oscillatory flow with a regular frequency, generating bubbles that are the size of the exit pores (Hanotu et al., 2012). The miniature bubbles are formed by fitting a diffuser to the bi-stable valve which ensures that the bubbles formed are approximately 10 times smaller than those originally dispersed in flotation methods (Zimmerman et al., 2009). Fine bubbles that are the size of the exit pores are generated by use of a fluidic oscillator (Hanotu et al., 2012). The bubbles formed attach to the hydrophobic cells suspended in solution and carry them to the surface. At the surface the bubble ruptures leaving the cells behind (Dai et al., 2000). This technique has been employed with microalgae by various authors.

The work performed by Hanotu et al. (2012) noted a microalgae recovery efficiency of 99.2% using microbubble generation at a pH of 5 with the aid of ferric chloride coagulant (150 mg/L). Elder (2011) reported a removal efficiency greater than 95% using micro-bubble flotation. Yap et al. (2014) noted a removal efficiency greater than 95% for removal of *Microcystis* and filamentous *Cylindrospermospsis* using micro-bubble flotation. However, not much research has been performed using this technique for microalgae recovery to deem it economically suitable for large scale recovery of microalgae cells (Milledge and Heaven, 2013).

2.2.6.2 Dissolved Air Flotation

The dissolved air flotation technique is the most widely used flotation technique in the treatment of industrial effluent (Matis et al., 1993; Rubio et al., 2002). This method requires a reduction in water pressure that is presaturated with air. The liquid is then injected into the flotation tank at atmospheric pressure (Rubio et al., 2002). Bubbles are generated from the diffuser nozzles and rise through the liquid carrying the microalgae cells in the suspended media to the surface of the tanks. The cumulated biomass at the surface can be skimmed off and collected. The clarified liquid portion is saturated with air and recycled back into the flotation tank (Rubio et al., 2002). The supply of air into the

system can be controlled by changing the saturator pressure or by changing the ratio that is recycled back into the tank. The size of the bubbles formed can be controlled by the saturator, operated above atmospheric pressure and by the injection flow rate (Edzwald, 2010; Uduman et al., 2010). The flow rate must be great enough to prevent backflow, provide a pressure drop and allow for bubble growth on the pipes surface (Uduman et al., 2010). Small bubbles ranging in size from 10 to 100 μm (with a mean size of 40 μm) are desirable (Edzwald, 2010).

The dissolved air flotation microalgae separation is usually coupled with the use of a chemical flocculation process. Edzwald (1993) investigated the use of dissolved air flotation for microalgae recovery and noted that this method required pretreatment by flocculation, but was more successful than the settling technique alone. Wiley et al. (2009) used dissolved air flotation for microalgae harvest and noted a SS concentrate of 5% with an energy consumption of 7.6 kWh/m^3. Goh (1984) noted that this method was effective in harvesting microalgae from pig slurry when coupled with the alum flocculant with a high dosage of 0.3 g/L.

2.2.6.3 Dispersed Air Flotation

Dispersed air flotation works by the injection of compressed air through the pores of a diffuser to produce bubbles, with a typical diameter range of 60 to 655 μm (Ramirez, 1979; Dafnopatidou & Lazaridis, 2008), and a bubble number of $2x10^5$ per cubic centimeter (Ramirez, 1979). The bubbles that float through the microalgae-liquid suspension attach to the microalgae cells, thereby carrying them to the surface (Uduman et al., 2010). The effectiveness of the flotation process depends on the instability of the suspended particles, where the air-particle contact is improved with lower instability (Shelef et al., 1984). Particle size is another important influence on the effectiveness of flotation, where particle sizes of less than 500 μm are most suited for this technique (Matis, Gallios, & Kydros, 1993). In addition to these factors, the particle capture by the bubble, plays a role on the effectiveness of the process (Matis et al., 1993). Furthermore, the flotation of dilute microalgae suspensions has also been improved through the use of surfactants (Levin, Clendenning, Gibor, & Bogar, 1962; Liu, Chen, & Ju, 1999; Csordas & Wang, 2004; Garg, Li, Wang, & Schenk, 2012; Coward, Lee, & Caldwell, 2014).

It is thought that the use of surfactants in dispersed air flotation can enhance the resistance of the bubbles to rupture by adsorbing onto the air/liquid interface (Figure 2.2), increase the hydrophobicity of the solid particles by migration to the solid surface (Chen, Liu, & Ju, 1998; Bulatovic, 2007; Garg et al., 2012), and aid in the linkage between the bubble and the particle through electrostatic interactions (Chen et al., 1998; Perea-Carpio, González-Caballero, & Bruque, 1988). As shown in Figure 2.2, there are two hypotheses that have been proposed to explain how surfactants improve the separation of microalgae through flotation (Coward et al., 2014). The first hypothesis is that the addition of surfactants modifies the bubble by forming a net positive charge, which results in electrostatic bonds between the algae's negatively charged surface and the positively charged bubble. This is a result of the hydrophobic tail ends on the surfactant embedding into the bubble, leaving the hydrophilic cationic heads at the bubble surface (Cho, Kim, Chun, & Kim, 2005; Coward et al., 2014; Edzwald, 2010). The second hypothesis is that the cationic surfactant adsorbs onto the microalgae cell surface by electrostatic interaction, and there is a hydrophobic attachment of the surfactant tail end into the air bubble (Chen et al., 1998; Liu et al., 1999; Phoochinda, White, & Briscoe, 2004; Coward et al., 2014).

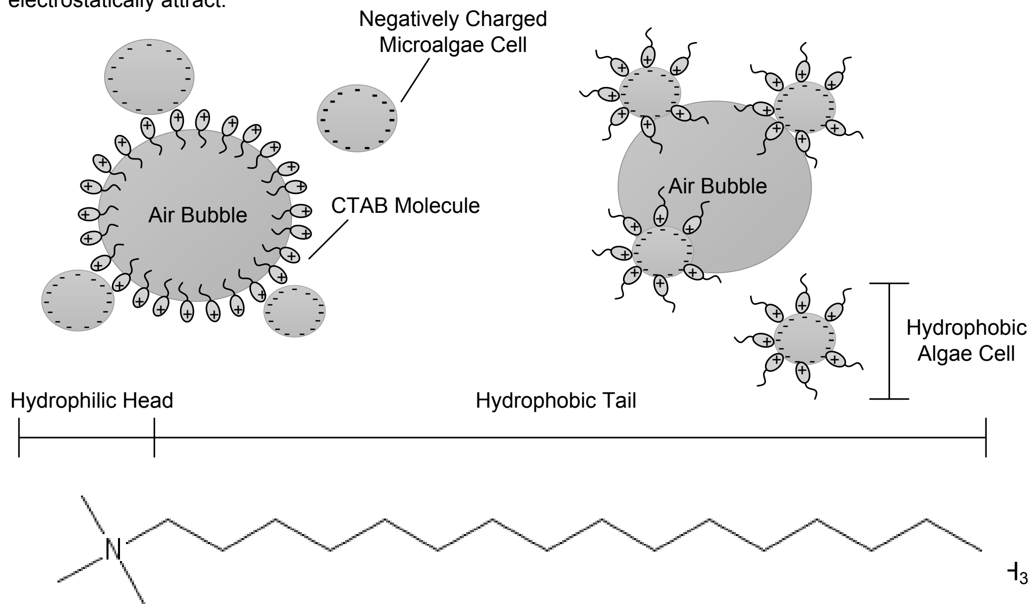
The combined process of surfactant and particle removal involving a column to allow drainage of liquid from the foam can be referred to as foam fractionation (Timmons, 1994). Foam fractionation is a separation technique that works by transferring particles (microscopic) in liquid suspension to flowing air or gas bubbles that gather at the air-liquid interface and collect at the top of the column (Burghoff, 2012). Foam fractionation apparatus (Figure 2.3) typically consists of a column that contains the suspended particles in liquid solution and a receiving vessel to collect the foam (Eldib, 1961). A gas is pumped into the system through a porous diffuser, creating bubbles which result in foam formation that rises up the column and is collected (Burghoff, 2012; Eldib, 1961). The surfactant plays an integral role in the foam fractionation process. Surfactants possess both a nonpolar and polar component which result in the hydrophobic interaction to the floating bubbles (Csordas & Wang, 2004). The desired particles are recovered from the top of the foaming unit (Coward et al., 2013). The foam column in the foam fractionation design, improves dewatering and results in higher concentration factors in the recovered biomass, as a result

1st Hypothesis
The positively charged bubbles electrostatically attract the negatively charged microalgae cells and carry them to the surface.

2nd Hypothesis
The microalgae cells adsorb the cationic surfactant.

The hydrophobic tail ends of CTAB imbed into the bubble surface leaving the hydrophilic polar heads exposed. This forms an overall positive bubble charge association whereby the negatively charged microalgae cells electrostatically attract.

Due to electrostatic attraction, the positively charged hydrophilic head groups of CTAB adsorb onto the microalgae cell surface. As a result, the microalgae possess a hydrophobic association which attaches onto the bubble.



Structure of Cationic Cetrimonium Bromide (CTAB)

Figure 2.2. Schematic diagram of proposed interaction between surfactant and microalgae cell in dispersed air flotation technology (Figure based on Phoochinda et al., 2004; Zhang, Amendola, Hewson, Sommerfeld, & Hu, 2012; Coward et al., 2014).

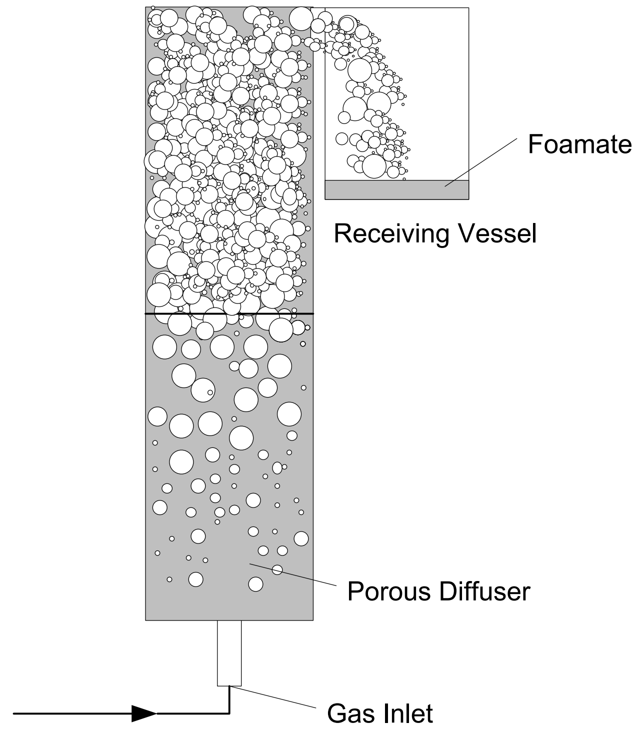


Figure 2.3. Schematic of foam fractionation experimental setup.

of greater foam residence time which allows for greater liquid drainage (Coward et al., 2013).

Most often in the recovery of microalgae cells by foam fractionation, performance is indicated in terms of the Recovery Percentage *R* from the original culture (Chen et al., 1998; Liu et al., 1999; Phoochinda et al., 2004; Phoochinda, White, & Briscoe, 2005; Kurniawati, Ismadji, & Liu, 2014). However, this indicator gives no assessment of the quality or concentration of the recovered biomass, since it does not give an indication of how effective the technique is in dewatering the biomass. Thus, the Enrichment Ratio (*ER*) or concentration factor also needs to be reported to assess performance.

The Recovery Percentage (*R*, %) is calculated from the mass of the microalgae in the foam that is recovered from the microalgae present in the initial suspension (Chan, Hossain, & Brooks, 2007; Mukhopadhyay, Khanam, & Nanda, 2010). It can be calculated using the equation below:

$$R = \frac{(Mass\ of\ Cells)_{recovered\ in\ foamate}}{(Mass\ of\ Cells)_{intial\ feed}} \times 100 \qquad (2)$$

The Enrichment Ratio (*ER*) is defined as the ratio of the concentration of microalgae present in the recovered foamate to the initial concentration of microalgae in suspension (Garg, Wang, & Schenk, 2015). It is determined using Equation 3 (Mukhopadhyay et al., 2010; Burghoff, 2012; Garg et al., 2015).

$$ER = \frac{[Algae]_{foamate}}{[Algae]_{initial\ culture}} \qquad (3)$$

Maximizing the recovery of microalgae in a foam fractionation process will often be at the expense of the Enrichment Ratio (Burghoff, 2012). Table 2.4 compares the operated parameters for Enrichment Ratio and Recovery of some studies on foam fractionation of microalgae and proteins. Garg et al. (2015) and Shea et al. (2009) demonstrated that operational conditions (i.e. pH, surfactant concentration and flow rate) varied for achieving either a high Enrichment Ratio or Recovery using foam fractionation. Csordas and Wang (2004) found that the Enrichment Ratio is a decreasing function of the foam volume, while the Recovery or harvesting efficiency was an increasing function of foam volume at

Table 2.4. Operational variables for influencing high enrichment and high recovery in foam fractionation separation processes.

Parameters	*Chlorella sp.* [1]		*Tetraselmis* sp. M8 [2]		*Chasetoceros* spp. [3]		Whey Protein [4]		Whey Protein [5]	
	ER_{max}=230 R= 2.3%	ER=1 R_{max}=23%	ER_{max}=10 R=55%	ER=6 R_{max}=93%	ER_{max}=130 R= 20%	ER= 3.6 R_{max}=90%	ER_{max}=5 R= 30%	ER= 1.86 R_{max}=91%	ER_{max}=48.2 R=96%	ER=48.2 R_{max}=96%
Surfactant	CTAB	CTAB	DAH	DAH	SS	SS	NM	NM	SDS	SDS
Surfactant concentration (mg/L)	10	15	10	25	NM	NM	NM	NM	333	333
pH	NM	NM	6	6	7.5-8.5	7.5-8.5	9	4.9	5	5
Flow rate (mL/min)	1666	1666	5,000	5,000	750-2,250	750-2,250	144	162	330	330
Starting volume (mL)	9500	9500	1,300	1,300	220,000	220,000	145	145	1,000	1,000
Foam column height (cm)	150	100	NM	NM	3.8-12.5	3.8-12.5	51	51	NM	NM
Foam Volume (L)	0.001	0.4	NM	NM	0.06	26.4	NM	NM	NM	NM

[1] (Coward et al., 2013); [2] (Garg et al., 2015); [3] (Csordas & Wang, 2004); [4] (Shea et al., 2009); [5] (Mukhopadhyay et al., 2010)

Where ER_{max} is maximum enrichment ratio
R_{max} is maximum recovery percentage
ER is enrichment ratio
R is recovery percentage
NM is Not Mentioned
CTAB is Cetyl trimethylammonium bromide
DAH is dodecyl amine hydrochloride
SS is self-secreted SDS is Sodium lauryl sulfate

varying experimental operational parameters. However, Mukhopadhyay et al. (2010) showed that both high protein Enrichment Ratio and Recovery can be obtained by the same experimental conditions. These conflicting results demonstrate the difficulties of applying foam fractionation as a harvesting technique (Burghoff, 2012). The effectiveness of the Recovery, is dependent on varying factors that can be interdependent (Burghoff, 2012).

2.2.6.4 Factors Affecting Air Flotation

There are a number of factors that have been noted in literature to affect the performance of microalgae harvesting techniques using air flotation. These include: pH, alkalinity, ionic strength, flow rate, growth stage/media, recycle rate, hydraulic loading, time, temperature, bubble size, and cell concentration. Other parameters that are specific to surfactant aided dispersed air flotation include: surfactant type and concentration, hydrophobicity, zeta potential and column size. All of which are discussed in the following section.

***pH*:** The pH of a solid-liquid suspension is an important parameter in flotation processes. The pH plays a role in the interfacial properties, surface charge (Mozes, Léonard, & Rouxhet, 1988; Matis & Mavros, 1991; Lin & Huang, 1994), changes in the chemistry of the water, and the solubility of the collectors (Fuerstenau, Jameson, & Yoon, 2007). The effectiveness of microalgae removal is influenced by the pH of the suspension (Phoochinda & White, 2003).

Chen et al. (1998) noted that the pH range of 5 to 8, resulted in *Scenedesmus quadricauda* removal efficiencies of 95%, as a result of effective adsorption between the anionic SDS surfactant and the positively charged microalgae association, at pH levels below 8; however, as the pH increased above 8 (8.5-9.5) the separation efficiency decreased to less than 10%, due to the negative microalgae surface association at these pH levels and anionic nature of SDS. Furthermore, they found that the electrostatic interaction between the cationic CTAB and the negatively charged algae, at levels above 3.5, was the primary reason for its effectiveness in adsorption onto the microalgae surface and in the flotation process. The optimum pH range for the CTAB surfactant resulted at a pH in the range of 5 to 8. Similarly, Liu et al. (1999) observed that the pH range of 5 to 8 resulted

in removal efficiencies of 85-90%, but that of 8.5 and 9 resulted in recoveries of 42% and 5%, respectively, for *Chlorella* sp. using a SDS surfactant concentration of 20 mg/L supplemented with 10 mg/L of chitosan. Additionally, they noted that the negatively charged algal surface, when the pH was greater than 3, made the electrostatic attraction between the cationic CTAB possible, with recoveries of 85 to 92 % in the pH range of 4 to 9.

Phoochinda et al. (2005) reported that decreasing the ambient pH value resulted in improved *Scenedesmus quadricauda* removal efficiencies, when using the anionic (SDS) surfactant. Garg et al. (2014) noted that the flotation recovery of *Tetraselmis* sp. was highest at a pH of 6 at any concentration of DAH (0 to 50 mg/L), compared to those achieved at a pH of 4 and 9.5. Garg et al. (2015) tested the removal efficiency of *Tetraselmis* sp. using four different surfactants at a pH of 6 and 9.5, and noted that the removal recovery was significantly higher at a pH of 6 compared to 9.5. Furthermore, Hosseini et al. (2016) reported cell recoveries for *Ochromonas danica* decreased from 93 to 22% as the pH was increased from 3 to 8, and that using waste cooking oil aided the flotation process. Zhang et al. (2016) reported that an increase in pH from 9.75 to 12.0 and an increase in magnesium concentration (collector) was necessary for achieving high harvesting efficiencies (94%) for *Chlorella zofingiensis*. The study performed by Kwon et al. (2014) reported that flocculant assisted air flotation technique required a pH of 7-8 for organic and 5-6 for inorganic flocculants in order to achieve removal efficiencies greater than 90%. The study of Hanotu et al. (2012) showed that acidic conditions were optimal for effective removal of microalgae.

***Alkalinity*:** The alkalinity of the media has been noted to impact the recovery efficiencies of the flotation process. In the case of the addition of a cationic surfactant to aid in the flotation of negatively charged suspended particles, competition between the adsorption of sodium and amine can occur, impeding the effectiveness of the process (Sayilgan & Arol, 2004). It has also been postulated that the reduction in recovery with increased alkalinity maybe due to the interaction between the cationic surfactant and the carbonate ions in solution (Sayilgan & Arol, 2004).

A few studies evaluated the effects of alkalinity on the effectiveness of the flotation process. These include the study by Chen et al. (1998), which reported that the removal efficiency (95%) of *Scenedesmus quadricauda* did not vary as the alkalinity increased from 0 to 50 mg/L of $NaHCO_3$. Sayilgan and Arol (2004) noted that the recovery of a negatively charged quartz sample (pH>2) decreased from 90% to 10% as the $NaHCO_3$ concentration increased from 0 to 8.4 g/L in the presence of both cationic and anionic surfactants in flotation processes. They also noted that effective recoveries could be achieved at a carbonate concentration of 8.4 g/L by further increasing the surfactant concentration. Furthermore, Luo et al. (2016) found that the increase in sodium carbonate (0 to 50%) resulted in decreased quartz recovery from 90 to 38% using flotation processes. However, Besson and Guiraud (2013) showed that the microalgae flotation recovery efficiency was improved by using sodium hydroxide at a concentration of 0.34 g/L (over the tested range of 0 to 1 g/L). Schlesinger et al. (2012) found that calcium hydroxide is most effective in flocculating microalgae that can be recovered using flotation techniques. Thangavel and Sridevi (2012) reported that carbonate salts can be used for microalgae flocculation to enhance the recovery efficiency of the microalgae. Variation between these studies maybe attributed to an optimal concentration that exists, in which case the study of Chen et al. (1998) explored concentrations below that point and other studies explored concentrations much higher than the 340 mg/L found to be effective by Besson and Guraud (2013).

***Ionic Strength*:** The ionic strength has been noted to affect the flotation efficiency. An increase in the concentration of inert salts in the solution has been reported in decreasing the flotation efficiency (Matis & Mavros, 1991; Lin & Huang, 1994). This occurs as a result of a decrease in zeta potential, and the weaker electrostatic interactions between the surfactant ions and the microalgae surface (Chen et al., 1998). Additionally, suspensions with a high ionic strength resulted in larger bubbles which are more easily ruptured (Lin & Huang, 1994; Matis & Lazaridis, 2002).

The work by Chen et al. (1998), showed that 100% removal efficiency of *Scenedesmus quadricauda* was obtained when the solution was very dilute with a $NaNO_3$ concentration of 0.005 M, but increasing the ionic strength by addition of $NaNO_3$ to a concentration of 0.1 M resulted in a decrease in removal efficiency (60%). Liu et al. (1999) also investigated the effect of ionic strength on the removal efficiency by flotation of *Chlorella* sp., and

found that solutions with $NaNO_3$ concentration of 0.1 M and 0.2 M resulted in efficiencies of 91% and 32%, respectively. However, Garg et al. (2012) found that the recovery of *Chlorella* sp. microalgae increased by 2% with the addition of NaCl at 35 ppt. Therefore, they concluded that ionic strength had little influence on the effectiveness of microalgae flotation.

***Flow rate*:** The gas flow rate has been identified as factor which influences the foam fractionation process. The gas flow rate and the design of the column are key determinates of the residence time of the bubbles. A long residence time in the column, which is achieved by low gas flow rates, improves the drainage of the liquid, thereby resulting in drier foams. However, increased residence time results in longer processing times. On the other hand, higher gas flow rates, result in larger bubble quantities, which means there is a larger hydrophobic surface for binding to surface active molecules. Consequently, the residence time of the foam in the column is reduced, resulting in wetter foams (Merz et al. 2011).

Studies investigating the effect of flow rate on microalgae recovery include the work by Chen et al. (1998) who found that the air flow rate over the tested range of 68 to 206 mL/min, did not have an effect on the removal efficiency of *Scenedesmus quadricauda*. Liu et al. (1999) noted that the flow rate affects the bubble size and the flow pattern in the column, however there was no significant difference in removal efficiencies of *Chlorella* sp. noted over the flow range of 68 to 206 mL/min. Phoochinda et al. (2005) reported that increasing the air flow rate only slightly improves the removal efficiencies of *Scenedesmus quadricauda*.

The effect of flow rate in other systems include the study by Merz et al. (2011) who noted a maximum enzyme recovery at a flow rate of 40 mL/min, over the tested range of 20 to 60 mL/min, but a maximum enrichment ratio was achieved at a flow rate of 20 mL/min, in flotation recovery of enzymes. Weeks et al. (1992) found that increases in air flow rate resulted in increased condensate production while decreasing foam concentration, during air flotation of suspended particles in fish culture tank. Rosa et al. (2007) found that the concentration of bovine serum protein in solution decreased from 100 mg/L to 5 mg/L in 22, 30 and 40 min at flow rates of 0.45, 0.30 and 0.15 cm/s, respectively.

Although, higher flow rates decreased the time necessary for recovery, the final volume of solution remaining after fractionation was 420, 350 and 320 mL for gas flow velocities of 0.15, 0.30 and 0.45 cm/s, respectively.

***Growth stage/media*:** Microalgae growth in a batch system generally increases until a stationary phase is reached where a peak culture density is achieved. This is followed by a drop in the culture density which occurs due to the aggregation of cells that settle out of algae suspension (Danquah et al. 2009; Coward et al. 2014) as a result of reduced electrochemical stability of the cells (Coward et al. 2014). The culture media also influences the flotation efficiency, since nutrients provide the necessary building blocks for cell surface functional groups (proteins and polysaccharides), that play an important role in stabilizing the surface charge which is required for effective air flotation recovery (Zhang et al. 2012). These two factors have been investigated in literature to determine air flotation separation performance.

Coward et al. (2014) noted that the highest concentration factor was achieved on day 12 of cultivation for *Chlorella sp*., over the tested range of 0 to 21 days using surfactant aided dispersed air flotation. In addition, Hosseini et al. (2016) reported that the cell recoveries for *Ochromonas danica* were highest during the stationary phase (91%) using waste cooking oil aided flotation, compared to those achieved during exponential-growth phase (78%), late exponential-growth phase (78%) and declining-phase (53%). However, Zhang et al. (2016) reported a harvesting efficiency of 90% irrespective of the growth phase (early exponential, late exponential or late stationary) for *Chlorella zofingiensis* using dissolved air flotation and a Mg^{2+} collector; but they did note that as the cultivation period increased, less Mg^{2+} collector was required (226 to 36 mg Mg^{2+}/g algae). Furthermore, Zhang et al. (2012) observed that more Alum coagulant was required for higher harvesting efficiency 90% in the exponential phase compared to the stationary and declining phases as the exponential phase requires more alum to destabilize the surface of the cells. They concluded that a linear correlation exists between the concentration of surface functional groups and alum dosage for a given harvesting efficiency (Zhang et al. 2012).

Nutrient limitation was studied by Zhang et al. (2012) where they found that microalgae cells that were nitrogen deprived had lower concentrations of surface functional groups as

they went from the exponential to the stationary and declining growth phases. Similarly, Schenk et al. (2008), reported that nutrient limitation in the media can improve the harvesting efficiency of microalgae flotation methods.

***Recycle rate*:** The recycle rate reported in the literature varied from 5 to 10%. Edzwald and Wingler (1990) obtained a 97-99% removal efficiency of *Chlorella vulgaris* species with a recycle rate of 8%. Vlaski et al. (1996) reported a removal efficiency of 94.5% for *Microcystics* with a recycle rate of 5-10%. Kempeneers et al. (2001) achieved a removal efficiency of 80% for *Melosira cyclotella* with a recycle rate of 6%. Teixeira and Rosa (2006) reported a 92-98% recovery efficiency of blue-green microalgae using a recycle rate of 8% and stated that the recycle system was vital for effective particle recovery but recycle rates past 8% illustrated little improvements. They also found that the addition of pressurized recycle system did not improve the recovery rate of the cells. This phenomena is attributed to the lack of particle destabilization since particle destabilization is vital to the effectiveness of dissolved air flotation as opposed to floc size. Gregory and Edwald (2010) reported a recovery efficiency of 90-99% using dissolved air flotation with a recycle rate of 10%.

***Hydraulic loading*:** The hydraulic loading rate for industrial air flotation applications ranges from 0.504 m/h to 40 m/h (Metcalf and Eddy, 1991; Corbitt, 1999). Edzwald (2007) reported that high rate dissolved air flotation techniques can be performed at hydraulic loadings of 20-40 m/h. The work of Haarhoff and Rykaart (1995) found that increasing the hydraulic loading lowered bubbles formation. It was stated by Dassey and Theegala (2012) that increased hydraulic loadings decrease the time for air to dissolve which results in poor bubble productions.

***Time*:** Operation time is another factor that influences the recovery efficiency (Csordas & Wang, 2004). Prolonged runs allow for increased chances in bubble particle collisions, which results in higher recovery efficiencies.

Csordas and Wang (2004) reported that increasing the run time during foam fractionation recovery of *Chaetoceros* spp., resulted in increased harvesting efficiencies, higher foam volume and greater dry weight yields. However, they noted that after 30 min run time, recoveries only increased by 10%, over the tested range of 15 to 60 min.

Similarly, Coward et al. (2014) noted that the air flotation technique effectively harvested microalgae within 30 min. Xu et al. (2010) noted a 93.6% recovery efficiency for *B. braunii* using air flotation in 14 min. In contrast, Edzwald and Wingler (1990) noted that the time required to remove 96-99% of *Chlorella vulgaris* using air flotation was 5 min. Variation in the operational times, maybe attributed to the different operational parameters used amongst the various studies.

***Temperature*:** Limited research has been performed on the impact of varying temperature on the effectiveness of microalgae flotation. Generally, it has been noted that there is no improvement in the flotation process through increased or decreased temperatures beyond ambient conditions (Phoochinda et al. 2003; Phoochinda et al. 2005). Operating flotation processes at increased temperatures may aid in cell wall degradation, however, care should be taken to avoid degradation of the desired end product.

In the study by Phoochinda et al. (2005), they reported that the removal efficiency of *Scenedesmus quadricauda* was unchanged with increasing temperature over the range of 20 to 40°C, using foam flotation separation. In a different study by Phoochinda and White (2003), they noted that algal removal efficiencies of *Scenedesmus quadricauda* using surfactant aided air flotation reduced at temperatures lower than 20°C. Merz et al. (2011) reported that the temperature significantly impacted the recovery of active enzymes, and optimal conditions were determined at 25°C, over the tested range of 22.6 to 32.4°C. However, O'Connor et al. (1984) noted that the temperature change over the tested range of 3 to 69°C, improved the rate of pyrite flotation and had little effect on the final recoveries.

***Bubble Size*:** The bubbles generated during flotation processes provide a means for lifting solid particles from liquid suspension, by adhering to a hydrophobic microalgae surface. The bubble size can increase the efficiency of the flotation process, increasing the surface area results in higher particle adsorption to the gas-liquid interface (Zhou, Xu, Finch, Hu, & Rao, 1997; Dai, Dukhin, Fornasiero, & Ralston, 1998; Dai, Fornasiero, & Ralston, 2000; Yoon, 2000; Burghoff, 2012).

In the study performed by Coward et al. (2015) and colleagues, it was found that higher biomass recoveries were achieved with smaller bubble sizes using oscillating airflow, as a

result of greater and more frequent bubble-algae collision and attachment efficiencies. Garg et al. (2014) noted that the use of micro bubbles for flotation (less than 500 µm) resulted in increased flotation recoveries in *Tetraselmis* sp. by at least 10%, compared to those achieved using bubbles that ranged 1-2 mm in size. Hosseini et al. (2016) reported that the cell recoveries (96.9 to 89%) of *Ochromonas danica* achieved using waste cooking oil aided flotation, decreased with increased diffuser pore size (4-8 to 25-50 µm). Furthermore, Merz et al. (2011) found that the optimal pore size for high enrichment and recovery of enzymes was 16 µm, over the tested range of 16 to 100 µm. In addition, Crofcheck and Gillette (2003) showed that the increase in gas flow rate results in increased bubble size.

Cell concentration**:** The effectiveness of flotation recovery technique, is dependent on the bubble-particle interaction. As the cell concentration increases in solution, more air bubbles are required for achieving effective cell removals through increased bubble-particle interaction (Zhang et al., 2014). However, it should be noted that although an increase in air flow rate would result in increased bubble formation, it may result in lower concentration factors as a result of wetter products recovered (Levin et al., 1962).

Levin et al. (1962) found that increased concentrations of *Chlorella* resulted in poor concentration factors using dissolved air flotation techniques, at both aeration flow rates of 300 and 650 cm^3/min. In addition they noted that increasing the aeration flow rates from 300 to 650 cm^3/min further decreased the recovery concentration factor. In another study by Zhang et al. (2014), they noted that increased cell concentrations for *Chlorella zofingiensis* from 1.2 to 7.4 g/L, decreased the cell recovery efficiency from 92 to 41%, using the same operating parameters in dissolved air flotation. Furthermore, Hosseini et al. (2016) reported that higher *Ochromonas danica* concentrations over the tested range of $2.3x10^8$ to $6.8x10^8$ cells/mL caused rapid foam formation, even when low air flow rates were used (50 mL/min), which resulted in poor dewatering of the recovered cells, during flotation using waste cooking oil as an aid.

Surfactant type**:** Microalgae surfaces vary from being hydrophilic to slightly hydrophobic at natural pH (Garg et al., 2014). The use of surfactants or collectors in air flotation has been noted in improving the hydrophobicity of the cells, making surfactants good collector

agents in the separation of microalgae from liquid suspensions (Chen et al., 1998; Phoochinda et al., 2004; Uduman et al., 2010; Garg et al., 2012). Surfactant molecules consist of two primary components, the hydrophilic head and the hydrophobic carbon chains (tail); the adsorption of these molecules onto the microalgae surface, improves the adherence of the cells to air bubbles (Bulatovic, 2010). The various classifications for surfactants are cationic, anionic, non-ionic, and zwitterionic, and are determined based on their hydrophobic and hydrophilic constituents (de Guertechin, 2001). Various types of surfactants have been studied for their effectiveness in improving microalgae recovery using flotation techniques (Table 2.5). Generally, cationic surfactants have been found to be better collectors, due to the negative charged microalgae cells.

Chen et al. (1998) found that the removal percentage of *Scenedesmus quadricauda* using Triton X-100 (nonionic) and sodium SDS (anionic) surfactants was less than 10%, but the use of 10 mg/L CTAB (cationic) resulted in 50% removal. Liu et al. (1999) noted that surfactant systems SDS and CTAB at concentrations of 40 mg/L resulted in removal efficiencies of 20 and 86%, respectively, for *Chlorella* sp. Phoochinda and White (2003) reported that the cationic surfactant (CTAB) was effective in recovering *Scenedesmus quadricauda* cells (90%), however the anionic (SDS) surfactant resulted in poor removal efficiencies (16%), using foam flotation separation. Garg et al. (2015) tested the flotation removal efficiency of *Tetraselmis* sp. using four different surfactants which included dodecyl pyridinium chloride (DPC), N-dodecylpropane-1,3-diamine hydrochloride(DN2), dodecyl hydrochloride (DAH), and sodium dodecyl sulfate (SDS) and found that DPC resulted in the highest recovery at a concentration of 25 mg/L. Zutic et al. (1981) noted that marine microalgae produced their own type of surfactant during the exponential growth phase, and that the concentration was related to the age of the culture. DeSousa et al. (2006) found that the flotation recovery of the yeast species, *Saccharomyces cerevisiae* (strain FTL-01), was 80% using SDS surfactant at a pH of 3, and 85.7% using CTAB in the pH range of 3 to 5.

***Surfactant concentration*:** The concentration of the surfactant in the media has been observed to impact the harvesting efficiency of the cells, as demonstrated in Figure 2.4 (Chen et al., 1998; Garg et al., 2014). When microalgae cells have a negatively charged cell surface (Cabral, 1992), they can bind with the positively charged head groups of

Table 2.5. Effectiveness of microalgae flotation using varying surfactant types and operational parameters.

Reference	Species	Cell Concentration (cells/ mL)	Surfactant Type (concentration)	pH	Flow Rate (mL/min)	Time (min)	Removal (%)
Chen et al. (1998)	*Scenedesmus quadricauda*	7.4×10^4	Triton X-100 (0-40 mg/L)	8.0	114	NR	<10
			SDS (0-40 mg/L)				<10
			CTAB (0-40 mg/L)				90
Liu et al. (1999)	*Chlorella* sp.	6.8×10^5	SDS (40 mg/L)	8.0	114	10	20
			CTAB (40 mg/L)				86
Phoochinda and White (2003)	*Scenedesmus quadricauda*	1×10^5	SDS (100 mg/L)	5.0	3500	15	16
			CTAB (100 mg/L)	4.9			65
Garg et al. (2015)	*Tetraselmis* sp.	NM	DPC (25 mg/L)	9.5	5000	NR	90
			DN_2 (25 mg/L)				57
			DAH (25 mg/L)				35
			SDS (25 mg/L)				22

Where NM is not mentioned

SDS is Sodium lauryl sulfate

CTAB is Cetyl trimethylammonium bromide

DPC is Dodecyl pyridinium chloride

DAH is dodecyl amine hydrochloride

DN_2 is N-dodecylpropane-1,3-diamine hydrochloride

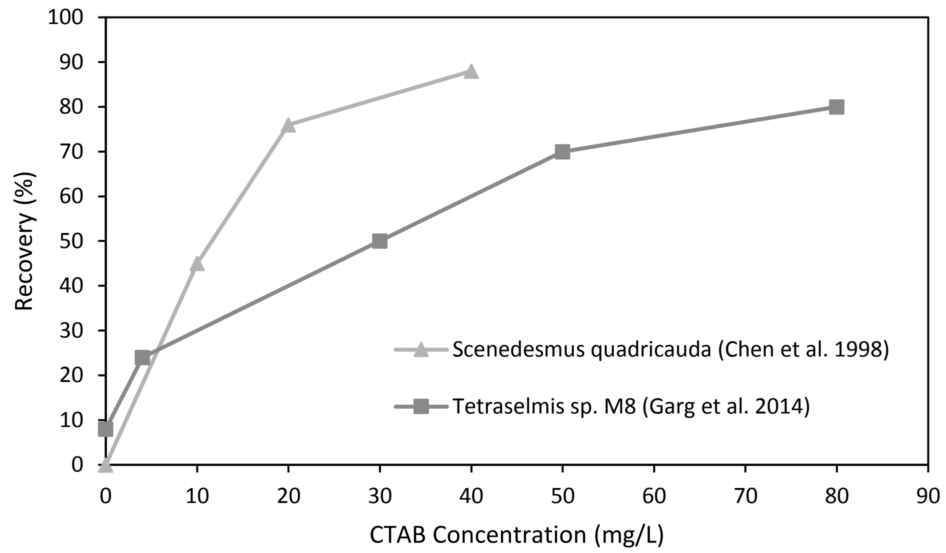


Figure 2.4. Cationic, CTAB, surfactant concentration correlation with microalgae flotation recovery efficiency.

cationic surfactants to form a cell-surfactant complex (Huang & Kim, 2013), Figure 2.2. When the net charge of the complex is close to zero, the cell-surfactant complex precipitates, but when a net negative or positive charge is present, they are dispersed in the medium (Huang & Kim, 2013).

In the study by Chen et al. (1998), they found that the removal efficiency of *Scenedesmus quadricauda* increased from 50 to 90 % with increasing concentrations of CTAB from 10 to 40 mg/L. Garg et al. (2014) noted that the increase in CTAB and DAH concentration from 0 to 50 mg/L resulted in increased cell recoveries from 6.4 to 70 % and 5 to 70 %, respectively, for *Tetraselmis* sp. at a pH of 9.5. In another study by Garg et al. (2012), they found that increasing the CTAB concentration from 0 to 3 mg/L resulted in microalgae removal efficiencies of 93 to 99 % and 6 to 23 % for *Chlorella* sp. and *Tetraselmis* sp., respectively.

Garg et al. (2012) tested the removal efficiency of *Tetraselmis* sp. using four different surfactants which included dodecyl pyridinium chloride (DPC), N-dodecylpropane-1,3-diamine hydrochloride (DN2), dodecyl amine hydrochloride (DAH), and sodium dodecyl sulfate (SDS) at two concentrations of 10 and 25 mg/L, and found that recovery efficiencies were higher at 25 mg/L for all surfactant types. Garg et al. (2015) also noted that the surfactants precipitated at a pH greater than 9.5 which resulted in lower recovery yields achieved without pH adjustments, however the degree of precipitation varied between different surfactants and the DPC resulted in the least precipitation, which consequently led to the highest recovery at that pH. Similarly, Dai and Laskowski (1991) found that the solubility limit for DAH decreased when the pH increased beyond 9.

***Hydrophobicity*:** The hydrophobicity of a particle surface, plays an integral role in determining its ability to be harvested effectively using flotation techniques (Garg et al., 2012; Garg et al., 2014). The hydrophobicity of an algal cell can be improved by the addition of surfactants, deeming them effective collectors for flotation processes (Xu & Yoon, 1990; Garg et al., 2012). The degree of the hydrophobic nature of the cell is dependent on the surfactant type and concentration, as well as the pH of the liquid suspension (Bulatovic, 2007; Garg et al., 2014).

Xu and Yoon (1990) found that hydrophobicity of the cell plays a critical role in flotation efficiency, and that increasing the hydrophobicity of the particles resulted in increased coagulation. Similarly, Garg et al. (2012) noted lower hydrophobicity of *Tetraselmis* sp. (1.3%) compared to that of *Chlorella* sp. (5.3%), and attributed this to the lower removal efficiencies of *Tetraselmis* sp. using flotation. In a different study by Garg et al. (2015), they reported that that highest hydrophobicity of *Tetraselmis* sp. achieved through the addition of surfactant and altering media pH, corresponded to the highest recovery yield.

Garg et al. (2014) noted that the increase in CTAB concentration from 0 to 80 mg/L resulted in increased cellular surface hydrophobicity from 1.2 to 25.0 % in *Tetraselmis* sp. and improved the recovery from 6.4 to 81.7 %. They also noted that the hydrophobicity of *Tetraselmis* sp. was highest at a pH of 6 compared to the hydrophobicity at a pH of 4 and 9, at any given concentration of dodecyl ammonium hydrochloride (DAH) surfactant. Furthermore, Somasundaran and Zhang (2006) found that the hydrophobicity index of alumina increased to 0.006 with increases in SDS surfactant concentrations of 7 to 70 mg/l.

It was observed by Garg et al. (2015), that the precipitation of surfactants at a pH of 9.5 resulted in decreased surface hydrophobicity, causing a decrease in recovery yields of *Tetraselmis* sp., as a consequence of reduced number of free molecules available for adsorbing onto the microalgae surface. This is in agreement with the findings of Dai and Laskowski (1991), that the solubility of DAH is low in solutions where the pH was greater than 9. Similarly using the yeast species *Saccharomyces cerevisiae* (strain FTL-01), DeSousa et al. (2006) found that the hydrophobicity generally decreased as the pH varied from 1.5 to 6, and that the highest values of cell recovery were achieved at the highest values of hydrophobicity, as a result of improved foam stability.

***Zeta potential*:** Zeta potential, or surface charge, is another parameter that can be used to determine the effectiveness of the bubble and particle interaction in flotation (Liu et al., 1999; Henderson, Parsons, & Jefferson, 2010; Coward et al., 2014; Xia, Li, & Song, 2016). The zeta potential or charge of the particle is obtained by determining the velocity of the particle, as it moves due to electrophoresis. Particles with a net charge will migrate towards an electrode when an electric field is applied. In other words, zeta potential is a measure

of electrophoretic mobility, which varies with varying pH, and amongst different microalgae species (Xia et al., 2016). Effective flotation of microalgae cells requires that the surface charge be close to neutral, which equates to a zeta potential measurement that ranges from -10 mV to +5mV (Henderson et al., 2008a). A positive bubble charge would adhere to the negatively charged particles in suspension, thereby improving the removal efficiencies of the flotation process (Bui et al., 2015).

Phoochinda and White (2003) noted that the zeta potential of *Scenedesmus quadricauda* microalgae suspended in distilled water decreased from 20 to -30 mV, as the pH increased from 4 to 12. They also noted that little change in zeta potential was observed in suspensions containing CTAB and SDS surfactant and that of the culture media. The zeta potential was positive for the system containing cationic surfactant (12 to 20 mV), and negative for both the anionic (-8 to -18 mv) and culture media (0 to -10 mV) suspensions, at varying pH levels.

In the study by Henderson et al. (2008a), they investigated the effectiveness of various surfactants using flotation on the removal of the freshwater cyanobacteria, *Microcystis aeruginosa*, and noted that cationic surfactants improved the recovery efficiencies. This cyanobacteria has a zeta potential of -23 mV at a pH of 7, which resembles that of freshwater microalgae, *Chlorella vulgaris* (-25 mV) (Aktas et al., 2012). Bui et al. (2015) also noted that the charge of the bubble and alga species are essential in the effectiveness of the flotation process; they reported that the zeta potential of *Microcystis* sp. varied from -28 to -37 mV as the pH increased from 4.5 to 7, respectively, and that of the bubble increased from -29 to +25 mV as the concentration of aluminum increased from 0 to 5 mg/L, respectively. While Xia et al. (2016) noted the zeta potential for six species of microalgae (*D. bijuga, M. dybowoskii, Chlorella sp., K. dianae, P. integrum* and *A. obliquus*) were all electronegative for the tested pH range of 2 to 8, with the exception of *P. integrum, A. obliquus* and *D. bijuga* which resulted in electropositive zeta potential below a pH of 3.3, 3 and 4.7, respectively.

Studies performed on the impact of surfactant concentration on the zeta potential of the cell include the work by Kurniawati et al. (2014), who reported that the zeta potential became less negative (-33 to -15 mV) for the *C. vulgaris* species, as the concentration of

CTAB increased from 0 to 60 mg/L, however only a slight change (-14 to -9 mV) was noted for *S. obliquus*. Henderson et al. (2010) noted that the zeta potential of *C. vulgaris* became less negative with increased concentrations of aluminum coagulant. It was stated by Xia et al. (2016) that aside from surface area, the functional group concentration present on cell surface, was directly related to the dosage of cationic reagent required for charge neutralization. This is in agreement with the work of Zhang et al. (2012) which reported that higher functional group concentrations required higher reagent dosage for effective recovery.

Column size**:** The column size plays an important role in the quality of the cells recovered during foam aided flotation. It influences the bubble rise time, which affects the cell capture, and the space available for foam drainage prior to exiting the apparatus (Hosseini et al., 2016). An increase in foam volume, results in longer drainage times (drier foams) and increased bubble coalescence, which result in higher enrichment ratios (Brown et al., 1999; Burghoff, 2012).

Hosseini et al. (2016) reported that an increase in the sample-to-column volume resulted in improved cell recoveries of *Ochromonas danica*, using waste cooking oil aided flotation, but also resulted in a wetter foamate with lower concentrating ratios. However, Merz et al. (2011) found that the enrichment ratio was not significantly affected by the increase in foam volume over the tested range of 65 to 120 mL. While Weeks et al. (1992) noted that an increase in overflow height resulted in decreased condensate production, and increased foam concentration. Csordas and Wang (2004) noted that harvesting efficiency of the marine *Chaetoceros* spp., recovered using foam fractionation, increased as a function of increasing foam volume. They noted that 70 % of the biomass was recovered in 2 % of the total reactor volume.

2.2.6.5 Energy Consumption

In general, flotation techniques have been regarded as energy intensive processes as a result of the high pressures required (Hanotu et al., 2012). However, dissolved air flotation is the most energy intensive (7.6 kWh/m^3) as a result of pressurized (390 kPa) microbubble formation needed for transporting suspended particles to the surface of the reactor, and requires flocculants to improve recoveries (Green et al., 1995; Haarhoff and Steinbach,

1996; Feris and Rubio, 1999; Liu et al., 1999; Wiley et al., 2009; Uduman et al., 2010). Fluidic oscillation does not require as much energy for operation and can consume 2-3 orders less than dissolved air flotation methods (Zimmerman et al., 2009; Hanotu et al., 2012). On the other hand, dispersed air flotation coupled with foam fractionation has significantly lower energy requirements (0.003-0.015 kWh/m^3) (Wiley et al., 2009; Coward et al., 2013). It works by applying a gas to the vessel that is dispersed using a low pressure sparger and uses foam (via added surfactants) to aid in cell collection (Wiley et al., 2009; Wiley et al., 2011; Coward et al., 2013), which is the least energy intensive of the three techniques.

2.2.6.6 Advantages and Disadvantages

Flotation methods are rapid, highly effective, and have been well established in microalgae recovery (Uduman et al., 2010; Edzwald, 2010). Compared to sedimentation, flotation methods are much faster and more effective for harvesting of microalgae (Singh et al., 2011). Dewatering microalgae using flotation methods (7 % SS) are much more rapid and efficient than the use of sedimentation which only concentrates to 1.5 % suspended solids (Mohn, 1988). Additionally, the use of surfactants has been shown to improve recovery of microalgae cells up to 90% (Chen et al., 1998; Phoochinda et al., 2004), by forming electrostatic links between the microalgae cell surface and the gas bubble (Henderson et al., 2008), and lowering the operational power necessary for achieving microbubbles for effective flotation (Wiley et al., 2009; Coward et al., 2013). However, depending on the type of technique applied, it may be energy intensive as a result of high pressurization for the formation of microbubbles (Mohn, 1988; Uduman et al., 2010).

2.2.7 Combination of Techniques

Cost effective methods for cell harvesting are vital for the economics of biodiesel production. Harvesting processes account for 20-30 % of the total biomass production costs (Grima et al., 2003). Selection of harvesting technique is dependent on the size and density of the microalgae cells, conditions of the culture, concentration of biomass and target product value (Demirbas, 2010).

The work of Schenk et al. (2008) reviews the various harvesting methods and notes that the combination of flocculation with sedimentation or flotation with filtration or flotation with centrifugation to be the most economical alternatives. Both Brennan and Owende (2010) and Uduman et al. (2010) reported that energy can be conserved and costs can be reduced by harvesting the microalgae using a process where two techniques are combined. In previous work, the microalgae were initially concentrated to 2-7% total suspended solids by flocculation and the cells were further concentrated into a paste (suspended solid concentration of 15-25%) by a secondary harvesting step such as filtration or electrophoresis (Uduman et al., 2010). As well, Funk et al. (1968) integrated dissolved air flotation with chemical flocculation (ferric sulfate) and noted increased recovery efficiency from 88% to 95% for *Chlorella vulgaris*.

The work performed by Kim et al. (2012) suggests that a new and innovative technique for improving the economics of the electrophoresis processes is the combination of electrolytic coagulation and electrolytic flotation into continuous electrolytic recovery of microalgae. In this method, the current direction (polarity exchanges) is exchanged for the continuous harvest of microalgae and their cultivation. The current direction creates two phases by using a pair of electrodes. The first phase works to destabilize the negatively charged microalgae cells forming flocs. The formation of flocs is mediated by metal ions that are released from the electrode dissolving in solution. In the second phase, the metal ion generation is halted and the bubbles formed from both electrodes lift the flocs to the top of the solution causing them to float.

The study of Xu et al. (2010) used electroflocculation integrated with dispersed air flotation and noted a harvesting efficiency of 98.9% in 14 min. They noted that the cell aggregate increased with the integrated system as opposed to those observed with electroflocculation. The use of dispersed air flotation increased the rate of aggregate formation. However, the stress from continued air supplementation into the system disturbed the up-floated flocs into algal aggregates. Thus disturbance was avoided by halting the supplementation of air into the system once the aggregate size reached its peak value.

2.3 Microalgae Pre-Treatment

Microalgae biomass is composed of proteins, carbohydrates, lipids and nucleic acid that vary widely in proportion (Demirbas, 2011) and is dependent on the species cellular response (Becker, 2007; Pimentel et al., 2008). There are numerous applications in which these components can be used such as health food additives for human consumption, animal feed, plant fertilizers and/or biofuels (Chisti, 2007; Becker, 2007).

Lipids are contained as small spherical droplets in the chloroplast and between the thylakoid membranes (Li-Beisson, 2015). They function in the structural support for the cell, the metabolic organelles in photosynthesis metabolism, the growth process of the cell and in the synthesis of lipoprotein membranes contained in the chloroplast (Hu et al., 2008). Biodiesel production is generated through the conversion of lipids into fatty acid methyl esters using a transesterification process (Gavrilescu and Chisti, 2005; Spolaore et al., 2006). Disruption of the cellular wall allows for easier recovery of the intracellular lipids, through enhancing the solvent/lipid contact, resulting in rapid and increased efficiencies in lipid extraction (Gouveia et al., 2007; Cooney et al., 2009; Lee et al., 2010; Greenwell et al., 2010).

Several techniques can be employed for disruption which include mechanical, thermal, pre-treatment, electromagnetic radiation, biological and chemical methods. Mechanical pre-treatment methods include beadmilling, ultrasound and high pressure homogenization. Thermal pre-treatment methods for microalgae include steam explosion, autoclaving and freeze drying. Electromagnetic radiation methods include microwave treatment. Biological and chemical treatments include enzyme hydrolysis and surfactants, respectively.

2.3.1 Bead Milling

Bead milling is a process that works to disturb the extracellular wall of microalgae by grinding and agitation of the cells on a solid surface of glass beads (Mercer and Armenta, 2011). Exciting the beads using a bead mill produces a high shear force that can destroy the microalgal cell walls (Munir et al., 2013). The optimal diameter size of the beads for effective microalgae cell wall disruption is 0.3-0.5 mm (Hopkins, 1991; Doucha and Livansky, 2008). These beads can be made of zirconia-silica, zirconium oxide or titanium

carbide (Hopkins, 1991). Bead milling vessels maybe one of two types: shaking vessels and agitated vessels.

The shaking vessel works to disrupt the cell walls through the shaking of the entire culture vessel (Kumar et al., 2014). The vessel or multiple vessels are placed on a platform that vibrates allowing the beads to move and collide with the cells. The use of this type of bead mill is limited to laboratory scale and is not as effective in damaging the cell walls as agitated beads method (Zheng et al., 2011; Kumar et al., 2014).

The study performed by Zheng et al. (2011), employed a bead milling vessel to extract lipids from *Chlorella vulgaris* and noted a recovery of 11%, which was lower than other methods tested. In another study performed by Shen et al. (2009a), they noted that the highest lipid recovery content of 18.8% from *C. protothecoides* was achieved using bead beater shaking vessel. Lee et al. (2010) noted that bead beating of *Botryococcus* sp. cells resulted in a lipid extraction of 28%. Prabakaran and Ravindran (2011) reported a lipid content recovery of 25-30% from *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp. species using bead beating. Similarly, Ryckebosch et al. (2011) noted a lipid recovery efficiency of 40% from *P. tricronutum* using a shaking bead beater.

In the agitated beads technique, both the beads and culture are agitated (Schilling, 2008). A rotating agitator inside the vessel supplies the heat. Agitating the beads provides better disruption of the cell walls which increases the extraction efficiency (Schilling, 2008). For heat-sensitive molecules, the vessel is equipped with cooling jackets. This technique provides agitation, collision and grinding of the biomass which results in effective disruption efficiencies (Lee et al., 2012).

In the study of Gouveia et al. (2012), they extracted 33 g of oil from 100 g of *Nannochloropsis* sp. using bead mill assisted techniques. Halim et al. (2012) found that agitated bead beating resulted in the disruption of 17.5% of *Chlorococcum* sp. microalga. In another study by Baldev et al. (2014), they reported a 2 fold increase in lipid yield in *Scenedesmus* sp. using agitated bead beater and mechanical grinding of the cells. Lee et al. (2010) used bead milling and reported an oil yield range from 7.9-8.1 g/L from *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. Shen et al. (2009a) noted an oil recovery of 20.5% using bead milling pre-treatment from *Chlorella protothecoides*. Ceron

et al. (2008) found that bead milling was the best method for lipid recovery from *Scenedesmus almeriensis*.

2.3.1.1 Factors Affecting Bead Milling

The degree of cell disruption is dependent on the strength of the microalgae cell walls, contact between the cells and the beads, as well as the shape, size and composition of the beads (Doucha and Livansky, 2008). However, the biomass concentration, residence time and agitator speed have been identified to be most influential on the cell wall degradation efficiency, processing time and on the energy consumption (Greenwell et al., 2010; Postma et al., 2015).

Biomass concentration**:** The biomass concentration is one of the primary factors affecting the efficiency of microalgae cell wall disruption (Postma et al., 2015). Increasing the biomass concentration in a pre-treatment bead mill leads to a higher fraction of microalgae cell disintegration as shown in Figure 2.5 (Postma et al., 2015). The authors found that increasing the biomass concentration in the bead milling of *Chlorella vulgaris* over the tested range of 25-145 g/L resulted in higher disintegration efficiency.

The work performed by Doucha and Livansky (2008) reported that increasing the *Chlorella* biomass concentration in bead milling resulted in increased disintegration of microalgae cell wall. Furthermore, that of Mogren et al. (1974) also noted that disintegration rates increased with increasing concentration of yeast biomass in bead milling pre-treatment. It was recommended by Greenwell et al. (2010), that the cell concentrations should range between 100 to 200 g/L for high efficiencies and suitable economics, energy-wise.

Agitator speed**:** The agitator speed is another primary determinant of the efficiency of microalgae cell wall disruption. Postma et al., (2015) stated that increasing the speed of agitation increases the impact force and the frequency which results in higher breakdown of cell wall, to a certain degree as shown in Figure 2.5. The authors found that increasing the agitator speed of bead milling in *Chlorella vulgaris* culture over the tested range of 9 – 12 m/s resulted in higher disintegration efficiency, but also noted an optimum speed in the range of 9-10 m/s, existed. In another study by Doucha and Livansky (2008), they noted that increasing the agitator speed in bead milling processing of microalgae resulted in

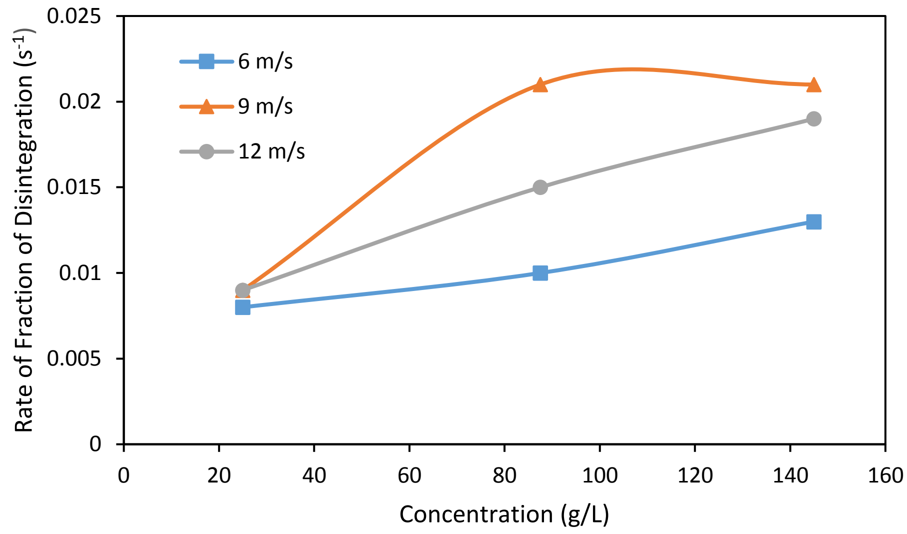


Figure 2.5. Fraction of cell disintegration as a function of concentration and agitation speed (adapted from the work of Postma et al., 2015).

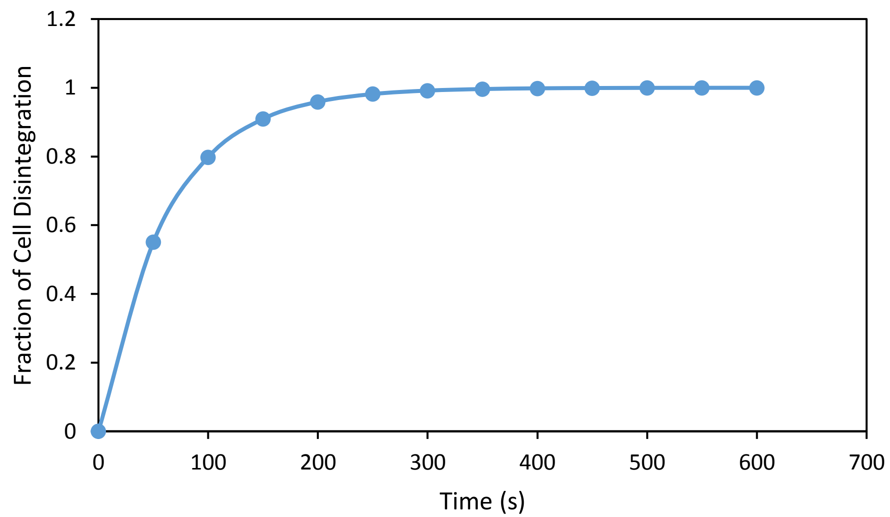


Figure 2.6. Microalgae fraction of cell disintegration as a function of time (adapted from the work of Postma et al., 2015).

increased cell wall destruction. That performed by Hedenskog et al. (1969) reported a cell destruction efficiency in *Scenedesmus quadricauda* biomass of 55% using a speed of 2800 rpm. Lee et al. (2010) noted lipid recovery efficiencies of 7.9-8.1 g/L from *Botryococcus* sp., *Chorella vulgaris* and *Scendesmus* sp. using a rotational speed of 2800 rpm. Similarly, Bert et al. (2011) also observed that increasing the agitator speed from 8 to 14 m/s increased the disruption efficiency of the agglomerate.

***Residence time*:** The residence time is another key factor of the efficiency of microalgae cell wall disruption (Greenwell et al., 2010). The fraction of microalgae cell disintegration as a function of time is graphically depicted in Figure 2.6 (Postma et al., 2015). It can be seen that increasing the time of the bead mill process leads to higher cell disintegration until a maximum disintegration is reached (Postma et al., 2015).

The study performed by Doucha and Livansky (2008) found that the breakdown of microalgae cell wall using bead milling increased from 67% to 95% as the treatment time increased from 30 min to 90 min, respectively. It was determined by Postma et al. (2015) that 500 s of bead mill pre-treatment of *Chlorella vulgaris* biomass resulted in 99% disintegration, but 90-95% disruption was achieved at 200-250 s. Hedenskog et al. (1969) reported a cell destruction efficiency in *Scenedesmus quadricauda* biomass of 87% in 5 min. Similarly the study of Safi et al. (2015) also reported that increasing the bead milling time increased the cell wall disintegration which led to greater protein and pigment recovery efficacies from *Chlorella vulgaris*, up to the maximum reached at 40 min.

***Bead density*:** Increasing the contact between the particles and the cells by increasing the bead density enhances the dispersion up to a limit of 85% (Bert et al. 2011). Doucha and Livansky (2008) reported that the cell wall destruction values of 65, 83 and 85% were achieved from *Chlorella* using chamber bead volumes of 60, 75 and 80%, respectively. Hedenskog et al. (1969) noted that 33 and 50% bead filling of the vessel resulted in a 55% and 90% cell disintegration of *Scenedesmus quadricauda* biomass, respectively. Liang et al. (2009) extracted 38% of lipids from *Chlorella vulgaris* using 71% bead filling volume. Schwenzfeier et al. (2011) used a bead filling volume of 65% for the algae *Tetraselmis* sp. biomass for effective cell disruption for protein recovery. The results of Bert et al. (2011) also indicated that the dispersion efficiency was improved with increasing bead filling

volumes over the tested range of 70-85%, but bead fill volumes greater than 85% significantly increased the wear on the mill.

***Bead type and size*:** Varying beads and bead sizes have different impact on the dispersion efficiency (Bert et al., 2011). Hedenskog et al. (1969) noted a cell disintegration of 55% using ballotini beads (0.35-0.5 mm) in *Scenedesmus quadricauda* biomass. The investigation of Doucha and Livansky (2008) found that zirconium dioxide beads (0.5 mm) and glass beads (0.42-0.58 mm) resulted in *Chlorella* biomass cell disintegration efficiencies of 98.5 and 99.9%, respectively. The work of Lee et al. (2010) found that the lipid recovery efficiencies ranged from 7.9-8.1 g/L for *Botryococcus* sp., *Chlorella vulgaris* and *Scendesmus* sp. using glass bead (0.1 mm diameter).

The study performed by Zheng et al. (2011) used glass beads (0.4-0.6 mm diatmeter) as a pretreatment for the recovery of lipids in *Chlorella vulgaris* biomass. That of Lee et al. (1998) reported a lipid recovery efficiency of 28.6% from *Botryococcus braunii* using 1 mm glass beads. Further, Bert et al. (2011) tested the effect of varying ceramic bead sizes (0.6-1.6 mm) on the agglomerate size and found that 0.8 mm beads were the most effective in achieving the lowest agglomerate size of 41 μm and that ceramic beads significantly improved the dispersion efficiency compared to glass ones.

2.3.1.2 Energy Consumption

Effective disruption of microalgae cell walls using bead mill pre-treatment has been noted to consume 35-810 Wh/kg of power (Doucha and Livansky, 2008; Greenwell et al., 2010; Postma et al., 2014). The large variation in power consumption is a result of varying microalgae species and operation parameters.

Lee et al. (2010) disrupted the microalgae *Botryococcus*, *Chlorella* and *Scenedesmus* using agitated bead mill with an energy consumption of 140 Wh/kg. That of Doucha and Livansky (2008) noted that the power consumption for rupturing *Chlorella* using an agitated bead mill was in the range of 35-250 Wh/kg. Greenwell et al. (2010) reported that the energy consumption for cell wall disruption using a bead beater ranged from 300-400 Wh/kg. While that of Postma et al. (2014) noted an energy consumption of 810 Wh/kg for bead milling of *Chlorella* sp. biomass.

2.3.1.3 Advantages and Disadvantages

The advantages of using bead milling technique are the simplicity, rapidness of the method, reproducibility of results and low labor intensity requirement (Kim et al., 2013; Gunerken et al., 2015). However, pre-treatment of microalgae cells using bead milling can be difficult to scale up and requires the use of a cooling jacket in order to prevent the degradation of the desired product (Grima et al., 2003; Lee et al., 2010; Munir et al., 2013; Kim et al., 2013). Additionally, bead mill pre-treatment is not a selective product recovery technique, which requires further processing to remove the undesired compounds (Gunerken et al., 2015). The biomass undergoing bead disruption techniques must be dry and concentrated in order to achieve high disruption efficiency (Biller and Ross, 2012; Gunerken et al., 2015).

2.3.2 Ultrasonication

Ultrasonication is another mechanical method that can be used for pre-treatment of microalgae prior to lipid extraction. In this method, algae are exposed to high intensity ultrasonic waves, creating tiny cavitation bubbles around the cells. The bubbles collapse and emit shockwaves that shatter the cell walls causing the intracellular lipids to enter the bulk of the solution (Singh and Gu, 2010; Mercer and Armenta, 2011). There are two types of ultrasound units: horns and baths (Hosikian et al., 2010). Both of these types are used for batch operations, but the addition of flow cells can alter them into continuous operational modes (Borthwick et al., 2005; Gogate et al., 2006).

Horn ultrasonic technology uses a piezoelectric generator that is composed of lead zirconate titanate crystals, that vibrate with an amplitude ranging from 10 to 15 μm (Minoletti et al., 2008). The formed vibrations travel down the titanium metal horn or probe, increasing in amplitude ranging from 100 to 150 μm at the tip. The power at the tip should be of high intensity in order to create cavitation with sufficient disruptive force since the energy dissipates rapidly with distance. The use of horn type sonicators is limited to laboratory scale, handling volumes of 10-100 mL (Lee et al., 2012). Setting the horn to vibrate laterally would increase the area of contact for larger cavitation, but a larger area would decrease the intensity of cavitation. The scale-up of horn sonication would require the use of multiple systems and the use of continuous flow of the cells (Lee et al., 2012).

Jeon et al. (2013) stated that disruption of microalgae biomass using horn sonicators is not suitable because the cavitation is localized. Similarly, the work performed by Wang et al. (2014) noted that the relative lipid increase rate for *S. dimorphus* (30 ml) using horn sonication was lower than that using bath sonication at operational times of 1 and 5 min. Furthermore, Menendez et al. (2014) reported that horn ultrasonic treatment did not increase the lipid yields in *Nannochloropsis gaditana* microalgae, compared to conventional extraction techniques. However, Cravotto et al. (2008) found that horn sonication of the marine microalgae *Crypthecodinium cohnii* (50 ml) was effective in increasing the lipid recovery.

Ultrasonic baths use transducers, placed as the bottom of the reactor, to generate the ultrasonic waves (Tovatech, 2015). The number and the arrangement of the transducers varies with the capacity of the reactor and the shape. Sonicator baths have a larger capacity (up to 3 L), but the reactor size is limited by the rapid rate of sonic energy dissipation with distance. Baths can be modified in flow cells for continuous industrial operation and higher efficiencies may be achieved using multiple transducers operating with 2 or 3 varying frequencies in the cell. A lipid recovery of 26% from *Nannochlorpsis* was noted by Wiyarno et al. (2011) using ultrasonic bath treatment. The disruption efficiency investigation performed by McMillan et al. (2013) on *Nannochloropsis oculata* biomass using microwave, mechanical force and ultrasonic bath treatments suggested that ultrasound was the least effective of the techniques. Neto et al. (2013) treated *Chlorella minutissima*, *Thalassiosira fluviatillis* and *Thalassiosira pseudonana* with ultrasonic bath waves and noted that ultrasonication was a necessary step for cell disruption in order to increase the lipid recoveries attained. Furthermore, Piasecka et al. (2014) found that sonication bath treatment of *Chlorella protothecoides* biomass resulted in an increase in lipid yields of 37%.

Other work performed by a number of researchers on microalgae suggests that ultra-sonication assisted microalgae lipid extraction significantly increases the yields and reduces the extraction time (Lee et al., 2010; Mercer and Armenta, 2011; Menendez et al., 2014). It was concluded by Pernet and Tremblay (2003) that the sonication technique for the extraction of oil from *Chaetoceros gracilis* increased the extraction rate which affects the recovery of lipid extracts. There are some contradictions in the literature regarding

scale up. Halim et al. (2012) noted that this technique is moderately suitable for scale up whereas Mercer and Armenta (2011) stated that ultrasound maybe difficult for upscale.

The study of Wiltshire et al. (2000) reported a 90 % extraction efficiency of fatty acids and pigments from the species *Scenedesmus obliquus* using ultrasound extraction. Cravotto et al. (2008) noted that ultrasound assisted lipid extraction from *Crypthecodinium cohnii* resulted in an increase in lipid yield of 21.1% as opposed hexane solvent extraction. It was observed by Ranjan et al. (2010) that ultrasound assisted lipid extraction from microalgae demonstrated more distorted clusters of biomass on micrographs, in comparison to cells with solvent penetration.

The comparison of various cell wall disruption techniques (autoclave, bead milling, microwave and sonication) on lipid extraction from *Botryococcus* sp. was investigated by Lee et al. (2010), where it was found that sonication was the least efficient in improving lipid yields, however, they were still higher compared to sole solvent extraction. Similarly, the study of Shen et al. (2009a) reported that the lipid content for *Chlorella protothecoides* was least using sonication treatment compared to bead beating and press methods. Zheng et al. (2011) tested the disruption treatment of *Chlorella vulgaris* biomass using microwave, ultrasound, enzyme lysis and bead beating, and noted lipid yields of 18, 15, 22 and 10%, respectively. De Souza Silva (2014) tested the pre-treatment of microalgae culture using microwave, autoclaving and ultrasonication technology for lipid extraction and found that ultrasound resulted in the lowest yields. In contrast, Prabakaran and Ravindran (2011) found that the lipid recovery from *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* sp. was highest using sonication pre-treatment compared with autoclaving, bead beating and microwave. Which was similar to the finding of Koberg et al. (2011), in that the lipid yields of 18.9% and 32.8% in *Nannochlorpsis* species were achieved using microwave and ultrasonication pre-treatment, respectively.

2.3.2.1 Factors Affecting Ultrasonic Assisted Extraction

The pre-treatment time, temperature of the reaction, cell concentration and microalgae type have been reported to influence the lipid yield in microalgae lipid extraction assisted with ultrasound technology.

***Extraction time*:** Increasing the time in which the cells are exposed to ultrasound can affect the recovery rate. Longer treatment time allows for increased cell disruption as a result of additional energy input (Lee et al., 2012). This effect was explored by Menendez et al. (2014), which found that an increase in extraction time from 5 to 20 min increased the lipid yield from 31 to 36%, respectively. Adam et al. (2012) noted that increasing the treatment time resulted in higher lipid recovery efficiencies. Additionally, Wiyarno et al. (2011) also reported that an increase in sonication time results in higher lipid yields. That performed by McMillan et al. (2013) suggested that increasing the sonication time resulted in greater cell disruption efficiencies. However, Tang et al. (2011) found that increasing the ultrasonic treatment time over the range of 15 to 90 min had no significant effect on the microalgae lipid recovery. In contrast however, Prommuak et al. (2012) noted that increasing the reaction time from 15 to 30 min resulted in decreased lipid yields from *Chlorella vulgaris* and *Haematococcus pluvials* species as a result of lipid oxidation with prolonged treatments.

***Reaction temperature*:** Altering the reaction temperature during ultrasonic treatment has been noted to influence the lipid recoveries from microalgae biomass. The study of Prommuak et al. (2012) found that an increase in temperature from 30 to 40°C recovered slightly higher lipids after 5-10 min. Adam et al. (2012) reported that increasing the temperature from 1 to 35°C in ultrasound assisted oil extraction from *Nannochloropsis oculata* resulted in increased oil yields by a factor of 1.5. Similarly, Wiyanrno et al. (2011) noted that increasing the reaction temperature from 23 to 60°C resulted in increases in lipid yields.

***Cell concentration*:** The effectiveness of microalgae cell disruption using sonication technique has been noted to change with cell concentration. It was stated by Lee et al. (2012), that increased cell fragment build up during treatment reduces the efficiency of the process, and concluded that sonication of concentrated biomass is less efficient than in dilute suspensions. Nowotarski et al. (2012) reported that sonication of the microalgae *Dunnaliella salina* was most effective using low densities. The work of Adam et al. (2012) indicated that the optimal concentration of microalgae cells for lipid extraction using sonication was 5% over the tested range of 5-30%. Natarajan et al. (2014) reported that the cell disruption efficiency increased with an increase in cell concentration up to 6.84 g/L

over the tested range of 0.07 g/L to 12.22 g/L. However, Gerde et al. (2012) found that varying the microalgae concentration over the tested range of 1.5-14.1 g cells/L did not require higher sonication energy for complete disruption.

***Microalgae type*:** The components of the microalgae cell wall coating are species specific and play an important role in the disruption efficiency using sonication techniques. Diatoms have siliceous cell wall coating called frustules and the degree of silicification of the frustules varies with cells of the same species as a result of nutrient availability (Tomas, 1997). It was reported by Neto et al. (2013) that for lipid extraction from diatom microalgae, sonication greatly impacted the cell disruption and improved the oil recovery. Joyce et al. (2013) reported that suspensions of *Nannochloropsis oculata* was unaffected by the ultrasound treatment as a result of varying microalgae cell wall thickness but *Dunaliella salina* and *Chlorella concordia* resulted in complete cell disruption with 4 min and 16 min sonication treatments, respectively. A similar finding was also noted by Nowotarski et al. (2012), in that *Dunnaliella salina* was effectively disrupted at low concentrations using sonication, while *Nannochloropsis oculata* was much more resistant even at longer sonication treatments. Furthermore, Natarajan et al. (2014) found that the *Chlorella* sp. cells released the lipids into the liquid suspension after sonication treatment but *Tetraselmis suecica* and *Nannochloropsis* sp. retained the lipids in the membrane after undergoing ultrasonication treatment.

Takeda (1991) noted that lipid extraction from *Chlorella minutissima* did not improve with sonication treatment as a result of cell fragility compared to diatoms. Sostaric et al. (2012) reported an increase in lipid yield of 6.02% in *Chlorella vulgaris* biomass using ultrasonic bath treatment because the *Chlorella* species possess a thin cell wall made up of sugar (Bicudo and Menezes, 2006). The work of Kaiwan-arporn et al. (2012) compared lipid yields from *Synechocystis aquatilis* using grinding and sonication techniques and found that the yields were 21.3% and 10.2%, respectively. Shen et al. (2009a) noted that the lipid yield using sonication is affected by cell size, shape and structure and reported lipid yields of 10.7 and 21.2% for *Chlorella protothecoides* and *Scenedesmus dimorphus,* respectively.

***Ultrasonic power*:** The ultrasonic power has been noted to have negligible effects on the oil yield of varying microalgae species. The study of Natarajan et al. (2014) tested ultrasonic power of 500, 750 and 1000 W on *Tetraselmis suecica* and *Chlorella* sp. oil yields and found that over the tested range no variations in yields were noted. Tang et al. (2011) tested the effects of varying ultrasonic power on the lipid yield of microalgae over the tested range of 80-200 W and found no significant difference in the oil yields with varying power. Similar findings were also noted by Cravotto et al. (2008), Singh and Gu (2010) and Adam et al. (2012), in that the sonication power had little effect on the oil yields of microalgae species.

2.3.2.2 Energy Consumption

Menendez et al. (2014) reported that the energy consumption using ultrasound assisted lipid extraction from *Nannochloropsis gaditana* increased from 1.7 to 6.7 Wh/g dried biomass when the reaction time was increased from 5 to 20 min which corresponded to increases in the lipid yields of 31 and 36%. Guldhe et al. (2014) reported that the energy consumption using ultrasound assisted lipid extraction from *Scenedesmus* sp. was 119 Wh/g. It was reported by Adam et al. (2012) that sonication resulted in an energy utilization of 16.66 Wh/g for lipid extraction from the microalga *Nannochloropsis oculata*. Halim et al. (2012) noted that the sonication energy consumption for *Chlorococcum* sp. was 36.66 Wh/g of dried biomass, but the lipid yields were only slightly higher than that from untreated biomass. Bigelow et al. (2014) found that sonication of *Chlamydomonas reinhardtii* microalgae biomass consumed 36 Wh/g. Wang et al. (2014) noted that sonication of *Scenedesmus dimorphus* and *Nannochloropsis oculata* consumed energy in the range of 17.3-86.8 Wh/g.

2.3.2.3 Advantages and Disadvantages

Pre-treatment of microalgae biomass using ultrasonication has several advantages which include: reduced extraction time, less solvent requirement, higher yields as a result of easier cell penetration, the biomass does need to be dry and easier release of intracellular components to the bulk of the solvent (Luque-Gracia and Castro, 2003; Lee and Shah, 2012). Chemat et al. (2011) and Wang and Weller (2006) stated that ultrasound assisted extraction can be operated at low temperatures (less thermal denaturation of biomolecules)

and is much more economical compared to other conventional extraction methods. However, ultrasound assisted techniques consume large amounts of power and pose scale up difficulties (Luque-Gracia and Castro, 2003; Lee and Shah, 2012). The use of high-intensity sonication can result in pressurization/heat that is damaging to the cells or tissue (Wang et al., 2014). This technique can be species specific since it has been noted to be ineffective in the destruction of diatoms with thick cellular coating (Nowotarski et al., 2012; Joyce et al., 2013; Natarajan et al., 2014).

2.3.3 High Pressure Homogenization

High pressure homogenization (HPH) is widely used in the food industry for processing of various products which include those of the dairy industry (Geciova, Bury, & Jelen, 2002) as well as microalgae (Lee, Lewis, & Ashman, 2012). This technique employs high pressures in the range of 150-400 MPa, by use of either one or two displacement pumps, forcing the cells through an orifice in order to collide with a valve seat (Halim, Harun, Danquah, & Webley, 2012a; Lee et al., 2012). After which, the cells are spread out on a seat surface and collide on an impact ring (Lee et al., 2012). The variation in the valve seat design influences the extent of cell disruption while minimizing the damage to the valve seat as a result of cavitation (Lee et al., 2012).

The mechanism for cell disruption using this technique is not exactly known, however, multiple theories have been proposed. These include: the impact as a result of hard surface collision with the valve seat and impact ring (Middelberg, 1995); high pressure exposure (Miller, Rogowski, & Kelly, 2002); cavitation (Clarke, Prescott, Khan, & Olabi, 2010); the pressure drop that suddenly occurs and the release of gas bubbles that result in the bursting of the bubbles (Clarke et al., 2010).

2.3.3.1 Factors Affecting High Pressure Homogenization

The major factors which influence the disruption efficiency, during high pressure homogenization, are pressure and the number of passes. Other factors include the temperature, the design of the orifice and valve, and the flow rate (Lee et al., 2012).

***Pressure*: The influence of pressure on the disruption efficiency of microalgae using HPH has been investigated for a number of species. The study of Wang et al. (2015) noted that

HPH of *Neochloris* biomass was significantly influenced by the change in pressure (40-80 MPa). A change in pressure from 40 to 60 MPa increased the disruption from 41 to 76 %, and further increases to 80 MPa only improved the disintegration by an additional 6.6 %. The authors noted a positive relationship between lipid yields and disintegration. Yap et al. (2016) found that the treatment of *Chlorococcum* sp. using HPH resulted in complete disintegration of the biomass when subjected to pressures of 35-40 MPa. However, Shene et al. (2016) found that increasing the pressure from 75 to 230 MPa did not enhance the lipid yield in *Nannochloropsis oculata.* This may be attributed to the higher pressures used, compared to those used in studies that noticed a variation.

***Number of passes*:** The effect of the number of passes on the disruption efficiency of microalgae using HPH was also investigated. It was reported by Halim et al. (2012a) that the disruption of *Chlorococcum* increased with the number of passes, and that after 5 passes (operating at 50 MPa) the ratio of intact cells to original number was less than 0.1. Similar, Shene et al. (2016) noted that increasing the number of passes from 1 to 6, using HPH increased the lipid yield from 8 to 14 % in *Nannochloropsis oculata* biomass.

2.3.3.2 Energy Consumption

The energy consumption of HPH for achieving 90 % cell disruption of the yeast *S. cerevisiae* was reported by Balasundaram and Pandit (2001) to be 26.8 Wh/g. In another study employing this technique on *Chlorococcum* sp., performed by Halim et al. (2012), an energy consumption of 147 Wh/g was noted, achieving an 80 % disruption efficiency.

2.3.3.3 Advantages and Disadvantages

The use of HPH is advantageous since it is scalable and is already employed at industrial levels (Geciova et al., 2002; Lee et al., 2012; Günerken et al., 2015). However, its implementation in microalgae bioprocessing is hindered by the dilute nature (0.01-0.85 % *w/w*) of microalgae suspension, therefore great amounts of energy would be required for processing (Günerken et al., 2015). Other drawbacks include the non-selective intracellular compound release, difficulties in disruption of rigid cell walls, and the presence of debris which may require an additional step for removal (Günerken et al., 2015). Furthermore, this technique maybe unsuitable for the recovery of fragile functional compounds (Janczyk, Wolf, & Souffrant, 2005; Günerken et al., 2015).

2.3.4 Steam Explosion

Steam explosion may also be used to disturb the microalgae cell wall so that the intracellular components maybe easily recovered. The treatment works by exposing the biomass to high temperatures that vary from 160°C to 260°C (Salvado et al. 2003; Velasquez et al. 2003; Cheng et al. 2011) and vapor pressure in the range of 1.03 and 3.45 MPa, followed by the return to ambient conditions through depressurization. The drop in pressure to ambient conditions results in cell wall rupture of the biomass (Kepp et al., 2000; Perez-Elveria et al., 2006; Morgan-Sagastume et al., 2011; Nurra et al., 2014; Lorente et al., 2015). Pre-treating microalgae biomass with steam technology should be performed at lower temperatures in order to prevent lipid degradation (Nurra et al., 2014).

In the study of Lorente et al. (2015), they reported that steam explosion pre-treatment of three types of microalgae biomass resulted in higher lipid extraction efficiencies compared to autoclaving, ultrasound and microwave techniques. Nurra et al. (2014) noted that the steam explosion pre-treatment of *Nannochloropsis gaditana* biomass resulted in increased lipid recovery from 0.3% to 3.6%. Furthermore, both Mosier et al. (2005) and Montane et al. (1998) reported that steam explosion is an economically efficient pretreatment technique for fractionating and modifying lignocellulosic materials which improves the feedstock quality for downstream processing.

2.3.4.1 Factors Affecting Steam Explosion

The factors which play an important role is steam explosion cell wall destruction of microalgae are temperature, pressure and microalgae species.

***Temperature*:** The temperature plays an important role in the effectiveness of microalgae cell wall rupture using this treatment (Nurra et al., 2014). The optimum temperature for hydrolysis of microalgae biomass depends on the microalgae species (Alzate et al., 2012).

The study performed by Nurra et al. (2014) demonstrated that increasing the temperature in steam explosion pre-treatment of *Nannochloropsis gaditana* biomass from 120 to 180°C increased the lipid yields from 3.6 to 8.8%. Also, Alzate et al. (2012) reported that increasing the temperature from 110°C to 170°C resulted in increased microalgae biomass biodegradability (10% to 27%). Furthermore, Mendez et al. (2014) observed an

increase in protein solubilisation in *Chlorella vulgaris* biomass of 16% at temperature of 140°C and a further increase in temperature in the range of 160 to 180°C resulted in solubilisation of 45%. Aguirre and Bassi (2014) noted that an increase in temperature from 104°C to 210°C in *Chlorella vulgaris* also increased the extraction efficiencies from 24 to 77%. However, that of Lorente et al. (2015) found that steam explosion of *Nannochloropsis gaditana* at 120°C and 150°C resulted in an increase in lipid extraction yields of 8.1 and 8.4%, respectively. In this case, the slight increase in temperature may not have been sufficient for significantly improving yields. It was noted by Du et al. (2012) that pre-treatment of microalgae biomass using steam explosion at a temperature of 150°C for 50 min was not sufficient in destruction of the microalgae cell wall.

Pressure**:** Altering the temperature in the steam explosion treatment vessel, changes the pressure of the system which effects the solubilisation of the microalgae biomass. It was stated by Aguirre and Bassi (2014) that temperature and pressure are correlated in steam treatment. Lorente et al. (2013) noted a slight increase in microalgae lipid recovery from 17.9 to 18.2% using steam explosion with increases in pressure from 2 to 4.7 bar which was achieved by increasing the temperature from 120 to 150°C. The study performed by Nuraa et al. (2014) found that an increase in lipid recovery from 3.6 to 8.8% was achieved using steam explosion pre-treatment of microalgae biomass, as the pressure was increased from 2 to 10 bar by increasing the temperature from 120°C to 180°C. Also, Mendez et al. (2014) reported that with increasing pressure from 3 to 10 bar in steam explosion of *Chlorella vulgaris* biomass, increased the carbohydrate solubilisation. Similarly, Robles Medina et al. (1998) noted an increase in carotenoid extraction from *Haematococcus* biomass of 15% when treated with high pressure compared to no pretreatment techniques.

Microalgae species**:** The lipid recovery from microalgae species using steam explosion varies with the type of species as a result of different cellular composition. It was noted by Lorente et al. (2013) that the lipid recovery using steam explosion resulted in increases of 8.7%, 9.5% and 2.1% compared to Bligh and Dyer extraction from *Nannochloropsis gaditana*, *Chlorella sorkiniana* and *Phaeodactylum tricornutum*, respectively. Similarly, the work of Alzate et al. (2012) found that steam explosion treatment for the production of biogas using *Chlamydomonas*, *Scenedesmus* and *Nannocloropsis* resulted in a

biodegradability of 70%, while *Acutodesmus obliquus*, *Oocystis*, *Phormidium* and *Nitzschia* sp. species resulted in a biodegradability of 30% using the same treatment. It was stated by Pandey et al. (2014) that the effectiveness of steam pre-treatment of microalgae is dependent on the cell wall composition which varies among microalgae species.

***Cell concentration*:** The biomass concentration has also been noted to influence the effectiveness of the steam explosion technique. Aguirre and Bassi (2014) found that the highest lipid yields from *Chlorella vulgaris* treated with steam, were achieved at concentrations lower than 5 g/L. The work of Kita et al. (2010) found that *Botryococcus braunii* treated with steam resulted in hydrocarbon recoveries of 90% or greater when operating at thermal temperatures of above 85°C with a biomass concentration of 1.5 g/L, while Frenz et al. (1989) noted a recovery of less than 1% using cell concentrations of 1 g/L for *B. braunii*, thus concentrations above 1 g/L were recommended. Further, it was mentioned by Menedez et al. (2014) that high pressure steam treatment at temperatures above 160°C for *Chlorella vulgaris* and concentrations of 16 g/L only solubilized 45% of the proteins, thus lower concentrations are required to achieve a higher degree of solubilisation.

2.3.4.2 Energy Consumption

Very little work has been done on the investigation of steam explosion as a pre-treatment technique on microalgae biomass. The energy consumption of this technique as reported by Keymar et al. (2013) consumed 2.92 Wh/g of volatile solid. The work of Ko et al. (2013) reported an energy consumption of 1.11 Wh/g for steam explosion pre-treatment of Ma bamboo for the production of 1 L of ethanol. That of Zhu and Pan (2010) on wood biomass indicated an energy consumption of 0.98 Wh/g for steam pre-treatment.

2.3.4.3 Advantages and Disadvantages

Microalgae steam explosion pre-treatment is advantageous as it disrupts that cellular wall as a result of sudden pressure release, making the lipids accessible for rapid recovery (Nurra et al., 2014) without the release of hazardous wastes (Cheng et al., 2011; Silva et al., 2013). Other advantages to steam explosion pre-treatment are its relatively low energy consumption (Zhu and Pan, 2010; Silva et al., 2013), low maintenance costs and its low corrosion potential (Silva et al., 2013). However, the effectiveness of this technique is

species specific (Pandey et al., 2014). To date steam explosion pre-treatment of microalgae biomass has only been recently studied and is under investigation at laboratory scale and has been primarily used for the production of biogas products (Pandy et al., 2014).

2.3.5 Autoclaving

Autoclaving microalgae biomass is a form of thermal treatment operating at a temperature of 121°C and pressure of 15 lbs (Surendhiran and Vijay, 2014). High thermal stress causes the cell walls to rapture forcing the release of the intracellular lipids (Prabakaran and Ravindran, 2011; Halim et al. 2012).

The study of Surendhiran and Vijay (2014) investigated the use of autoclave pre-treatment on *Nannochloropsis oculata* biomass and noted higher lipid yields compared to untreated biomass. Lee et al. (2010) also found that maximum oil recoveries from *Chlorella vulgaris* were achieved with autoclave pre-treatment. Prabakaran and Ravindran (2011) reported that the lipid yield recovered from *Chlorella vulgaris* was higher with autoclave pre-treatment than that without pre-treatment, but the lipids were not higher than microwave treated biomass. De Souza Silva et al. (2014) noted that autoclave and microwave pre-treatment of microalgae biomass resulted in lipid yields of 15.4 and 33.7%, respectively.

2.3.5.1 Factors Affecting Autoclave Assisted Extraction

The efficiency of autoclave technology is dependent on time elapsed of the treatment as well as the type of microalgae.

***Time*:** Altering the autoclave treatment duration has been reported to effect the effectiveness of microalgae lipid recoveries. The study of Surendhiran and Vijay (2014) pretreated *Nannochloropsis oculata* biomass using autoclave operating at 121°C and a pressure of 15 lbs at 10, 20 and 30 min, and achieved the highest lipid recovery of 29.34% at 30 min. That of Prabakaran and Ravindran (2011) noted an increase in lipid content of 22% in *Nannochloropsis oculata* with autoclave pre-treatment at 121°C for 5 min, but higher yields were achieved using microwave pre-treatment. Lee et al. (2010) reported an increase in lipid recovery of 4% in *Botryococcus* when autoclaved for 5 min. Further,

Rakesh et al. (2015) found that the autoclave pre-treatment of *Botryococcus* sp. for 15 min was effective in increasing the lipid recovery from the cells.

***Microalgae type*:** The species type has been noted to effect the autoclaves ability to effectively disrupt the cell wall. Rakesh et al. (2015) reported that autoclaving microalgae biomass for 15 min effectively increased the lipid recoveries in *Botryococcus* sp., but resulted in a 50% reduction in total lipid recovered from *Chlorella sorokinana* species. The work of Prabakaran and Ravindran (2011) indicated higher lipid recoveries in *Chlorella* sp., *Nostoc* sp. and *Tolypothrix* when autoclaved, however higher recoveries were achieved using microwave, sonication and bead beating since they are more abrasive disruption techniques. Lee et al. (2010) found that autoclave pre-treatment was effective in increasing the lipid recovery in *Scenedesmus* sp., but was not effective on *Botryococcus* sp. and *Chlorella vulgaris* compared to other pre-treatment methods investigated. Yu et al. (2015) reported that autoclaving *Chlorella sorokiniana* species resulted in the lowest lipid yields compared to other techniques such as bead beating, microalgae and sonication. The study of Miranda et al. (2012) noted that autoclave treatment of *Scenedesmus* biomass was the most efficient technique for sugar recovery. The effectiveness of autoclaving treatment on different microalgae spices varies as a result of different cell wall structures that can be tough and unaffected by autoclave disruption techniques (Russell, 1995; Miranda et al. 2012).

2.3.5.2 Energy Consumption

The energy consumption for autoclave pre-treatment of microalgae biomass is high as a result of high operational temperatures and pressures (King, 2014). This technique has been shown to consume higher amounts of energy than those of bead beating techniques (Lee et al., 2010). Lopata et al. (1999) reported that the costs of autoclave machinery range from high to very high. The power consumption as reported by Panasonic (2014) for their laboratory size (50 L) autoclave (Model No. MLS-3751L-PA) is 1900 watts. That reported by SciCan (2015) is of the order of 1700 watts for their Bravo laboratory scale (17 L) autoclave. A bigger autoclave with a volume capacity of 85 L (Market Forge STM-E autoclave), was reported to operate at a power of 9000 watts (BME, 2015).

2.3.5.3 Advantages and Disadvantages

The use of autoclave pre-treatment of microalgae biomass prior to lipid extraction is advantages because it disturbs the extracellular cell membrane allowing for easier recovery of lipids due to increased penetration (King, 2014). Autoclaving cells at high temperatures over a short duration can reduce the degradation of the desired product (Luo et al., 2010). However, this technique is difficult to upscale, long duration of time required, and large scale use would require high energy consumptions (King, 2014).

2.3.6 Freeze Drying

Freeze drying microalgae biomass is one of the most preferred drying techniques as a result of its mild operating conditions and the ease of lipid extraction after pre-treatment (Grima et al., 2003). The solvent extraction of lipids from wet biomass can be difficult, thus prior freeze drying of the biomass will overcome this difficulty (Grima et al., 2003). However, microalgae lipids are susceptible to degradation under thermal drying techniques and can result in evaporative loss of lipids (Pourmortazavi and Hajimirsadeghi, 2007). Further milling of the freeze dried microalgae would enhance the efficiency of lipid extraction as a result of increased surface area for biomass-solvent contact and diminishes the diffusion pathway (Halim et al., 2012).

A number of researchers have investigated the use of freeze dried microalgae biomass. These include the work of Guldhe et al. (2014), which states that water needs to be removed from microalgae biomass in order to increase the lipid extraction efficiency. That of Pasquet et al. (2011) found that freeze drying of the microalga species *Dunaliella tertiolecta* preserved the integrity of the cell. Arora (2012) noted that freeze dried microalgae biomass is more effective in the recovery of microalgae lipids as opposed to the solvent recovery using wet biomass. Belarbi et al. (2000) reported that solvent extraction of oil from wet biomass can be difficult, but prior freeze drying of the biomass can extract lipids more readily. Fajardo et al. (2007) also used freeze dried biomass for the solvent recovery of oil from *P. tricornutum* microalga species. However, Guldhe et al. (2014) noted that solvent lipid extraction from dried *Scenedesmus* sp. using freeze-dryer, oven and sun drying techniques did not significantly differ from one another. The work of Balasubramanian et al. (2013) also noted that the drying type (freeze-drying, oven and sun

drying) of *Nannochloropsis* sp. biomass has no significant influence on the lipid recovery using solvent extraction.

2.3.6.1 Energy Consumption

The energy consumption reported in the literature for freeze-drying of microalgae biomass has only been investigated on a laboratory scale. Guldhe et al. (2014) noted that the energy consumption for freeze-drying *Scenedesmus* sp. biomass was 17.7 kWh for every gram lipid recovered. The work of Green and Perry (2008) reported that the energy consumptions for drying microalgae using a freeze dryer was 5.56 Wh/g. Bennion (2014) found that the energy consumption for freeze drying microalgae biomass was 5.27 Wh/g. While the work of Ratti (2001) calculates the industrial freeze drying of biomass would be 38.88 Wh/g of biomass. Variation in the energy usage is a result of varying operational parameters and operational duration.

2.3.6.2 Advantages and Disadvantages

Freeze drying of microalgae biomass breaks up the biomass cells and turns them into fine powder, eliminating the need for homogenization (Ahlgren and Merino, 1991). Freeze drying is one of the most commonly used technique for the production of high value products because it is gentle and does not have adverse effects on the cellular components (Ryckebosch et al., 2011). Recovery of lipids from freeze-dried microalgae biomass does not require the use of a prior pre-treatment technique (Belarbi et al., 2000). However, the process can be time consuming and the costs can be high (Taher et al., 2011; Lee et al., 2012). Freeze drying has been deemed unsuitable for large scale operation as a result of high costs associated with scale up (Grima et al., 2003; Taher et al., 2011).

2.3.7 Sun Drying

The extraction of lipids from wet biomass can be difficult or ineffective as a result of water interference. Therefore it is necessary to dry the biomass prior to oil extraction for some treatment processes in order for effective recovery. Drying techniques include sun drying, solar drying, oven drying, freeze drying, spray drying and drum drying.

Sun drying of microalgae biomass is one of the easiest and low cost drying option available. It is performed by spreading the biomass on a bed lined with plastic sheets and

exposing them to sunlight in an area that is dust-protected (Becker, 1994; Lam and Lee, 2012). Layers of microalgae biomass that are greater than 0.75 cm in thickness have been noted to require more than 24 h of sun exposure. Single-day sun drying of microalgae has been noted effective on biomass placed on the plastic sheets with no more than 0.5 cm thickness (Becker, 1994). Gulde et al. (2014) dried *Scenedesmus* sp. biomass on a bed lined with plastic that is 1500 µm in thickness and exposed it to ambient temperatures that ranged from 25-30°C for 72 h. The study of Tiburcio et al. (2007) noted that sun drying of *Spirulina* biomass resulted in the lowest lipid pre-oxidation when compared with solar and oven drying techniques.

2.3.7.1 Advantages and Disadvantages:

Sun drying of microalgae biomass is advantageous since no input of energy is required for drying and oxidation of lipids is limited since the temperatures achieved are much lower compared with other methods (Becker, 1994; Glude et al., 2014). It is regarded as the only low cost and practical process for drying microalgae biomass (Lam and Lee, 2012). However, this technique is weather dependent, requires prolonged dehydration periods, prone to contamination and spoilage (Becker, 1994; Lam and Lee, 2012). Additionally, increased free fatty acid content has been reported in biomass subjected to natural sunlight drying, and a high free fatty acid content could decrease the transesterification efficiency deeming it unsuitable for biodiesel production (Chen et al., 2015).

2.3.8 Solar Drying

Similarly to sun drying, solar drying uses sunlight energy for dehydrating the biomass. However, this technique uses a closed solar device which allows for higher temperatures to result which in turn increases the drying rate (Iamronen, 2013). Rwehumbiza et al. (2012) effectively dried *Nannochloropsis salina* biomass using a solar dryer by placing the biomass on a polyethylene sheet with a thickness of 1 cm. The study of Prakash et al. (1997) effectively dried *Scenedesmus* biomass using a solar drier in 3-5 h. They stated that an ambient temperature of 35°C resulted in temperatures of 75-80°C in the solar devices. Tiburcio et al. (2007) reported that sun drying of *Spirulina* resulted in much lower peroxidation of lipids compared to solar drying and that solar drying and oven drying

resulted in similar peroxidation of lipid values. Kadam (2002) reported that solar drying of microalgae biomass is a feasible and cost effective method for industrial use.

2.3.8.1 Energy Consumption

Energy utilization is very little and is only required for the automated machine that mix and convey the biomass from one end of the solar device to the other, which has been calculated to be 20-40 kWh per ton of water evaporated (Delrue et al., 2012). Kadam (2002) stated that the power needed for fan in solar drying operation are estimated at 5 kWh/t CO_2.

2.3.8.2 Advantages and Disadvantages

Solar drying of the biomass is advantages since contamination is limited by the closed structure of the device and by the higher temperatures achieved in the panels allowing for increased rate of drying (Prakash et al., 1997). Additionally, solar drying provide a low cost method for drying microalgae biomass (Delrue et al., 2012). However, the lipids dried using solar dryers are susceptible to lipid oxidation as a result of long drying times (Lardon et al., 2009), the technique is weather dependent and the low temperatures achieved are not sufficient to sterilize the product of induce cell decomposition (Becker, 1994).

2.3.9 Oven Drying

Microalgae biomass slurry can also be dried using a convective drying apparatus such as an oven (Prakash et al., 1994; Desmorieux and Decean, 2005; Oliveira et al., 2009; Guldhe et al. 2014; Dissa et al. 2014). Gulde et al. (2014) effectively dried microalgae biomass at 60°C for 12 h, and noted that the free fatty acid content in the oil achieved from the oven dried biomass was almost as much as that achieved using freeze drying method. Ehimen et al. (2010) tested the conversion of *Chlorella* microalga oil to biodiesel efficiency using biomass that was oven dried at 80°C (0% moisture) with biomass that of partially dried and noted higher efficiencies with completely dried biomass. The work of Liang et al. (2009) effectively dried *Chlorella vulgaris* biomass using an oven operated at 80°C for further lipid extraction. That of Chen et al. (2015) noted that oven drying or solar drying are viable methods for biodiesel production and that these techniques do not have adverse effects on the lipids. In another study by Mandal and Mallick (2009), they effectively dried

Scenedesmus obliquus biomass at 60°C and noted a lipid recovery of 43% of dry cell weight. Cakmak et al. (2012) dried *Chlamydomonas reinherdtii* biomass by hot air oven and noted a lipid recovery efficiency of 41% dry cell weight.

2.3.9.1 Energy Consumption

The energy consumption for oven drying of microalgae has only been reported at lab scale. The study of Guldhe et al. (2014) noted that oven drying of *Scenedesmus* sp. biomass consumed 6 kWh. That of Bagchi et al. (2015) reported that drying of *Scenedesmus* sp. biomass over a period of 15 h consumed 0.033 kWh using a laboratory scale hot air oven. The Thermo Scientific Heratherm general laboratory oven is reported to consume 3.36 kW of power (Thermo Scientific, 2010).

2.3.9.2 Advantages and Disadvantages

Some of the advantages for using an oven to dry the microalgae are its simplicity, and rapidness compared to sun drying. This technique does not show significant differences in lipid yields compared to that of solar drying, but its drying efficiency, is however noted to be substantially better than solar drying techniques (Guldhe et al., 2014). Despite this, the content of free fatty acids in the naturally dried microalgae biomass (sun dried) is much higher than that dried using hot air convection. Increased amounts of free fatty acids in the biomass can result in decreased transesterification efficiency using alkaline catalyst, thus making natural sunlight drying of the biomass unsuitable for biodiesel production (Chen et al., 2015).

2.3.10 Spray Drying

Spray drying is a technique that works rapidly for continuously drying solutions, emulsions and slurries. A fine spray of solution, generated by pressure or centrifugal atomizers or gas-liquid jets, is brought into continuous contact with hot air in a large vessel (1-10 m). The small droplet size and the large droplet surface area ensure high evaporation rates so that drying times are measured in seconds. Air flow is usually cyclonic. The dimensions of the spray drier are such that the droplets only reach the walls when sufficiently dry to prevent sticking and burning on (Belter et al., 1988). Spray drying technology is most commonly used for the extraction of high value added products as a

result of its high costs (Desmorieux and Decaen, 2006; Milledge et al., 2013). Leach et al. (1998) effectively dried *Dunaliella salina* biomass using spray drying technology. Chen et al. (2015) noted that the products achieved with oven drying techniques result in 10 to 20 % loss in proteins compared to spray drying method.

2.3.10.1 Energy Consumption

The energy consumption of spray drying microalgae has been reported to utilize 0.43 kWh per kg of dry microalgae powder (Terma Vefechrenshrik, 2014). Liang et al. (2015) estimated the energy demand for spray drying of microalgae to be 6.4 kWh/kg of product.

2.3.10.2 Advantages and Disadvantages

Spray drying technique is advantageous because of the extremely short drying periods with limited contact to heat and oxygen which results in significantly lower peroxidation of lipids (Tiburcio et al., 2007). Additionally they are continuous in operation, the dried powder product does not require further size reduction and the product is of good quality (Belter et al., 1988). However, this technique is high in cost and can account for up to 30% of total algal production costs (Tiburcio et al., 2007). This method is not suitable for low value added products such as biodiesel (Brennan et al., 2010; Mata et al., 2010).

2.3.11 Drum Drying

Drum dryer technology works by placing the wet biomass into the rotating chromium-plated heating drum, where it is heated and dried (Becker, 1994). To ensure that the material does not stick onto the drum surface it should be highly polished. Compositional cell studies performed on the microalgae biomass after drum-drying revealed that it is excellent and highly digestible (Becker, 1994). Ben-Amotz and Avron (1987) reported that drying of *Dunaliella* biomass using a drum dryer did not have adverse effects on the intercellular components. Furthermore, Becker and Wolfgang (1983) effectively extracted lipids and proteins from *Chlorella*, *Scenedesmus* and *Spirulina* microalgae algae biomass using drum drying method. Both, Mahadevaswamy and Venkataraman (1981) and Saleh et al. (1985) reported that the use of drum drying of *Scenedesmus* sp., sterilized the sample and disrupted the cell wall simultaneously.

2.3.11.1 Energy Consumption

The energy consumption of various drum dryers have been noted to vary as a result of different operation conditions. Delure et al. (2012) noted that the energy consumption for drying of microalgae using a drum dryer is 900 kWh/ ton of water evaporated. That of Mineralit (2014) reported that the drum drying of microalgae biomass consumes 0.09 kWh of energy per kg of dry biomass. Liang et al. (2015) found that the energy demand for drum drying of microalgae was 5.2 kWh/kg and attributed that low efficiency as a result of low drying temperatures. In the study of Ryan (2009) it was noted that the energy required to dry microalgae biomass to a solid content up to 90% is 420 kWh/ha/d using a drum dryer.

2.3.11.2 Advantages and Disadvantages:

Drum drying technology is employed as a result of its convenience and dependability (Becker, 1994; Prakash et al., 1997). Additionally, it is reliable in achieving a product that is fully digestible and bacteriologically safe (Becker, 1994). However, this technique suffer from high operational and capital costs (Prakash et al., 1997).

2.3.12 Microwave

Microwave pretreatment processes enhance the kinetics of the lipid extraction process by disruption of the cellular wall (Wang and Weller, 2006; Menendez et al., 2014). This technique provides large amounts of thermal energy from electromagnetic radiation with a certain frequency to the cells (Hemwimon et al., 2007; Balasubramanian et al., 2011). The process consists of a feeding tank, microwave unit, water bath and a peristaltic pump that generates feed circulation through the system. Tubes with a diameter of 0.953 cm were used to carry the microalgae biomass from the feed-tank to the microwave unit. In the microwave chamber the mixture is heated using microwave radiation within seconds. Varying the radiation time alters the cell wall disruption efficiency. After treatment, the processed fluid is released into a 50 mL beaker that is held in a constant temperature water bath (Balasubramanian et al., 2011).

The thermal energy is a result of frictional forces that are caused by inter and intra-molecular movements (Amarni and Kadi, 2010). This energy causes temperature and

pressure effects on the cell wall that result in cell wall rapture (Halim et al., 2012). Disruption of the cell walls allows for the cell components to be released which increases the efficiency of the extraction process by overcoming the concentration gradient associated with solvent extraction (Lee et al., 2010; Cheng et al., 2013).

The work of Balasubramanian et al. (2011) investigated the use of microwave assisted solvent extraction for lipids from *S. obliquus* and noted that higher oil yields were achieved with solvent system hexane compared to the hexane extraction without microwave radiation at all temperatures and reaction times investigated. That of Lee et al. (2010) tested beadmilling and microwave cell disruption techniques on lipid extraction from *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. with chloroform/methanol (1/1 *v/v*) solvent system and found that microwave assisted method resulted in the highest the lipid yield for all three species. Sostaric et al. (2012) reported that pre-treatment of microalgae biomass using microwave irradiation results in higher oil yields. It was noted by Cheng et al. (2013) that the use of microwave irradiation resulted in a 31% increase in oil yields. It was postulated by Sostaric et al. (2012) that pre-treatment of microalgae biomass resulted in higher bio-oil yields due to micro-cracks present in the cell wall.

2.3.12.1 Factors Affecting Microwave Assisted Extraction

Temperature, reaction time and energy input can impact the efficiency of microalgae lipid extraction using microwave pre-treatment technology.

Temperature**:** High amounts of thermal energy in the form of heat disrupt the cellular wall of microalgae organisms and allows for easier recovery of intracellular lipids (Wang and Weller, 2006; Menendez et al., 2014). Increases in temperature result in rapid destruction of the cellular wall which results in increased lipid recoveries (Prommuak et al., 2012; Menendez et al., 2014). Figure 2.7 depicts the effect of microwave pre-treatment temperature on the lipid recoveries of varies microalgae species (adapted from the work of Biller et al., 2013).

The study of Menendez et al. (2014) found that increasing the reaction temperature from 60 to 90°C in microwave assisted lipid extraction from *Nannochloropsis gaditana* resulted in increased extraction efficiency from 29 to 40%, respectively. The of Prommuak et al. (2012) noted that the lipid extracts from *Chlorella vulgaris* and *Haematococcus*

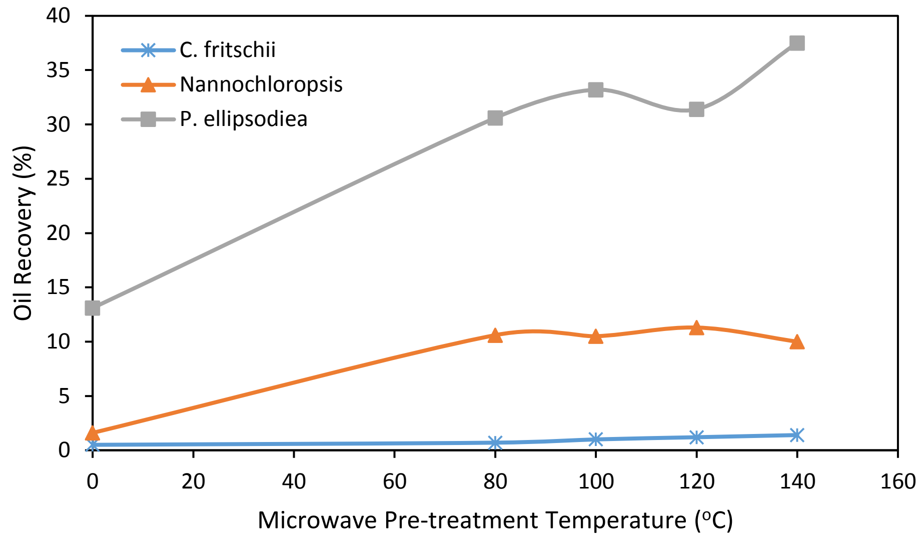


Figure 2.7. Effect of increasing microwave treatment temperature on the lipid extraction of microalgae (Biller et al., 2013).

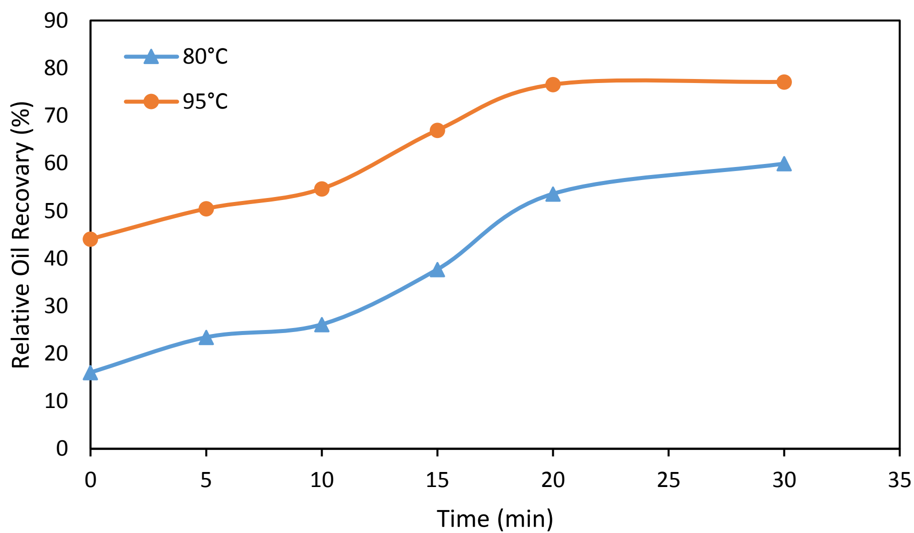


Figure 2.8. Varying microwave treatment time on *Scenedesmus obliquus* biomass with oil recovery efficiencies (Balasubramanian et al., 2011).

pluvialis using microwave assisted techniques at high temperatures resulted in higher lipids. Further, Balasubramanian et al. (2011) reported that increasing the temperature from 80 to 95°C in microwave assisted lipid extraction resulted in higher lipid yields (23-30% dry weight) from *Scendesmus obliquus*. In another study by Passos et al. (2013), they noted increased solubilisation of microalga biomass with increasing temperatures over the tested range of 50 to 98°C. Cheng et al. (2013) reported an increase in microalgae cell wall destruction with microwave pre-treatment as the temperatures increased from 80-120°C.

***Time*:** The length of time in which cells are exposed to microwave radiation has significant influence on the effectiveness in the destruction of the cell wall, which determines the recovery efficiency of the lipids present in microalgae biomass (Patil et al., 2011). Figure 2.8 depicts the relationship between varying the length of microwave treatment with the oil recovery (Balasubramanian et al., 2011).

The study performed by Patil et al. (2011) found that the reaction time has a significant effect on the effectiveness of the microwave assisted pre-treatment and that increased duration of microwave exposure increased the oil yields. Menendez et al. (2014) noted that increasing the reaction time from 10 min to 20 min in microwave assisted lipid extraction from *Nannochloropsis gaditana* at a temperature of 60°C resulted in increased extraction efficiency from 29 to 40%. Balasubramanian et al. (2011) reported that increasing the extraction time from 10 to 20 min in microwave assisted lipid extraction resulted in an increased lipid yield from 10% to 22% for *Scendesmus obliquus*. In addition, Dai et al. (2014) noted that increased microwave extraction time from 10 to 40 min resulted in increased microalgae lipid recovery 14 to 18%.

***Applied energy*:** The amount of microwave energy applied has been noted to effect the microalgae solubilisation. It was noted by Dai et al. (2014) that an increase in microalgae lipid recovery was attained with increasing microwave treatment power from 400 to 1000 W. Qv et al. (2014) reported an increase in microalgae extraction efficiency with increases in microwave power from 140 to 560 W. However, the authors noted that further increase to 700 W decreased the extraction recovery. Biller et al. (2013) found that increasing the microwave power from 25 to 61 Wh/g resulted in increased lipid yield from *Nannochloropsis* biomass from 1.6 to 10%. Furthermore, Passos et al. (2013) noted that

increasing the microwave energy from 300 to 900 W resulted in increased microalgae biomass solubilisation.

2.3.12.2 Energy Consumption

The energy consumption of microwave pre-treatment depends on the temperature and duration of the treatment. Menendez et al. (2014) reported that the energy consumption using microwave assisted lipid extraction from *Nannochloropsis gaditana* increased from 0.9 Wh/g to 1.6 Wh/g of dried biomass, as the reaction temperature increased from 60°C to 90°C which corresponded to lipid yields 29% and 40%. The work of Balasubramanian et al. (2011) demonstrated 76-77% oil extraction from *Scenedesmus obliquus* using microwave radiation with an energy consumption of 60 Wh/g of dried biomass. Guldhe et al. (2014) found that the energy consumption for microalgae lipid extraction from *Scenedesmus* sp. was 98 Wh/g of dried biomass at a temperature of 100°C. In another study by Bermudez et al. (2012), they noted that the energy consumption for microwave assisted lipid extraction from microalgae over 60 min period increased from 441 Wh/g fatty acid to 588 Wh/g fatty acid, as the temperature increased from 60°C to 120°C. Additionally, they found that reducing the treatment time to 20 min at 90°C consumed 204 Wh/g fatty acid and did not vary significantly from a higher treatment time. Passos et al. (2013) noted an increase in microwave power from 300 W to 900 W resulted in increased temperatures from 50°C to 98°C, and that increasing the time from 3 to 9 min at 300 W resulted in temperature increases from 50 to 95°C. The study of Biller et al. (2013) found that increasing temperature from 80°C to 140°C resulted in increased microwave power consumption from 25 Wh/g to 61 Wh/g. Additionally, Azcan and Yilmaz (2012) also noted that an increase in microwave temperature radiation consumed higher amounts of energy.

2.3.12.3 Advantages and Disadvantages

Microwave assisted lipid extraction from microalgae is one of the simplest methods and most effective amongst other extraction methods (Lee et al., 2010). This technique is moderately suitable for scale up (Halim et al., 2012). Microwave microalgae assisted lipid extractions has been noted to be the most applicable method for large scale use due to its simplicity and effectiveness (Lee et al., 2010). The rapid extraction time, high heating rates, low operating costs, environmentally friendly nature, lesser solvent requirements,

high product purity and high efficiency make it an attractive method for microalgae lipid recovery (Hemqimon et al., 2007; Refaat et al., 2008; Balasubramanian et al., 2011; Sostaric et al., 2012; Kumar et al., 2014). However, the disadvantages include the maintenance costs associated with large scale applications, an additional step required after pre-treatment for lipid recovery, high temperatures can result in lipid degradation and lengthy cooling times are required to avoid lipid loss (Wang et al., 2010; Kumar et al., 2015).

2.3.13 Enzyme Hydrolysis

Enzymes are used to hydrolyze the microalgae cell walls, releasing the intercellular components (such as lipids) into the bulk of the medium, making their recovery much more rapid and effective (Mercer and Armenta, 2011). Numerous types of enzymes have been reported to be effective in the cell wall degradation of microalgae. These include sanilase, cellulase, neutral protease, alkaline protease, papain and lysozyme (Table 2.6). Addition of these enzymes to the biomass will work to degrade the polymers on the cell surface which will allow easier recovery of the lipids from the biomass (Natarajan et al., 2014; Taher et al., 2014).

Freshwater microalgae possess highly resistant, non-hydrolyzable aliphatic biopolymers that are made up of even carbon numbered long-chain (30 to 34 carbon atoms) ω^9-unsaturated ω-hydroxy fatty acid monomers (Blokker et al., 1998). Intermolecular ester links of the monomers form linear chains that act as the starting position of ether cross-linking. These algaenans are highly resistant against degradation as a result of their polyether nature (Blokker et al., 1998; Burczyk et al., 1999; Allard and Templier, 2000). Blokker et al. (1998) predicted the structure of these algaenans consisting of linear polyester chains that are cross-linked by ether-bonds (Blokker et al., 1998).

The effectiveness of sanilase was tested by Zheng et al. (2011), on the hydrolysis of *Chlorella vulgaris* biomass, and they noted a lipid recovery of 7% which was much lower than that achieved using lysozyme and cellulase. The study of Liang et al. (2012) reported a lipid recovery of 34% using the enzyme snailase on microalgae biomass consisting of *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp. species. That of Lu et al. (2011) reported that the snailase enzyme was more effective in disrupting *Chlorella*

Table 2.6. Enzymes-assisted hydrolysis of microalgae for lipid recovery.

Enzyme	Microalgae	Lipid Yield (%)	Reference
Snailase		34	
Trypsin	*Chlorella vulgaris*	34	
Cellulase	*Scenedesmus dimorphus*	17	Liang et al. (2012)
Neutral protease	*Nannochloropsis* sp.	11	
Alkaline protease		8	
Neutral protease	*Neochloris oleoabundans*	24.3	Wang et al. (2015)
Cellulase Neutral protease	*Chlorella vulgaris*	66	Liu et al. (2013)
Neutral protease Alkaline protease	*Chlorella vulgaris*	85 80	Ying (2012)
Cellulase Novozyme 188	*Chlorella vulgaris*	73	Cho et al. (2013)
Papain	*Spirogyra* sp.	18	Reddy and Majumder (2014)
Cellulase	*Chlorella* sp.	63	Fu et al. (2010)
Cellulase Papain	*Neochloris oleoabundans*	10	Wang et al. (2015)
Lysozyme Cellulase	*Scenedesmus* sp.	16.6 15.4	Taher et al. (2014)
Lysozyme Cellulase	*Scenedesmus* sp.	12	Taher et al. (2014)
Cellulase	*Nannochloropsis oculata*	32.7%	Surendhiran & Vijay (2014)
Cellulase	*Chlorella vulgaris*	65	Liu et al. (2013)
Cellulase Alkali protease	*Chlorella vulgaris*	66	Liu et al. (2013)
Cellulase	*Chlorella vulgaris*	35	Zheng et al. (2012)
Sanilase		7	
Cellulase	*Chlorella vulgaris*	24	Zheng et al. (2011)
Lysozyme		22	

protothecoides cell wall than cellulase as a result of higher protoplast concentrations achieved. Cheng et al. (2012) also noted effective recovery of *Schizochytrium* protoplasts using the combination of snailase and cellulase enzymes for cell wall hydrolysis.

Liang et al. (2012) noted that lipid recoveries greater than 30% were achieved from *Chlorella vulgaris* using trypsin and snailase enzymes while lower yields resulted from neutral protease, alkaline protease and cellulase. The study of Sander and Murthy (2009) found that effective cell wall degradation in microalgae cells can be achieved using enzymatic cellulase. Further it was reported by Cho et al. (2013) that the presence of cellulose enzymes increased the cell hydrolysis which resulted in higher solvent lipid recoveries. The work of Fu et al. (2010) reported that cellulose enzyme hydrolyses of *Chlorella* sp. increased the lipid recovery to 56% compared to 32% prior to treatment. Liu et al. (2013) reported a lipid recovery of 65% from *Chlorella vulgaris* using cellulase assisted enzyme hydrolysis. Moreover, Zheng et al. (2011) indicated a lipid recovery of 24% from *Chlorella vulgaris* using cellulase cell wall hydrolysis.

In the study of Liang et al. (2012), they noted that a lipid recovery of 11% using neutral protease microalgae hydrolysis of *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp. In another study by Liu et al. (2013) they reported a lipid recovery of 66% from *Chlorella vulgaris* using neutral protease enzyme hydrolysis. Wang et al. (2015) noted a cell disintegration that ranged from 10.9% to 24.3% in *Neochloris oleoabundans* using neural protease corresponding to enzyme concentrations of 1% to 6%. That performed by Ying (2012) tested neutral protease enzyme extraction of lipids from *Chlorella vulgaris* biomass and noted a recovery efficiency of 85%.

Khomova et al. (1993) noted a complete recovery of lipids (37%) from *Monochrysis* biomass using alkaline protease enzyme treatment. The work of Liu et al. (2013) demonstrated a lipid recovery of 66% from *Chlorella vulgaris* biomass using a mixture of alkali protease and cellulase. Liang et al. (2012) attained a lipid recovery of 8% when a mixture of microalgae biomass (*Chlorella vulgaris*, *Scenedesmus dimorphus* and *Nannochloropsis* sp.) was treated with alkaline protease. Further, Ying (2012) reported an extraction efficiency of 80% using enzymatic alkaline protease in *Chlorella vulgaris* biomass.

It was observed by Wang et al. (2015) that a disintegration efficiency in *Neochloris oleoabindans* of up to 45.2% using papain enzyme for cell wall hydrolysis was achieved. Horst et al. (2012) noted that the papain enzyme assisted cell hydrolysis of *Phaeodactylum tricornutum* biomass was effective in lipid extraction. Further, Morris et al. (2009) used the enzyme papain to hydrolyze *Chlorella vulgaris* biomass and found that it resulted in a 16% disintegration efficiency. Reddy and Majumder (2014) used papain to hydrolyze *Spirogyra* sp. biomass and noted a lipid recovery of 18%.

Gerken et al. (2013) noted that the enzyme lysozyme was effective in the digestion of *Chlorella vulgaris* cell wall. Similarly, Taher et al. (2014) reported a lipid recovery from *Scenedsmus* sp. of 16.6% using the enzyme lysozyme for cell wall hydrolysis. The work of Zheng et al. (2011) also recovered a lipid concentration of 22% from *Chlorella vulgaris* biomass using lysozyme enzyme hydrolysis. That of Natarajan et al. (2014), stated that microalgae cell wall can be effectively degraded using lysozyme enzyme hydrolysis. Cuellar-Bermudez et al. (2014) reported that lysozyme was effective in disrupting microalgae cell wall.

2.3.13.1 Factors Affecting Enzyme Assisted Extraction

A number of factors have been noted to influence the efficiency of enzymatic pre-treatment for microalgae lipid extraction. These factors include: treatment time, enzyme concentration, cell concentration, pH and temperature.

***Time*:** Prolonged exposure of enzyme to microalgae biomass allows for increased cell wall degradation (Liang et al., 2012). The rupture of microalgae cell wall allows for easier recovery of the intracellular lipids (Fu et al., 2010; Surendhiran and Vijay, 2014).

The study of Liang et al. (2012) found that lipid yields increased with increasing reaction time from 2 to 12 h for *Chlorella vulgaris*, but reached a plateau at 12 h. Conversely, Surendhiran and Vijay (2014) noted that increasing the reaction time from 8 to 12 h resulted in increased lipid yield from *N. oculata* species. In another study by Reddy and Majumder (2014) they also found that increasing the reaction time of enzyme hydrolysis from 1 to 4 h resulted in increased oil yield from 12 to 18%. However further increase in incubation time to 8 h did not show any increases in oil yield and 10 h incubation time resulted in reduction of yield to 17%. Furthermore, Cho et al. (2013) noted that

increasing the enzymatic reaction time from 1 to 72 h resulted in increased *Chlorella vulgaris* oil yield from 18 to 85%. Fu et al. (2010) found that the microalgae lipid content increased with increasing reaction time from 1 to 70 h.

Enzyme concentration**:** The enzyme concentration affects the efficiency of microalgae cell wall degradation. Greater enzyme concentration allows for rapid cell wall degradation by increasing the enzyme to biomass content (Liang et al., 2012).

It was noted Liang et al. (2012) that the lipid recoveries from *Chlorella vulgaris* using Trypsin and snailase enzymes at concentrations of 4 to 8% did not vary from one another, but increasing the enzyme concentrations from 0.5 to 2% significantly increased oil yields. Similarly, Wang et al. (2015) noted that increasing the enzyme concentration from 1 to 6% in *Neochloris oleoabundans* resulted in increased cell disintegration from 48.2% to 64.4%, 38.1% to 45.2% and 10.9% to 24.3% using cellulose, papain and neutral protease, respectively. Additionally, Morris et al. (2008) reported that increasing the enzyme concentration from 0.55 to 4.5% resulted in increased cell hydrolysis from 10 to 25%. Kose and Oncel (2015) found that the highest protein recovery, as a result of effective cell wall hydrolysis, was achieved at an enzyme to biomass ratio of 8% over the rested range of 1-20%.

Cell concentration**:** The microalgae biomass concentration also plays a role in the efficiency of microalgae cell wall degradation. The greater the cell concentration the lower the contact of enzyme to biomass (Cho et al., 2013).

The work of Cho et al. (2013), found that increasing the microalgae cell concentration while holding the concentration of enzyme, resulted in decreased hydrolysis yields in *Chlorella vulgaris* as a result of increased mass transfer with higher microalgae concentrations. Fu et al. (2010) achieved the highest cell wall hydrolysis in *Chlorella* species at the lowest cell concentration of 20 g/l (20 to 40 g/l). Moreover, Morris et al. (2008) noted that the highest protein yield, as a result of cell wall hydrolysis, resulted at a biomass concentration of 10 or 15% over the tested range of 5-20%. Furthermore, Kose and Oncel (2015) found that the increasing the biomass concentration (lowering the enzyme to biomass ratio) resulted in lower protein yields which is attributed to lower cell wall hydrolysis. Similarly, Ho et al. (2013) noted that increasing the microalgae biomass

concentration (10 – 40 g/L) while holding the enzyme concentration constant, resulted in decreased glucose production yields.

***pH*:** The pH of the reaction medium plays an important role in the effectiveness of the enzyme (Table 2.7) as the function of the enzyme is strongly influenced by the pH (Liang et al., 2012).

The study of Cho et al. (2013) noted that an optimal pH exists for maximum enzymatic (Cellulase and Novozyme 188) hydrolysis of *Chlorella vulgaris* of 4.8 over the tested range of 3.8-5.8. That of Liang et al. (2012) found that snailase and trypsin enzymatic lipid extraction from microalgae was pH dependent and that the increases in pH above 4 (4-9) resulted in lower lipid yields. Fu et al. (2010) achieved the highest cell wall hydrolysis in *Chlorella* species at a pH of 4.6 using cellulase over the tested range of 3.6 to 7.6. Conversely, Harun and Danquah (2011) investigated the hydrolysis of *Chloroccum* sp. using cellulase over the pH range of 2.5 to 7.5 and found an optimal pH between 4 to 6, as a result of enzyme impairment under acidic and alkaline conditions. Reddy and Majumder (2014) used papain assisted enzyme cell wall hydrolyses on *Spirogyra* sp. and found it effective at a pH of 6.

***Temperature*:** The temperature of the reaction is crucial for efficient microalgae cell wall degradation. Low temperatures can slow down the reaction rate and high temperatures can result in lipid degradation. Thus the temperature of the reaction must be optimized for the enzymatic activity in order to achieve high lipid recoveries.

Cho et al. (2013) noted that a temperature of 50°C resulted in the highest hydrolysis efficiency in *Chlorella vulgaris* over the tested range of 40-60°C. Similarly, Reddy and Majumder (2014) found that increasing the reaction temperature in enzyme hydrolysis from 30 to 60°C resulted in increased oil yields from 14 to 27%, but further increase to 70°C resulted in a decreased oil yield (25%). In addition, Fu et al. (2010) found that the cell wall hydrolysis rate was highest at a temperature of 50°C over the tested range of 40-60°C in *Chlorella* species. Harun and Danquah (2011) found that an optimal temperature of 40°C existed for cellulase activity during over the tested range from 28 to 60°C. Both the works of Saha and Cotta (2007) and Mtui et al. (2009) also reported an optimum temperature range of 30-45°C for cellulase cell wall hydrolysis.

Table 2.7. Optimal pH for enzyme assisted microalgae cell wall hydrolysis.

Enzyme	**Microalgae**	**Tested pH range**	**Optimal pH**	**Reference**
Cellulase Novozyme 188	*Chlorella vulgaris*	3.8-5.8	4.8	Cho et al. (2013)
Snailase Trypsin	*Chlorella vulgaris* *Scenedesmus dimorphus* *Nannochloropsis* sp.	4-9	4	Liang et al. (2012)
Cellulase	*Chlorella* sp.	3.6-7.6	4.6	Fu et al. (2010)
Cellulase	*Chloroccum* sp.	2.5-7.5	4-6	Harun and Danquah (2011)
Papain	*Spirogyra* sp.	6	-	Reddy and Majumder (2014)
Cellulase Papain	*Neochloris oleoabindans*	6.5	-	Wang et al. (2015)
Lysozyme Cellulase	*Scenedesmus* sp.	7.48	-	Taher et al. (2014)

2.3.13.2 Advantages and Disadvantages

Microalgae lipid extraction using enzymes is a highly specific and rapid which make it desirable for specific biproducts (Bhat, 2000; Liang et al., 2012). This method requires low operational temperatures and has high specificity/selectivity of the lipid class and no corrosion issues associated, making it a desirable technique for microalgae cell wall hydrolysis compared to chemical and physical methods (Balat et al., 2008; Taher et al., 2014). However, efficiency is affected by the lipid composition and the type of microalgae (Liang et al., 2012) and enzymes can be high in cost (Kumar et al., 2014). These limit the use of enzyme hydrolysis on industrial scale (Dincer and Telefoncu, 2007; Fu et al., 2010).

2.3.14 Surfactant Disruption

Surfactants are amphipathic molecules that possess both hydrophilic and hydrophobic entities (Middleberg, 1995; Gilbert and Moore, 2005). The hydrophilic components are often times ionic, while the hydrophobic portions are made up of hydrocarbon chains (Belter et al. 1987; Middleberg, 1995). There are various classifications for surfactants, which include cationic, anionic, non-ionic, and zwitterionic. The basis of classification is determined by their hydrophobic and hydrophilic constituents (Arachea et al., 2012).

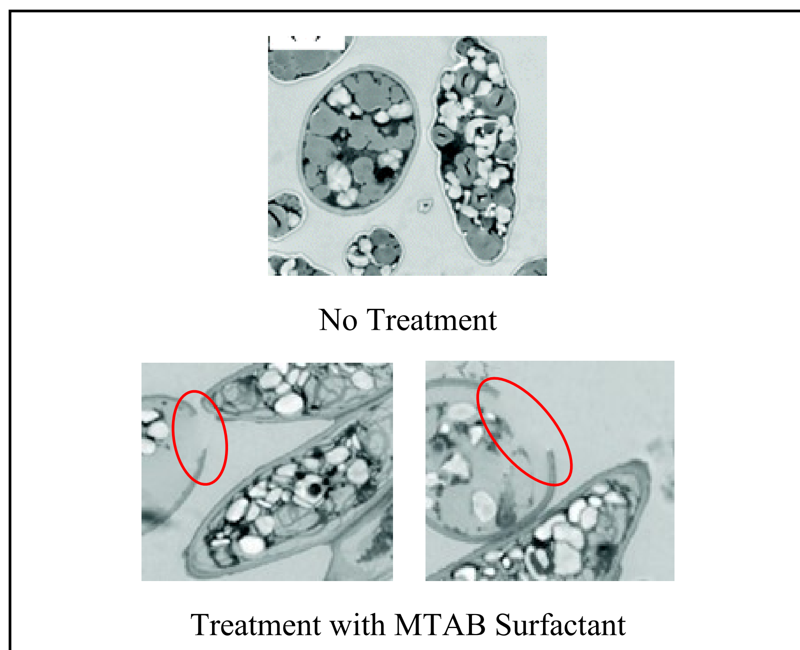
Surfactant use for protein separation has been well established. They work by binding to the hydrophobic components of the cell, thereby disrupting its form and allowing for the release of the proteins (Arachea et al., 2012). Following this mechanism, surfactants have found their way into the cell wall disruption of microalgae biomass (Corre et al., 1996; Huang and Kim, 2013; Coward et al., 2014; Lai et al., 2016). Microalgae cell surface has a negatively charged association as a result of the carboxyl, hydroxyl, phosphate, amino and sulfhydryl groups present on membrane surface (Monteiro et al., 2012), thus detergents of the opposite charge (cationic) are most suited for forming the initial attraction between the cell and compound.

The mechanism for surfactant cell disruption is hypothesized as two main interactions, electrostatic and hydrophobic (Huang et al. 2013). Electrostatic interaction is the adsorption process (Huang and Kim, 2013), whereby the positively charged cationic surfactant head groups, electrostatically bind to the negatively charged microalgae surface (Rupprechet and Gu, 1991; Gilbert and Moore, 2005). Hydrophobic interactions are where

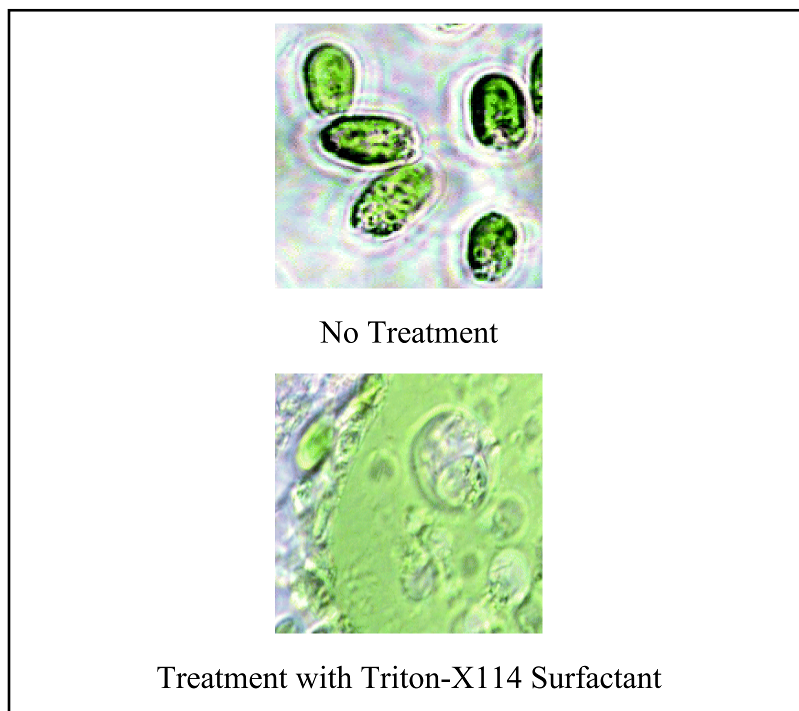
the surfactant interacts with the phospholipid components of the cell walls which results in micelle formation that distorts the cell wall (Chen et al., 2000; Huang and Kim, 2013). Additionally, it is thought that the hydrophilic-lipophilic interplay of the microalgae and surfactant can bring about the cell disruption as indicated by Ulloa et al. (2012). Whereby, the more hydrophobic surfactants result in greater interaction with the lipidic cell walls of microalgae (Ulloa et al., 2012). Thus, cell disruption occurs as a result of the interaction between the cells and the surfactant, and the biocidal effect noted with some surfactants (Salton, 1951; Isquith et al., 1972). The disruption of microalgae cell walls using surfactants was shown by various studies using transmission electron microscopy (TEM) and light microscopy, Figure 2.9.

A surfactant's ability to achieve cell disruption can be evaluated by its critical micelle concentration (CMC). The CMC is a measure of the threshold concentration of a surfactant for achieving a mixed lipid surfactant micelle, which, as mentioned earlier, results in cell disruption (le Maire et al., 2000). However, cell wall disruption through the use of surfactants may also be achieved at concentrations below the CMC value, the effectiveness depends on the surfactants composition and arrangement (le Maire et al., 2000). The effectiveness of disruption can also depend on the surface charge or hydrophobicity of the cell (Gerken et al., 2013), as well as the interplay of the hydrophilic-lipophilic components (Ulloa et al., 2012), and the pH of the media, since it could alter the charge present on the microalgae cell surface and surfactant sorption (Phoochinda and White, 2003).

As mentioned previously, there are various types of detergents which can be used for cell wall disruption, including ionic, non-ionic and zwitterionic (Brown and Audet, 2008). Selection of an appropriate detergent is crucial since it plays an important role in the time required for effective disruption. For example, the ionic detergent, SDS, is capable of disrupting the cell membrane in a matter of seconds, as a result of its strong nature (Brown and Audet, 2008). However, this detergent is less desirable in applications where the proteins are required to remain intact, since it has adverse effects on their activity. For protein preservation, it is suggested that milder (non-ionic) surfactants such as Triton X-100 be used in order to reduce the effect of protein degradation. Additionally, zwitterionic surfactants such as (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate),



(a) *Scenedesmus* Biomass



(b) *Tetraselmis suecica* biomass

Figure 2.9. Impact of surfactant cell disruption on (a) *Scenedesmus* biomass (treated with MTAB surfactant using TEM (adapted from Lai et al., 2016)), and (b) *Tetraselmis suecica* biomass (treated with Triton-X114 surfactant, using light microscopy (adapted from Ulloa et al., 2012)).

may also be used for cell wall distortion, with no net change in the protein charge (Brown and Audet, 2008).

2.3.14.1 Factors Affecting Surfactant Treatment:

Numerous factors have been investigated to determine the effectiveness of microalgae cell disruption using surfactant treatment addition. These include surfactant type (Ulloa et al., 2012; Park et al., 2014; Lai et al., 2016), growth stage (Coward et al., 2014; Lai et al., 2016), exposure time (Corre et al., 1996; Huang and Kim, 2013; Lai et al., 2016), surfactant concentration (Park et al., 2014; Lai et al., 2016), and the microalgae cell wall structure (Corre et al., 1996; Simpson et al., 2003; Lai et al., 2016).

Based on the literature citing's of the effects of these parameters on microalgae product recovery, it can be seen that the surfactant type (cationic, anionic, non-ionic, and zwitterionic) used has significant influence on the effectiveness of microalgae cell wall disruption, which intern effects the product recovery yields. In the study by Lai et al. (2016) they noted the effects of varying surfactant type, concentration, and microalgae growth stage on the effectiveness in disruption of the algaenan containing cell wall of *Scenedesmus* species. They noted that the FAME recovery of *Scenedesmus* biomass improved by 10-16 folds using 3-Decyldimethylammonio)propanesulfonate (3-DAPS), Myristyltrimethylammonium bromide (MTAB), and Sodium dodecyl sulfate (SDS), compared to untreated cells extracted using the same conditions. Furthermore, they found that surfactants without a charge (non-ionic) were less effective in improving FAME recoveries using isopropanol solvent. Generally, increasing the surfactant concentration resulted in higher FAME recoveries for all surfactant systems and prolonged growth resulted in greater FAME extractions using 3-DAPS and MTAB systems. However, the SDS resulted in the lowest FAME yields with increasing growth, for all concentrations tested. The structure and the integrity of the cellular matrix, and composition of the cell, change with growth phase. Characterization of the extracellular wall would indicate changes that take place, and consequently, give inference on how effective the bond formation between the cell and surfactant will be.

Similarly, Park et al. (2014) noted that the anionic surfactant tested on the polysaccharide extracellular wall of *Chlorella vulgaris*, resulted in lower lipid recoveries

compared to that of the control and sulfuric acid treatment. While the cationic surfactant (CTAB) resulted in an increase of 2.3 folds in FAME (Huang and Kim, 2013) and 7% phospholipid (Coward et al., 2014) recovery, compared to the control for the same species. This can be explained by the change in microalgae extracellular structure with time (as it is formed), which alters the charge of the surface effecting the bonds formed between the cell and surfactant (Gerken et al., 2013; Lai et al., 2016). In other words, the components of the surfactant must complement those present on the microalgae cell surface, in order to achieve proper electrostatic and hydrophobic interactions, which are the driving force for the disruption process (Rupprecht and Gu, 1991; Gilbert and Moore, 2005; Huang and Kim, 2013).

Conversely, Corre et al. (1996) reported that the presence of an outer trilaminar layer in *Chlorella emersonii* acted as a barrier against anionic (dodecylbenzene sulfonate) and nonionic (Tritron-100) detergent degradation, though the comparison of photosynthetic capacity of surfactant treated and untreated biomass. However, *Chlorella vulgaris* species, lacking the trilaminer structure, was susceptible to both types of detergents, which was noted by the drop in photosynthetic capacity. Furthermore, they observed negative photosynthetic capacity values for *Chlorella vulgaris* which were attributed to the consumption of intracellular storage materials. The degree of toxicity on *Chlorella vulgaris* varied significantly between the two detergents, DBS and Tritron X-100 resulted in negative values at concentrations up to 450 and 70 mg/L, respectively. This finding is in agreement with the inference made by Lai et al. (2016) that charged surfactants are more effective in disrupting the cell wall compared to non-ionic surfactants, since the toxicity of non-ionic detergents is high enough to cause the cells to consume their intracellular products, consequently reducing product recovery yields. Additionally, Corre et al. (1996) found that the growth stage significantly impacted the effect that the detergents had on *Chlorella vulgaris*, however, no variation was noted on *Chlorella emersonii*. *Chlorella vulgaris* cells in the stationary phase, exhibited lower sensitivity to the impact of Triton X-100, where the photosynthetic capacity became negative at concentrations closer to 90 mg/L as opposed to 70 mg/L in the high growth phase. However, the stationary phase cells of *Chlorella vulgaris* were not as effected by the DBS detergent, there was a slight inhibition (photosynthetic capacity of 80 %) noted at concentrations of 500 mg/L, but

overall it resembled a similar profile as that of the species possessing the trilaminer outer layer.

Increasing the surfactant exposure time (Corre et al., 1996; Huang and Kim, 2013; Lai et al., 2016), has been linked with greater product recovery, as a result of increased surfactant-cell contact.

2.3.14.2 Advantages and Disadvantages:

The use of surfactants has been noted effective in pre-treatment of certain species to increase product recoveries (Coward et al., 2014), where specificity is determined by the composition of the microalgae cell wall structure and type of surfactant used. Organisms lacking the algaenan (trilaminer structure), are most susceptible to cell wall degradation (Corre et al., 1996; Burczyk et al., 2014). Additionally, the surfactant concentration must be high enough to form surfactant-complex micelles (le Maire et al., 2000), and consideration must be made for the presence of surfactant in recovered products depending on the technique used. However, the use of surfactants as a cell disruption technique, is most promising since it can couple as both an agent in recovery (flotation processes) process of microalgae cells, and in disruption of the cellular matrix. In addition, the use of flotation techniques would recover both the surfactants and microalgae biomass, therefore limiting environmental hazards and increasing the concentration of surfactant in the recovered biomass; which eliminates the need for additional amounts for cell lysing processes. Moreover, the energy input requirement is low, and the cationic surfactant, CTAB, has been used as a food grade chemical in the extraction process of pigments for animal/human consumption (Thimmaraju et al., 2003). Therefore, the presence of surfactants in the biomass does not limit the types of value added products that can be formed (Coward et al., 2014), i.e. viable for cosmetics and pharmaceutical industries.

2.4 MICROALGAE OIL EXTRACTION

There are several mechanical and chemical oil extraction methods for processing microalgae biomass. However, lipid extraction technology should be selective towards acylglycerol in order to reduce the downstream costs associated with fractionation/purification. Lipid fractions such as polar lipids and nonacylglycerol neutral

lipids such as free fatty acids (FFA), hydrocarbons, sterols, ketones, carotenes and chlorophylls should not be extracted with the desired lipids (Medina et al., 1998). Furthermore, the technology should also be cheap and both energy and time efficient, nonreactive with the lipids and safe (Kates, 1986).

There are various chemical extraction methods that have been employed, which include: the addition of solvent to a closed vessel containing the microalgae biomass, closed Soxhlet apparatus which works by recycling the solvent, accelerated solvent extraction process which extracts lipids using solvents at elevated temperatures and pressures, and supercritical carbon dioxide extraction which uses CO_2 at critical temperature and pressure.

Mechanical microalgae lipid extraction techniques are thought to be an effective approach because they do not depend on the type of microalgae species used. Additionally, these modes of extraction offer no lipid contamination. Nonetheless, mechanical extraction methods suffer from higher energy requirements than those of chemical or enzymatic ones (Kumar et al., 2014). Mechanical extraction methods include the expeller press (Ramesh, 2013).

2.4.1 Solvent Extraction

Solvent extraction of lipids is a simple method that works based on the concept that compounds with similar polarity dissolve into one another as a result of the interactions between their hydrophobic fatty acid chains (Medina et al., 1998; Ramesh et al., 2013). Solvents can extract up to 98% of the fatty acids present in the biomass (Richmond, 2004). In the presence of an organic solvent (chloroform or hexane), the some cell membranes are permeable to the solvent allowing it into the cytoplasm. The lipids present in the cytoplasm are soluble in the organic solvent, forming a lipid-solvent complex (Mercer and Armenta, 2011). The lipid-solvent complex diffuses across the cell membrane by concentration gradient. This results in the extraction of the lipids out of the cell membrane and remain dissolved in the solvent (Halim et al., 2012). The remaining cell debris are removed from the solution by simple filtration technique (Halim et al., 2012). The steps required for lipid extraction using solvents is schematically depicted in Figure 2.10. The addition of a non-polar organic solvent to the system will induce a biphase separation in the single phase

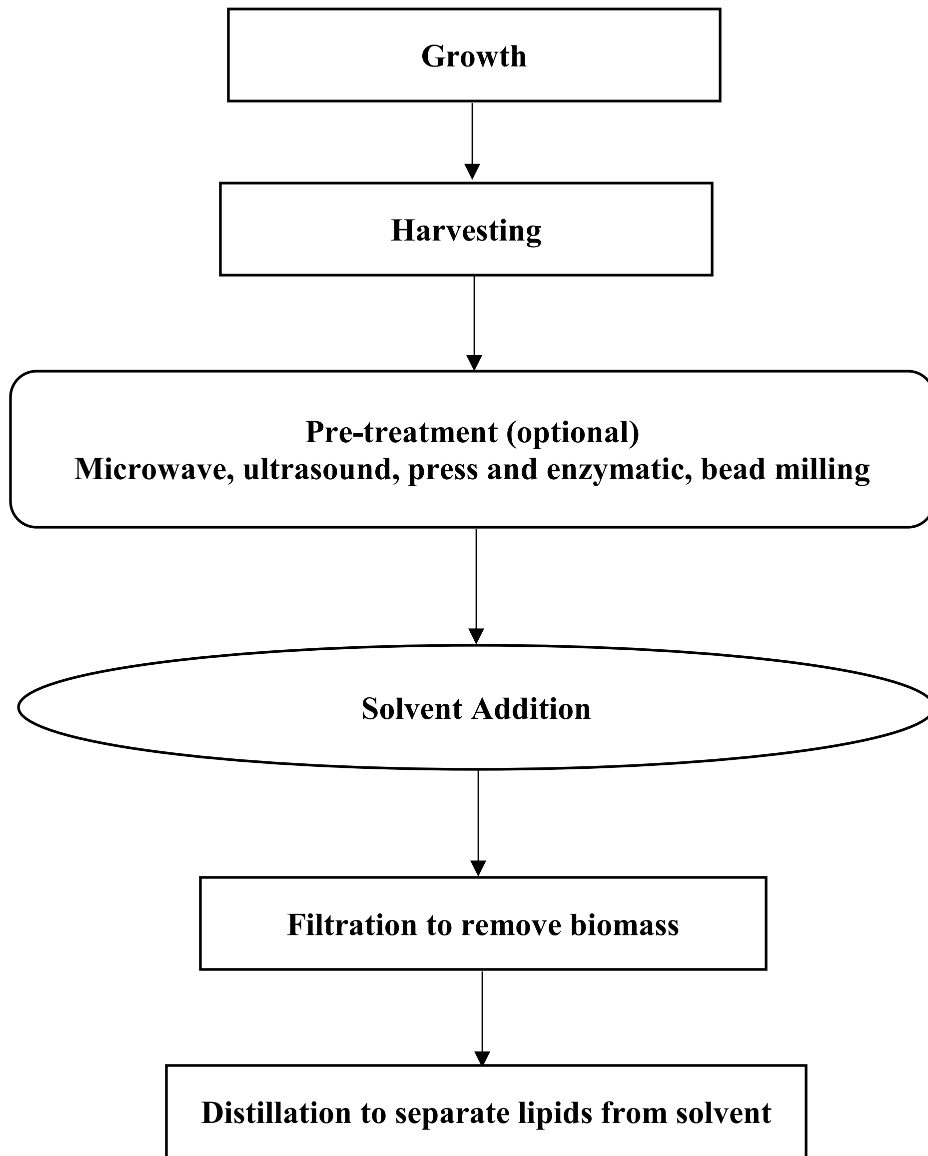


Figure 2.10. Schematic for the process of solvent lipid extraction from microalgae (adapted from Halim et al. (2012)).

organic solvent mixture (both polar and non-polymer organic solvents). This forms an organic phase that consists of both natural and polar lipids, and an aqueous phase consisting of water mixture and non-lipid contaminants such as proteins and carbohydrates (Medina et al., 1998). The organic phase is decanted and the lipids are recovered from the organic phase by simple distillation using a rotary evaporator (Medina et al., 1998; Araujo et al., 2013; Ramesh et al., 2013; Munir et al., 2013).

Different types of solvent or solvent systems can be used for the extraction of microalgae lipid (Table 2.8). The degradation of microalgae cell wall and the extraction of oil from the cells have been shown effective using chloroform, hexane, alcohols and the combination of solvent systems, (Harun et al., 2010; Ramesh et al., 2013). Ideally the organic solvents should be water insoluble, but lipid soluble, with a low boiling point and distinguishable difference in density from that of water for rapid recovery. The organic solvent should also be cheap and effective when recycled in order to decrease processing costs (Banerjee et al., 2002).

Chloroform: One of the most widely used organic solvent mixture for lipid extraction is chloroform/methanol (1:2 *v/v*). This mixture enables complete removal of both neutral and polar lipids from the cell. Biphasic separation is induced by additional chloroform/water after the removal of cell debris. The organic phase (lower phase) consisting of the lipids is recovered (Medina et al., 1998).

In the study of Lee et al. (1998), they found that the chloroform/methanol (2:1 *v/v*) solvent mixture resulted in the highest total lipid yield of 0.29 g/g (dry weight) for *Botryococcus braunii*. That of Guckert et al. (1988) reported an extraction efficiency of 11.1% (dcw) from *Chlorella* sp. using chloroform/methanol/50 mM phosphate buffer (35:70:28 *v/v/v*). Prommuak et al. (2012) noted that the polar and nonpolar mixture using chloroform/methanol system allowed for higher lipid extracts compared to systems using hexane alone, however not all lipids were suitable for conversion into biodiesel. In another study by Mendes et al. (2006), they found that the lipid recovery using chloroform/methanol combination yielded the highest lipid recovery compared to hexane and ethanol. Chen et al. (2009) effectively extracted 38% of lipids from *Chlorella vulgaris* using chloroform/methanol solvent system. Similarly, Prommuak et al. (2012) found that

Table 2.8. Solvent systems used for lipid recovery from microalgae biomass.

Solvent	Microalgae	Lipid Recovered (%)	Reference
Chloroform: Methanol (2:1 *v/v*)	*Botryococcus braunii*	29	Lee et al. (1998)
Chloroform: Methanol (1:2 *v/v*)	*Chlorella vulgaris*	38	Chen et al. (2009)
Chloroform: Methanol (2:1 *v/v*)	*Chlorella vulgaris*	38.9	Prommuak et al. (2012)
Chloroform: Methanol (2:1 *v/v*)	*Scenedesmus dimorphus*	13	Axelsson and Gentili (2014)
Hexane:isopropanol: water (70:47:3 *v/v/v*)	*Chlorella* sp.	5.8	Guckert et al. (1988)
Hexane:ethanol (3:1 *v/v*)	*Tetraselmis* sp.	6.5	Li et al. (2014)
Hexane	*Scenedesmus dimorphus*	29.7	Shen et al. (2009a)
Hexane: Ethanol	*Scenedesmus dimorphus*	25	Shen et al. (2009a)
Hexane	*Chlorella protothecoides*	23.5	Shen et al. (2009a)
Hexane: Ethanol	*Chlorella protothecoides*	17.7	Shen et al. (2009a)
Hexane	*Nannochloropsis gaditana*	0.73	Menendez et al. (2014)
Hexane	*Chlorella vulgaris*	20.3	Prommuak et al. (2012)
Butanol Hexane/2-propanol Ethanol	*Chaetoceros muelleri*	90 73 78	Nagle and Lemke (1990)
Ethanol/hexane	*Phaeodactylum tricornutum*	80	Fajardo et al. (2007)
Ethanol/hexane	*Picochlorum* sp.	33	Yang et al. (2014)
Methanol Ethanol 1-butanol	*Chaetoceros gracilis*	3.1 20.2 18.9	Wahlen et al. (2011)
1-butanol Chloroform	*Chlorella* sp.	1.96 0.64	Ehimen et al. (2009)

solvent system using chloroform/methanol yielded 38.9% lipid from *Chlorella vulgaris* while hexane resulted in a yield of 20.3%. Axelsson and Gentili (2014) reported a lipid recovery in *Scenedesmus dimorphus* of 13% using chloroform: methanol (2:1 *v/v*) solvent mixture. Ryckebosh et al. (2011) noted that the lipid recovery from *Chlorella vulgaris* using 2:1 chloroform: methanol was 76.5% of that achieved using 1:1 chloroform: methanol mixture.

Hexane: Hexane solvent extraction has been widely used for oil extraction. It can be used alone or coupled with other physical extraction processes such as expeller/press (Lee et al., 2014). Hexane/isopropanol (3:2 *v/v*) has been suggested as a substitute to chloroform/methanol because of its low-toxicity (Halim et al., 2011). Separation works similar to the system using chloroform/methanol, but the organic phase will be the upper portion upon biphasic separation. This mixture is more selective to the recovery of neutral lipids than those recovered using chloroform/methanol mixture (Lee et al., 1998). The most common solvent for microalgae oil extraction is hexane as a result of its low cost, low boiling point and high extraction efficiency (Mercer and Armenta, 2011; Ramesh, 2013).

A lipid extraction efficiency of 5.8% (dcw) using hexane/isopropanol/water (70:47:3 *v/v/v*) for *Chlorella* sp. was reported by Guckert et al. (1988). It was noted by Li et al. (2014) that hexane lipid extraction from *Tetraselmis* sp. was three times lower compared to the hexane/ethanol (3:1 *v/v*) solvent system, the reason being that ethanol is a polar solvent extracting much of the polar lipids which allows more of the non-polar hexane to extract the non-polar lipids. Similarly, Ryckebosch et al. (2012) and Lewis et al. (2000) found that solvent systems made up of both polar and non-polar mixtures resulted in higher lipid extracts. However, Shen et al. (2009a) found that the hexane/ethanol (1:1) solvent system resulted in lower oil yields than hexane alone on *Scenedesmus dimorphus* and *Chlorella protothecoides* species. Menendez et al. (2014) noted that the use of hexane for *Nannochloropsis gaditana* lipid extraction resulted in a 0.73% yield which was unsatisfactory. Further, Prommuak et al. (2012) noted that the crude extract from microalgae using hexane (20.3%) was much lower than that using chloroform/methanol mixture (38.9%). Widjaja et al. (2009) noted that the use of hexane solvent resulted in lower lipid yields than that using chloroform/methanol mixture.

Alcohol: Alcohols such as butanol, isopropanol, and ethanol can be used to extract lipids, but are limited to the interaction with free-standing neutral lipids as a result of its highly polar nature (Halim et al., 2011). The polar and non-polar nature of alcohols (ethanol) make it suitable for lipid extraction, however a purification step is necessary for the removal of undesired lipids (Fajardo et al. 2007; Yang et al., 2014).

Nagle and Lemke (1990) noted that of the three organic solvent (butanol, hexane/2-propanol, ethanol) systems tested for lipid extraction of *Chaetoceros muelleri*, butanol was the most efficient in lipid recovery compared to the other solvent systems. In the study of Fajardo et al. (2007), they effectively extracted lipids from *Phaeodactylum tricornutum* dry biomass using ethanol followed by the addition of hexane to remove non-lipid fractions such as protein and carbohydrates. That of Yang et al. (2014) achieved a lipid extraction of 33% from *Picochlorum* sp. wet biomass using ethanol solvent followed by hexane for purification. Wahlen et al. (2011) noted that the triacyelglyceride contact obtained from 100 mg of *Chaetoceros gracilis* was 3.1, 20.2 and 18.9 mg using methanol, ethanol and 1-butanol, respectively. The investigation performed by Ehimen et al. (2009) found that the lipid extracted from *Chlorella* sp. biomass was 1.96 and 0.64% (dry biomass) using 1-butonal and chloroform, respectively. Mendes et al. (2006) found that the lipid recovery using ethanol solvent, only extracted 73% of the lipids that were extracted using chloroform/methanol solvent combination.

2.4.1.1 Selection of Solvent Extraction System

Different solvent polarities have varying lipid recovery abilities. The selection of an appropriate lipid recovery solvent should be determined by considering the costs, solvent toxicity, extraction efficiency and ability to form a two phase separation with water. Table 2.9 provides a comparison of the chloroform, hexane and alcohol solvent systems. Based on these assessment criteria it can be seen that hexane solvent system is much more suitable for large-scale lipid extraction. Although chloroform has a higher lipid recovery efficiency than hexane, it is unsuitable for large scale use because large volumes of chloroform is hazardous. The use of alcohols for large scale microalgae lipid recovery can be difficult since they do not form a biphasic separation with water, thus the lipids will be recovered in large volumes of liquid without quick and easy separation techniques.

Table 2.9. Assessment of different solvents for microalgae lipid extraction (Cequier-Sanchez et al., 2008; Halim et al., 2011; Shang et al., 2011; Grima et al., 2013; Yang et al., 2014).

Assessment Criteria	Solvent Type		
	Chloroform	Hexane	Alcohols
Toxicity	Toxic solvent, residue cannot be reused for extraction of compounds without purification	Low toxicity, biomass residues maybe used for further processing of other value added products	Low toxicity, biomass residues maybe used for further processing of other value added products
Cost	Low cost	Low cost	Low cost
Extraction Efficiency	High extraction efficiency	Somewhat efficient in lipid recovery	Somewhat efficient in lipid recovery
Bi-phasic separation with water	Effective biphasic separation with water	Effective biphasic separation with water	No biphasic separation with water
Recovery of Solvent	Low boiling point, relatively easy recovery of solvent	Low boiling point, relatively easy recovery of solvent	Low boiling point, relatively easy recovery of solvent

2.4.1.2 Factors Affecting Solvent Extraction

The effectiveness of lipid extraction from microalgae using solvents can be dramatically altered by various parameters including the type of solvent, extraction time, solvent concentration and the temperature of the reaction.

Extraction time: The rate of lipid recovery has been noted to decrease with elapsed extraction time, Figure 2.11 (Fajardo et al., 2007). The majority of the lipids are extracted in the beginning of the extraction run as a result of higher concentration gradient. Continued lipid disposition into the bulk of the solvent reduces the concentration gradient, thus slowing the extraction process (Halim et al., 2012).

The effect of extraction time was investigated by Yang et al (2014), where they found that prolonged extraction times resulted in higher lipid yields in *Picochlorum* sp. biomass. In another study by Menendez et al. (2014), they noted that the lipid extraction yields from *Nannochloropsis gaditana* increased from 31 to 38% as the reaction time increased from 15 to 45 min, respectively. Further, Prommuak et al. (2012) noted that increasing the extraction time resulted in higher lipid recoveries. Cho et al. (2012) noted a linear increase between lipid yield and reaction time for the marine microalga *Scnedesmus*. Similarly, Fajardo et al. (2007) extracted the majority of the lipids (60-70%) in the first 8 h and had no significant improvement after 20 h of extraction time.

Solvent concentration: The amount of organic solvent required for effective lipid extraction is dependent on the amount of cellular lipids and the interaction between the lipid and solvent (Halim et al., 2012). The higher the solvent availability the higher the degree of lipid extraction from the biomass (Yang et al., 2014). It should also be noted that lipid content varies with species strain and with the cultivation parameters used, Figure 2.11 and 2.12 (Fajardo et al. 2007; Halim et al. 2012; Yang et al. 2014).

The study of Yang et al. (2014) achieved a lipid extraction of 96% from *Picochlorum* sp. wet biomass using ethanol with a solvent to biomass ratio of 4:1 over the tested ratio range of 2:1-6:1. That of McConnell (2013) reported that increasing the ratio of hexane to algae biomass from 5:1 to 30:1 resulted in increased lipid recovery from 2.75 to 3.9 g oil/g dry algae, respectively. Further, Chen et al. (2012) noted that increasing the solvent: biomass ratio from 5:1 to 10:1 while using the co-solvent hexane: ethanol (3:1) system

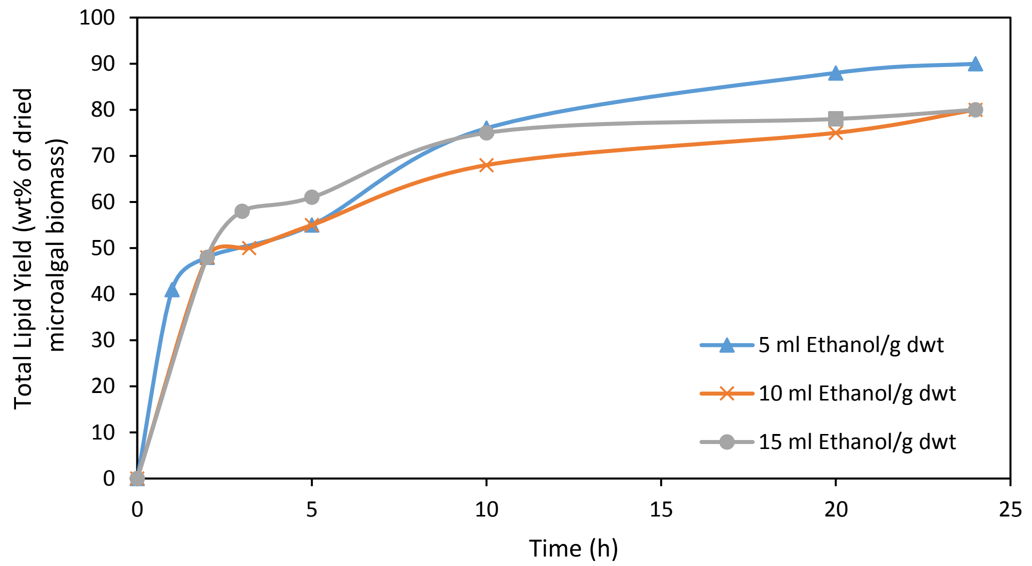


Figure 2.11. Effect of extraction time on the lipid recovery of *Phaeodactylum tricornutum*, using varying concentrations of solvent (adapted from Fajardo et al., 2007).

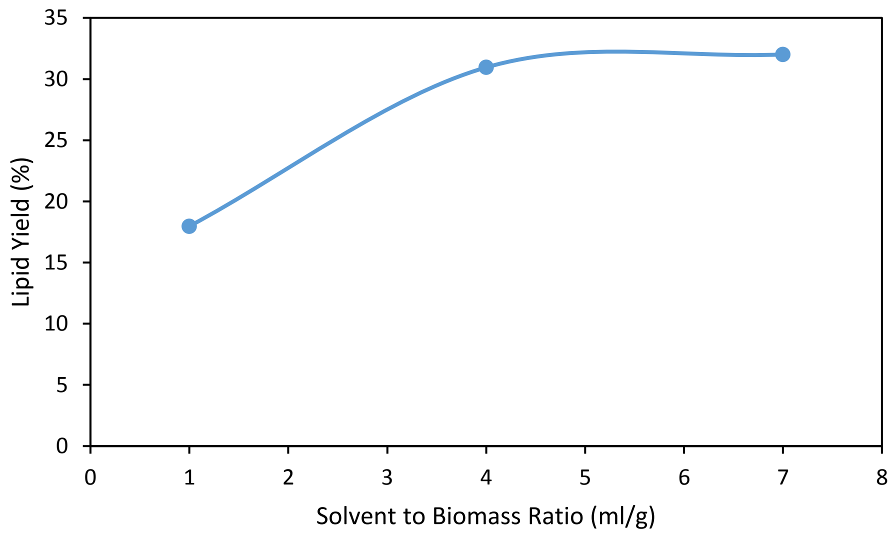


Figure 2.12. Effect of solvent concentration on the lipid yield *Picochlorum* sp. (adapted from Yang et al., 2014).

resulted in increased lipid yields from 55.9 to 88.2%. Ibanez-Gonzalez et al. (1998) reported that decreasing the ethanol to biomass ratio from 19.86 to 1.05 mL/g algae resulted in a 4% decrease in lipid yield from *Phaeodactylum tricornutum*. While, Li et al. (2014) noted that hexane/ethanol (3:1) solvent system resulted in higher lipid extract from *Tetraselmis* sp. than hexane alone. The investigation of Shen et al. (2009a) found that the hexane/ethanol (1:1) solvent system resulted in lower biomass yields than hexane alone on *Scenedesmus dimorphus* and *Chlorella protothecoides* species. Ryckebosch et al. (2012) demonstrated that chloroform/methanol (1:1) and (2:1) solvent systems extracted 24 and 18% of lipids from *Chlorella vulgaris,* respectively. Fajardo et al. (2007) noted that decreasing the concentration of hexane/hydroalcoholic (1:1) solution resulted in higher lipid extracts from *Phaeodactylum tricornutum* up to a hexane to hydroalcoholic ratio of 3:2. Wiyarno et al. (2011) noted that increasing the ethanol concentration during Soxhlet lipid extraction resulted in lower oil yields using the microalgae *Nannochloropsis* sp.

Reaction temperature: The reaction temperature has been noted to affect the lipid extraction yields. Due to increased mass transfer kinetics, higher lipid yields are achieved with increased temperatures (Balasubramanian et al., 2010).

The study of Fajardo et al. (2007) reported that an increase in the extraction rate of lipids as the temperature increased from 30°C to 60°C, but temperatures above 70°C resulted in lipid degradation (decreased lipid yield). That performed by Balasubramanian et al. (2010) noted a significant increases in total lipid yield from *Scenesdesmus obliquus* with increased reaction temperature. However, Yang et al. (2014) found that increasing the reaction temperatures from 20 to 50°C in *Picochlorum* sp. using ethanol solvent were not significantly vary from one another. Further, Menendez et al. (2014) noted that the lipid extraction yields from *Nannochloropsis gaditana* increased from 33 to 38.3% as the temperature increased from 23 to 60°C, respectively.

Stirring: Stirring of the cells during extraction has been suggested to improve the extraction efficiencies. Stirring of the solvent and biomass mixture during lipid extraction allows for the lipids to be carried away from the cell walls by the slight agitation (Yang et al., 2014). This was investigated by Gonzalez-Delgado and Kafarov (2013), whom noted that stirring the extraction mixture allows for cell wall rupture by the mechanical action.

In addition, Ranjan et al. (2010) stated that the lipid extraction mechanism using organic solvents is diffusion and disruption, whereby diffusion is a result of the solvent and the disruption is attributed to the friction caused by the solid during stirring.

The study of Chen et al. (2012) stated that stirring at 100 rpm during extraction of lipids from *Nannochloropsis* sp. promoted the contact between the lipids and solvent. Reis et al. (1998) extracted lipids from microalgae biomass using various solvents at room temperature with stirring rate of 100 rpm. Samori et al. (2013) extracted lipids from microalgae biomass using organic solvent with a stirring rate of 250 rpm for 24 h in order to ensure contact between the solvent and biomass. The study performed by D'Oca et al. (2011) extracted lipids from microalgae biomass operated at room temperature with magnetic stirring rate of 700 rpm. Garcia et al. (2010) found that stirring at a rate of 500 rpm for 24 h during solvent lipid extraction of microalgae promoted the contact between solvent and biomass which resulted in higher lipid recovery efficiencies.

2.4.1.3 Advantages and Disadvantages

Solvent lipid extraction is advantageous because it is moderately suitable for scale up, large quantities can be used (Halim et al., 2012), drying of the biomass is not necessary (Medina et al., 1998), solvents can be cheap, reproducible results can be obtained (Herrero et al., 2004) and the extraction process requires a lower energy input (Lee et al., 2014). However, some of the disadvantages include: the requirement of large amounts of chemicals, solvent toxicity, solvents are highly inflammable, long reaction times are required, energy intensive removal of solvent techniques and traces of organic solvent maybe present in the end product (Herrero et al., 2004; Galloway et al., 2004; Sahena et al., 2009; Mercer and Armanta, 2011; Halim et al., 2012; Ramesh et al., 2013; Lee et al., 2014; Kumar et al., 2014). Another disadvantage for using chemical lipid extraction include the added costs for solvent recovery for reuse (Lee et al., 2014), the efficiency of lipid recovery using solvent extraction techniques is dependent on the lipid mass transfer equilibrium (Wang and Weller, 2006), the selectivity of the lipids is low, and thus additional processing is required for biodiesel products (Crespo and Yusty, 2005) and solvent extraction efficiencies are also dependent on the species type (Kumar et al., 2014).

2.4.2 Soxhlet Solvent Extraction

Soxhelt solvent extraction works by continuously recycling the solvent through evaporation and condensation in order to limit the amount of solvent used (Wang and Weller, 2006). The microalgae biomass can be in the form of dried powder, concentrate or disrupted concentrate and is placed in the Soxhlet extractor (De Castro and Ayuso, 2000). The solvent is placed in the round bottom flask placed at the bottom of the vessel. The flask is heated and the organic solvent makes its way up to the condenser, where it recondenses and enters the Soxhlet extractor containing the biomass. The lipids in the biomass dissolve into the solvent and the thimble prevents the biomass from getting carried away. Once the organic solvent overflows, it is siphoned back into the round bottom flask. The process continues where the organic solvent evaporates leaving the lipids in the round bottom flask. The reaction is allowed to continue until there are no more lipids left in the biomass by multiple solvent extraction from the biomass (Wang and Weller, 2006).

2.4.2.1 Factors Affecting Soxhlet Solvent Extraction

Soxhlet microalgae lipid extraction efficiency has been noted to be affected by a number of factors which include: species type, extraction time, sample moisture content and the type of solvent used for the extraction.

Species: Different species type has been noted to yield different lipid contents as a result of varying cellular composition (Mandal et al., 2013; Rios et al., 2013).

In the study performed by McNichol et al. (2012), they noted that lipid extraction from *N. granulate* and *Phaeodactylum tricornutum* was highest using ethanol Soxhlet extraction while *B. braunii* lipid recovery was greatest using chloroform/methanol solvent mixture. Frigo-Vaz and Wang (2014) tested the lipid extraction from *Chalamydomonas reinhardtii* and *Dunalieela tertiolecta* and found that higher yields using acetone were obtained for the *C. reinhardtii* species compared with *D. tertiolecta*. That of Mandal et al. (2013) studied the Soxhlet lipid extraction efficiency of 13 different microalgae and noted that *Scenedesmus obliquus* resulted in highest extraction yield using Soxhlet chloroform: methanol mixture. They also noted that for *Scenedesmus obliquus* hexane, chloroform and petroleum ether solvents resulted in higher yields at room temperature than those operated in Soxhlet extraction. Furthermore, Xiaobin et al. (2012) noted that hexane Soxhlet lipid

extraction from *Chlorella pyrenoidosa* was least effective compared to 4 other solvent extractions at room temperature. Rios et al. (2013) tested *Phaeodactylum tricornutum*, *Nannochlorpsis gaditana* and *Chaetoceros calcitrans* species in hexane Soxhlet extraction and found that there was no significant difference in the yield between species. Additionally, Gog et al. (2012) noted that *Nannnochloropsis oculata* lipid extraction was highest using hexane Soxhlet extraction compared to hexane lipid extraction.

Time: During Soxhlet lipid extraction, the duration of the extraction plays a critical role. As the reaction time is increased more lipids are extracted as a result of increased exposure of fresh solvent with the biomass (Wiyarno et al., 2011; Long and Abdelkader, 2011). However, prolonged periods of time have been noted to decrease the lipid recovery as a result of lipid denaturation (Manirakiza et al., 2001).

It was noted by Wiyarno et al. (2011) that ethanol oil extraction from *Nannochloropsis* sp. resulted in greater oil yields with increased treatment time over the tested range of 80 to 220 min. Similarly, Tanzi et al. (2012) found that increasing the time from 30 to 450 min resulted in increased lipid yields from *Chlorella vulgaris* microalgae using hexane solvent. Furthermore, Long and Abdelkader (2011) noted that hexane Soxhlet lipid extraction resulted in yields that ranged from 15.7-17% (8 replicates) from *Nannochloropsis* sp. Li et al. (2014) extracted lipids from *Nannochloropsis* microalgae species by means of Soxhlet in the range of 9.4-15.1% operating for 6 h. Molnar et al. (2013) found that increasing the treatment time in Soxhlet chlorophyll extraction from *Chlorella vulgaris* microalgae species. In another study by Manirakiza et al. (2001) noted that increasing the time from 60 to 150 min in Soxhlet lipid extraction from margarine resulted in higher recoveries, but further increase in treatment resulted in lower yields. Deng et al. (2012) noted that in Soxhlet lipid extraction of *Synechoccus* sp. the optimal time of extraction was 4 h over the tested range of 2 to 6 h.

Moisture content: The moisture content in the biomass has been noted to effect the oil recovery yields form microalgae biomass (Topare et al., 2011).

The work of Topare et al. (2011) found that microalgae biomass moisture content of 0, 25 and 50% resulted in oil yields of 1.92, 1.58 and 1.13 g, respectively, using hexane Soxhlet extraction. That of Bi and He (2013) reported that increasing the moisture content

of 2.9 and 7.7% in *Nannochloropsis salina* resulted in a slightly lower saturated and monounsaturated fatty acid recoveries. Furthermore, Naghdi et al. (2014) found that lipid extraction of *Tetraselmis* sp. biomass with an 80% moisture content resulted in 32% less lipid yield compared to dried microalgae biomass. Deng et al. (2012) noted that in Soxhlet lipid extraction of *Synechoccus* sp. the optimal liquid to solid ratio for maximum lipid recovery was achieved at 6 ml/ 10^9 cell over the tested liquid addition of 2 to 10 ml in 10^9 cell.

Solvent: The solvent type also plays a critical role in the Soxhlet extraction of lipids from microalgae. The different polarities of the solvent are capable of extracting different types of lipids (Long and Abdelkader, 2011; Halim et al., 2011).

In the investigation of McNichol et al. (2012), they noted that Soxhlet lipid extraction from *N. granulate, B. braunii* and *Phaeodactylum tricornutum* using chloroform/methanol, ethanol, acetone and hexane was highest using ethanol solvent for *N. granulate* and *Phaeodactylum tricornutum,* but lipid extraction from *B. braunii* was highest using chloroform/methanol mixture. Additionally, Long and Abdelkader (2011) tested hexane, hexane/cyclohexane, cyclohexane/2-prponal, hexane/2-propanol and cyclohexane/1-butonal solvent systems for the lipid extraction of *Nannochloropsis* sp. and noted the highest lipid yield of 31.2-35.4% was obtained using cyclohexane/1-butanol Soxhlet extraction. Li et al. (2014) reported a 3-fold increase in Soxhlet lipid recovery from *Tetraselmis* sp. using hexane/ethanol (3:1 *v/v*) solvent system compared to hexane alone.

The study of Halim et al. (2011) reported that the Soxhlet hexane lipid extraction was significantly more efficient than its batch counterpart using the microalgae species *Chloroccum* sp. That of Long and Abdelkader (2011) noted that the lipid extraction from *Nannochloropsis* using hexane/2-propanol solvent system at room temperature resulted in lipid yields of 11.9-21.8%, while the same solvent system in Soxhlet apparatus resulted in yields in the range of 25-31%. Further, Sanchez et al. (2014) found that Soxhlet microalgae oil extraction resulted in yields of 6.1 and 9.8% using hexane and acetone solvent systems, respectively. In that of Naghdi et al. (2014), they noted that the lipid recovery from *Tetraselmis* sp. using hexane and hexane-ethanol (3:1 *v/v*) was 7.7 and 14.3%, respectively. D'Oca et al. (2011) found that lipid extraction from *Chloroella pyrenoidosa* using soxhlet

extraction was highest with ethanol solvent system. The works of Mandal et al. (2013) tested hexane, diethyl ether, chloroform, petroleum ether, chloroform: methanol, cyclohexance: 2-propanol, acetone: hexane and dichloromethane: hexane solvent systems in Soxhlet lipid extraction of *S. obliquus* and noted that chloroform: methanol combination resulted in the highest lipid recovery efficiencies.

2.4.2.2 Energy Consumption

The energy consumption for lipid recovery of microalgae using Soxhlet solvent extraction is high in energy demand. The biofuel production was assessed form microalgae by Lardon et al. (2009), where it was found that 90% of the processing costs were accounted for in the drying and solvent steps required for Soxhlet extraction. It was reported by Tanzi et al. (2013) that an energy consumption for Soxhlet microalgae (*Nannochloropsis oculata* and *Dunaliella salina*) lipid extraction of 8.84 kWh/g was consumed, which accounts for the drying of the biomass that is required for this process. The work of Ghosh et al. (2013) noted that the Soxhlet extraction of lipids from *Chlorella varabilis* biomass consumed 5.22 kWh for condenser chilling and 4 kWh of energy for the heating mental. That of Fabiano-Tixier et al. (2011) reported that the separation of certain compounds from the plant *Cinchona* using Soxhlet solvent extraction consumed 3 kWh of energy solely for heating and evaporation of the solvent.

3.2.3. Advantages and Disadvantages

This extraction method is advantageous because of the low solvent requirements and continued recycling of the solvent (Wang and Weller, 2006). High extraction temperature with heat from the distillation flask is maintained and no additional filtration is required for the extract (Tandon and Rane, 2008). However, this method is difficult to scale-up and suffers from high energy requirements (Wang and Weller, 2006; Halim et al., 2011). This technique is time-consuming and can result in degradation of certain lipids (Cravotto et al., 2008; Lam et al., 2012). In addition, agitation of the mixture is not possible in the Soxhlet apparatus and the decomposition of the lipids may occur as a result of high temperatures to transform the solvent from liquid to vapour form (Tandon and Rane, 2008).

2.4.3 Accelerated Solvent Extraction (ASE)

Accelerated solvent extraction (ASE) is a solid-liquid process that is performed at temperatures of 50 to 200°C and pressures of 10 to 15 MPa (Cooney et al., 2009). The solid sample is enclosed within a sample cartridge and is submerged with the extraction solvent at elevated temperatures and pressures for short durations of time (5-10 min). The sample is purged using compressed gas and the extract from the cells is released into a collection vessel (Richter et al., 1996). Elevating the temperature and pressure of the solvent extraction system above the solvents boiling point enhances the lipid extraction efficiency (Richter et al. 1996; Cooney et al., 2009). The higher temperatures accelerate the kinetics of the process and the increased pressure allows the solvent to remain in the liquid state (Herrero et al., 2004; Rulong et al., 2012). Such conditions result in the breakdown of cellular structures (Herrero et al., 2005).

The study of Chen et al. (2011) extracted lipid from *Nannochloropsis* sp. wet biomass using subcritical ethanol and noted a lipid recovery of 90.2%. The work of Rulong et al. (2012) found that lipid extraction from microalgae using ASE method resulted in an increase in lipid yield of 39-47% compared to extraction using Soxhlet apparatus. That performed by Mulbry et al. (2009) used ASE to recover 85-95% of the fatty acids from microalgae. Likewise, Andrich et al. (2006) reported a higher PUFA extraction yield from the species *Spirulina platensis* using supercritical fluid extraction as oppose to solvent extraction method.

2.4.3.1 Factors Affecting ASE

Accelerated solvent extraction techniques effectiveness in microalgae lipid extraction depends on the following factors: the type of solvent used, the extraction time, pressure and temperature of the reaction.

Type of solvent: Different solvents vary in the nature of their polarity which dictates the type of analytes they are capable of extracting (Jaime et al., 2005). It is for this reason that the type of solvent used in accelerated solvent extraction can affect the extraction yields obtained.

This factor was investigated by Rulong et al. (2012), and it was found that the ASE using hexane, petroleum ether and chloroform demonstrated poor lipid extraction effects from *Nannochlorpsis oculata*, but ethanol and acetone solvents proved to be good extraction solvents. Additionally, Herrero et al. (2005) used ASE of lipids from *Spirulina platensis* using hexane, petroleum ether, ethanol and water for the extraction of antioxidants and noted that ethanol resulted in the highest yield of 19.7% at 170°C using an extraction time of 9 min. Zheng et al. (2012) used four different solvents for ASE of lipids from *Spirulina* and ranked the efficiency of the solvents as chloroform: methanol> dichloromethane: methanol> ethanol> hexane. The study of Mulbry et al. (2009) tested various solvents in ASE of lipids from microalgae and recovered 85-95% fatty acids, and stated that chloroform/methanol resulted in better yields than isopropanol/hexane and hexane systems.

Extraction time: Prolonged extraction times have been correlated with increases in lipid recovery from microalgae cells. Increasing the reaction time allows longer solvent penetration periods and lipid-solvent interaction, thus resulting in higher lipid recoveries (Cooney et al., 2009; Rulong et al., 2012).

It was reported by Canela et al. (2002) that the pressure and temperature of ASE do not influence the yield of the extracted compounds, but instead influence the extraction rate of the reaction. Similarly, Rulong et al. (2012) noted that the ASE of lipids from *Nannochloropsis oculata* species increased with an increase in extraction time over the tested range of 4 to 20 min. Additionally, Cooney et al. (2009) stated that the use of pressurized solvents under high temperatures would shorten the extraction time that would be required with conventional solvent extraction systems. The work of Islam et al. (2014) noted that increasing the reaction time in ASE from 5 to 15 min resulted in a lipid recovery increase of 3.1%. That of Herrero et al. (2005) found a slight increase in the antioxidant yields using ASE from *Spirulina platensis* species as the extraction time increased from 3 to 15 min. Jaime et al. (2005) found that the extraction yields of antioxidants from the microalgae *Spirulina platensis* increased slightly with increasing extraction time over the tested range of 3 to 15 min.

Pressure: Increasing the pressure in ASE allows the solvent to fill the cellular matrix at a much faster rate, resulting in rapid extraction of the lipids from the solid matrix (Tandon and Rane, 2008). Elevating the pressure of the reaction reduces the dielectric constant to values that make the polarity of the lipids similar to those of the solvent, which leads to easier recovery (Richter et al., 1996; Herrero et al., 2006).

Richter et al. (1996) noted that increasing the pressure in ASE promotes increased transport of the solvent to the substances that are trapped in the pores and matrices of the cells that would not be reached under atmospheric conditions. The work of Huie (2002) showed that increasing the extraction pressure so that the solvent remains in the liquid state at high temperatures, enhances the solvents capacity to dissolve the desirable compounds. It was stated by Ju and Howard (2005) that with increasing pressure the polarity of the solvent is reduced, thus polar solvents are capable of dissolving nonpolar compounds. Zheng et al. (2012) successfully extracted fatty acids from *Spirulina* using a pressure of 10 MPa in ASE. Rulong et al. (2012) effectively extracted lipids from microalgae using ASE operated at 10 MPa using ethanol solvent.

Extraction temperature: Increasing the temperature in ASE increases the kinetics of the overall process, which leads to higher extraction efficiencies (Rulong et al., 2012). The diffusion coefficient of the solvent increases with increased temperatures which allows for better penetration into the solid microalgae particles favoring the desorption of compounds from the solid matrix (Richter et al., 1996; Herrero et al., 2005).

The work of Rulong et al. (2012) indicated that the lipid yield increased with an increase in ASE temperatures over the tested range of 75 to 175°C from *Nannochloropsis oculata* species. That of Islam et al. (2014) showed that maximum microalgae lipid yields using ASE were achieved at temperatures of 90-120°C, over the tested range of 70-120°C. In another study by Herrero et al. (2005), they found that increases in temperature from 60 to 170°C in ASE resulted in significant increases in antioxidant yields from *Spirulina platensis* species. Jaime et al. (2005) found that the extraction yields of antioxidants from the microalgae *Spirulina platensis* increased significantly with increasing temperatures over the tested range of 60 to 170°C. Similarly, Plaza et al. (2010) found that the antioxidant yields from *Himanthalia elongata* and *Syechocystis* sp. species increased

significantly with increasing temperature over the tested range of 50 to 200°C. Further, Santoyo et al. (2009) noted that increasing the reaction temperature in ASE from 50 to 200°C increased the antioxidant yields from *Haematococcus pluvialis*.

2.4.3.2 Advantages and Disadvantages

ASE technique is non-toxic, simple in operation and no traces of organic residue are left behind in the extract. The small amount of solvent required for lipid extraction (Rulong et al., 2012) and the rapid extraction time (Cooney et al., 2009) add to its appeal. However, this method suffers from intensive energy consumption and has not been operated for large scale processing of microalgae biomass. This technique requires large amounts of energy and high operating costs, thus it faces scale up difficulties (Macias-Sanchez et al., 2005; Wang and Weller, 2006; Cooney et al., 2009). In addition, the biomass needs to be dry for extraction using subcritical conditions (Cooney et al., 2009).

2.4.4 Supercritical CO_2

Supercritical CO_2 (SC-CO_2) is the point at which the fluid is raised above its critical temperature and pressure values (critical temperature is 31.1°C and pressure of 72.9 atm) (Pourmortazavi and Hajimirsadeghi, 2007; Macias-Sanchez et al., 2007; Cooney et al., 2009). The physical properties of supercritical fluids lay in between the liquid and the gas phase (Mercer and Armenta, 2011). These properties allow for quick penetration of the fluid through the cellular matrix of microalgae, which results in higher lipid yields and reduces the extraction time (Taylor, 1996).

In this method, an extraction vessel equipped with a heating unit houses the microalgae biomass with packing material (diatomaceous earth or diatoms). CO_2 is supplied into the vessel using a feed pump at pressures that are higher than the critical value. Heating of the vessel above the critical temperature results in the conversion of CO_2 into the supercritical state and begins the lipid extraction process from the biomass (Halim et al., 2012). The lipids are desorbed from the biomass forming a SC-CO_2-lipid complex. The formed complex diffuses across the SC-CO_2 film formed around the cell, and enters the bulk of the SC-CO_2 flow. At the end of the vessel, frits are placed to serve as filters in order to prevent the biomass and the packing material from being carried with the SC-CO_2 flow. The complex enters the collection vessel and a micrometering valve is placed to depressurize

the fluid. After depressurization the SC-CO_2 returns to its gaseous state leaving the lipids to precipitate in the collection vessel (Halim et al., 2012). The gaseous nature of CO_2 at room temperature ensures that it can be fully removed after the completion of lipid extraction, making it safe for food based products (Sahena et al., 2009). Lipid recoveries can be as high as 100% using this technique (Popoola and Yangomodou, 2006; Demirbas and Demirbas, 2010).

The work of Aresta et al. (2005) found that SC-CO_2 extraction resulted in higher amounts of long chain fatty acids (FA) as opposed to liquefaction methods. That of Andrich et al. (2005) reported that over the temperature range of 45-55°C and pressure of 400-700 bar, there was no impact on the extraction of bioactive lipids (PUFA) from the species *Nanochloropsis*. Further, both Demirbas (2009) and Demirbas and Demirbas (2011) reported that supercritical fluid process is capable of extracting 100% of oils. However, Couto et al. (2000) reported that lipid extracts from *Crypthecodinium cohnii* using SC-CO_2 were lower than those using the conventional solvent extraction techniques. Also, Li et al. (2014) found that solvent systems dichloromethane/methanol, propan-2-ol/cyclohexane and chloroform/methanol resulted in higher lipid yields from *Tetraselmis* sp. than SC-CO_2 extraction.

2.4.4.1 Factors Affecting SC-CO_2

The efficacy of the SC-CO_2 extraction is depended on the extraction time, pressure, temperature and the flow rate of the fluid (Pourmortazavi and Hajirsadeghi, 2007; Xu et al., 2008; Sahena et al., 2009; Harun et al., 2010).

Extraction time: The efficiency of the lipid extraction process using SC-CO_2 has been noted to decrease with elapsed time. The reason for this is that lipids released out of the cells are controlled by concentration gradients between the microalgae biomass and the SC-CO_2 (Halim et al., 2012).

The work of Andrich et al. (2005) noted that 80% of the total lipids were extracted within the first 83 min and that continued extraction run to 166 min did not significantly increase the total lipids extracted. That of Halim et al. (2011) reported an extraction of 0.058 lipid/g from *Chloroccum* sp. using SC-CO_2 for 80 min. Similarly, Santana et al. (2012) demonstrated that the lipid extracted from *Botryococcus braunii* increased from 5

to 10.5% as the extraction time was increased from 5 to 10 min. Couto et al. (2010) found that SC-CO_2 extraction of lipids from *Crypthexodinium cohnii* increased with increasing reaction time over the tested range of 10 to 180 min.

Pressure: Pressure plays an important role in the SC-CO_2 lipid extraction process (Taylor, 1996). The solvents power is increased with pressure since higher pressure increases the fluid density.

The impact of increasing pressure (316-484 bar) was investigated by Sajilata et al. (2008), in the SC-CO_2 lipid extraction from *Spirulina platensis* and reported an increase in yield with pressure increase Additionally, Andrich et al. (2005) reported that increasing the pressure (400-700 bar), increased the lipid extraction rate in *Nannochloropsis*. Likewise, Cheung (1999) found that an increase in pressure (241-379 bar) resulted in increased lipid yields from *Hypnea charoides*. Furthermore, Mendes et al. (2003) noted an increase in lipid yields with pressure increases from 100 to 350 bar in *Spirulina maxima*. Santana et al. (2012) noted lipid increases from 6 to 14% in *Botryococcus braunii* as the pressure was increased from 200 to 250 bar, respectively, using an extraction temperature of 80°C. The work of Dejoye et al. (2011) indicated that increasing the reaction pressure from 200 to 280 atm increased the lipid yields in *Chlorella vulgaris* from 2.2 to 4.86%. Couto et al. (2010) found that SC-CO_2 extraction of lipids from *Crypthexodinium cohnii* increased with increasing pressure over the tested range of 20 to 30 Mpa.

Temperature: Supercritical carbon dioxide has the ability to extract fatty acids. Changes in the reaction temperature alters the properties of the supercritical carbon dioxide which impacts its ability to penetrate certain molecules and extract specific compounds (Dunford et al., 2003).

It was reported by Andrich et al. (2005) that a slight increase in the lipid yield was attained with increased temperature (40-55°C) in *Nannochloropsis*. The work of Mendes et al. (2003) noted a decrease in lipid yields with temperature increase (50-60°C) from *Spirulina maxima*. Similarly, Cheung (1999) also noted a decrease in lipid yield with increase in temperature (40-50°C) from *Hypnea charoides*. Santana et al. (2012) noted higher lipid yields from *Botryococcus braunii* at a temperature of 50°C as opposed to 80°C. Dejoye et al. (2011) found that increasing the reaction temperature from 40 to 70°C did not

significantly increase the lipid yield (4.73-4.86%) for *Chlorella vulgaris*. The work of Taher et al. (2014) found that the lipid yields of *Scenedesmus* sp. for systems operating at temperatures of 35, 50 and 65°C were highest at 50°C. That of Couto et al. (2010) noted that SC-CO_2 lipid extraction from *Crypthexodinium cohnii* operating at 99°C resulted in higher yields than that operating at 40°C.

Flow rate: The kinetics of the lipid extraction process are affected by the flow rate of the SC-CO_2 in the extraction vessel. Increasing the flow rate of SC-CO_2 allows for increased contact between the lipids and the extraction fluid. However, increased flow rate leads to poor fluid penetration within the vessel (Pourmortazavi and Hajimirsadeghi, 2007).

The investigation of Taher et al. (2014) found that the lipid yields from *Scenedesmus* sp. decreased with increases in flow rate over the range of 1.38 to 4.02 g/min, and that the effect was greater as the temperatures increased from 35 to 65°C. That of Nobre et al. (2013) noted that change in the CO_2 flow rate from 0.35 to 0.62 g/min had no influence on the lipid yield. Similarly, Safi et al. (2014) found that CO_2 flow rate of 30 g/min extracted 10% of the lipids in *Chlorella vulgaris* microalgae biomass. That of Mouahid et al. (2012) reported an oil extraction yield from *Nannochloropsis oculata* of 1.14% (dcw) using dried biomass and a CO_2 flow rate of 8.33 g/min.

Moisture content: The moisture content in the biomass sample can affect the efficiency of the SC-CO_2 lipid extraction. High moisture contents reduce the contact time between the sample and the solvent. The reason being is that moisture acts as a barrier limiting the CO_2 and lipid diffusion into and out of the cell. It is for this reason that samples are typically dried before undergoing SC-CO_2 extraction (Sahena et al., 2009).

In the study of Mouahid et al. (2012), they found that the higher the moisture content (4-20%) in the microalgae species *Nannochlorophsis oculata* and *Dunaliella salina* resulted in increased extraction times, but had no effect on the oil yields. That performed by Crampon et al. (2013) noted that increasing the water content to 20% in *Nannochlorophsis oculata* biomass improved the extraction kinetics, but did not affect the oil yields. The works of Halim et al. (2011) found that SC-CO_2 lipid extraction from *Chlorococcum* sp. was higher using wet biomass as opposed to dry. In that of Dunford and

Temelli (1997) reported that moisture content plays a significant factor in sc-CO_2 lipid recovery as it acts as a barrier to the diffusion of SC-CO_2 into the sample.

2.4.4.2 Energy Consumption

It was reported by Quinn et al. (2014) that an energy consumption for lipid extraction from *Nannochloropsis salina* using SC-CO_2 was 0.03 kWh per g of extracted oil. The work of Pellerin (2003) determined that SC-CO_2 extraction consumes 0.8 kWh of energy per kg of plant extract. That of Kim and Lee (2015) noted that the energy consumption of lipid extraction from microalgae biomass using SC-CO_2 is significantly lower than solvent extraction, since additional operational costs for removal and recovery of the lipids are not necessary. However, Halim et al. (2012) reported that SC-CO_2 lipid extraction of microalgae suffers from high energy consumption as a result of fluid compression and heating in order to reach supercritical state.

2.4.4.3 Advantages and Disadvantages

Utilizing SC-CO_2 for extraction of FAME is great from an environmental perspective because it can be recycled, reduces environmental impacts and proposes an alternate route for carbon dioxide recycling (Herrero et al., 2006). SC-CO_2 extraction method does not require elevated temperatures and pressures, is nontoxic and rapid (Crespo and Yusty, 2005: Halim et al., 2011; Mercer and Armenta, 2011). These characteristics make it suitable for extraction of thermolable compounds that decompose at high temperatures (Aresta et al., 2005). SC-CO_2 extraction has a high affinity towards lipids that are desirable for biodiesel production. Additionally, the lipids recovered are solvent free which means no costs associated with solvent removal (Mendiola et al., 2007; Sahena et al., 2009; Halim et al., 2012; Ramesh et al., 2013). This technique is moderately suitable for scale up (Halim et al., 2012).

The drawbacks associated with this method are that it is sensitive to the water content present, which needs to be addressed in order to increase the contact between the SC-CO_2 and the microalgae cells (Aresta et al., 2005; Ramesh, 2013), otherwise residual water can result in plugging of the restrictor (Crespo and Yustry, 2005). Additionally, high costs are associated with installation and compression of the fluid using high pressures and

temperatures (Crespo and Yustry, 2005; Halim et al., 2012; Munir et al., 2013). This lipid extraction technique suffers from difficulty in scale up (Mercer and Armenta, 2011).

2.4.5 Expeller Press

In this method the microalgae biomass is dried and the lipids are contained in the cells (Munir et al., 2013). Expeller/press mechanical extraction technique is simple and works by applying pressure to crush the dry microalgae cells in order to release the oil from the cells (Demirbas, 2009; Mercer and Armenta, 2011). The lipids extracted from the cells typically range from 70-75% of the total lipids (Demirbas, 2009). Extraction efficiency can be improved by varying the applied pressure. However, increases in pressure can result in color alteration of the oil (darkening) and low-quality oil grade (Ramesh, 2013). Despite its simplicity, this method is energy intensive and the oil recovery is low (Popoola and Yangomodou, 2006; Williams, 2007). The expeller works by pressing the oil from the biomass using a screw in a closed vessel. The microalgae enter the press from one end and the oil is pressed and exists the other end of the vessel. The screw press compresses the algae by use of friction and application of continuous pressure. The pressed algae residues form a cake that contains some oil residues (Topare et al., 2011).

Numerous studies have employed this technique in the lipid extraction from microalgae biomass. The work of Shah et al. (2012) reported an oil recovery of 115 ml from 500 g of microalgae using expeller press method. That of Packer et al. (2009) used an oil expeller followed by solvent extraction to recover 95% of the total oil present in microalgae biomass. In another study by Popoola and Yangomodou (2006) they reported that 75% of the microalgae oil can be extracted using expeller/press oil extraction methods. The investigation of Dembiras (2011) found that simple press method can extract 70-75% of microalgae oil. Additionally, Topare et al. (2011) reported that the press was capable of extracting 1.92, 1.58 and 1.13 g of the oil in microalgae biomass using 100%, 75% and 50% dry samples, respectively.

2.4.5.1 Energy Consumption

The energy consumption of small sized expeller press technology has been reported to consume 10-30 kWh/ tonne of dry biomass (CANMET, 2005; Huber Technology, 2013). The work of Poku (2002) noted that pressing of one tonne of fresh fruit bunches consumes

12 kWh of energy. That performed by Mulder et al. (2012) reported an energy consumption that ranged from 10 to 20 kWh per tonne of biomass using pressing technology. Yan and Modigell (2012) reported that the energy consumption for treatment of one tonne of wet wood chips was 84 kWh. Kristoferson and Bolkalders (1986) reported that oil pressing technologies consume anywhere between 0.1 to 1 kWh/l of oil. In that by Wang (2015), they noted that expeller pressing of oil from Jatropha curcas L. seeds (3-5 tonnes) was achieved with an energy consumption of 7.5 kWh operating for 8 h period.

2.4.5.2 Advantages and Disadvantages

The advantages with expeller pressing are its simplicity, no solvents required, product is solvent free and the oil is safe for storage (Popoola and Yangomodou, 2006; Ramesh, 2013). However, some of the disadvantages are the large amounts of sample required, long periods of extraction time, high energy requirements, high maintenance costs and the inadequacy in complete lipid recovery from the biomass (Popoola and Yangomodou, 2006; Boldor et al., 2010; Ramesh et al., 2013). Additionally, this method requires the samples have a low moisture content and drying adds to the costs of the process (Kumar et al., 2014). Pigments are also a major drawback to this method and must be removed before conversion to oil either using solvent extraction or activated carbon adsorption (Ramesh, 2013).

2.4.6 OriginOil Single-Step Extraction

A novel single-step microalgae lipid extraction method has been developed by OriginOil (2010). This technique combines the dewatering of the cells, cell disruption and oil extraction in a single step (Halim et al., 2012; Lee et al., 2014). This technique is known as the OriginOil Single-Step Extraction.

In this technique the microalgae concentrate is exposed to Quantum Fracturing that combines fluid fracturing, pulsed electromagnetic fields and pH adjustments. After undergoing Quantum Fracturing the cells are disrupted and the intracellular lipids are released from the microalgae biomass. In the gravity clarifier, the lipids rise to the top for skimming, lipid fractionation and transesterification, while the remaining biomass residues settle to the bottom of the gravity clarifier. The three components to the OriginOil Single-Step Extraction are 1) injection of CO_2 to lower the pH in order to optimize electromagnetic

field delivery for cell disruption assistance, 2) Quantum Fractioning mechanically stresses the microalgae cells and 3) Pulsed electromagnetic fields deliver a force that disturbs the microalgae cells (Halim et al., 2012).

2.4.6.1 Energy Consumption

The energy utilization of the single-step OriginOil extraction unit has been noted to consume very little energy. It was reported by Portillo (2014) that the OriginOil technology consumes 0.22 kWh per barrel which accounts for the pumping and membrane energy. In the work of OriginOil (2010) they reported that biomass sludge consisting of a concentration of 1 g/L utilized 5625 kWh energy for extraction from 10 million L and 179 kWh for post-extraction dewatering of 10 million L of microalgae biomass, which is translates to cost 0.20 $/kg.

2.4.6.2 Advantages and Disadvantages

The advantages of using this technique are the substantial cost reductions associated with biodiesel production since OriginOil Single-Step extraction technology combines dewatering, cell disruption and lipid extraction processes. The technology does not require the use of any solvents that maybe toxic and consequently does not require a solvent recovery step (Halim et al., 2012; Lee et al., 2014), heavy machinery or the dewatering of the feedstock (Lee et al., 2014). In addition the lipid extraction method is highly effective when applied to wet feedstock or concentrated biomass which eliminates the need for an initial dewatering step (Halim et al., 2012).

FOREWORD TO CHAPTER 3

The literature review performed in Chapter 2 identifies the various techniques that can be used for microalgae harvesting, pre-treatment and oil extraction, highlighting their efficiency, as well as the impact of processes parameters. The energy consumption and advantages and disadvantages of each technique are also reviewed within the sections. This information serves as the knowledge base for performing the comparative analyses presented in Chapters 3 and 5, to identify suitable harvesting and oil extraction techniques, respectively, and to provide background in cell disruption techniques, which are examined in relation to microalgae cell structure in Chapter 4.

In Chapter 3, the seventeen harvesting techniques identified in Chapter 2 are evaluated to determine the most suitable techniques for large scale operation. Evaluation is based on dewatering efficiency, cost, toxicity, suitability for industrial scale, time, species specificity, reusability of media, and maintenance. The results of this chapter are used to select a harvesting technique for further experimental investigation in the later chapters of this thesis.

CHAPTER 3 COMPARATIVE ANALYSIS OF MICROALGAE HARVESTING TECHNIQUES

Parts of this chapter have been published in the Journal of *Fundamentals of Renewable Energy and Applications* and are being reproduced here with permission from the publisher (Appendix A). The following is the full citation of the article used.

Al hattab, M., Ghaly, A. & Hammoud A. (2015). Microalgae harvesting methods for industrial use: critical review and comparative analysis. *Journal of Fundamentals of Renewable Energy and Applications*, 5(2):154-179.

3.1 Abstract

The major obstacle for using microalgae biomass on an industrial-scale is the harvesting step which can account for more than 30% of the total downstream processing costs (i.e. cultivation, harvesting, extraction and conversion). The aim of this study was to perform a comparative analysis on the microalgae harvesting techniques, in order to determine the most efficient and economically viable methods for large scale processing. The harvesting methods investigated consisted of various physical, chemical, bio-flocculation, auto-flocculation and electrophoresis processes. Evaluation was based on their dewatering efficiency, cost, toxicity, suitability for industrial scale, time, species specificity, reusability of media, and maintenance. Each criterion was assigned a score between 7 and 15 based on its degree of importance, where higher values were given to those deemed most important for the development of an efficient and economic large scale dewatering method, and lower values to criteria that were deemed necessary, but less critical. The results indicated that five techniques scored values of 80/100 or greater, deeming them more suitable for harvesting microalgae on an industrial scale, because of their effectiveness, low operational costs, suitability for numerous species, rapidness, minimal maintenance required and being environmentally friendly. These included three physical techniques (disc stack centrifuge (87/100), cross flow filtration (84/100), decanter centrifugation (82/100)) and two chemical based methods (organic flocculation (80), and surfactant aided dispersed air flotation (83)). Other methods were deemed less suitable because they were less effective in dewatering a wide array of microalgae species, not suited for large volumes, costly and are associated with high maintenance costs.

3.2 Introduction

To address the issues regarding cost effective and scalable microalgae harvesting techniques, the following comparative analysis was undertaken to sieve through the various techniques, identified in the literature, to determine the most efficient and economically viable dewatering methods for large scale harvesting of microalgae biomass.

The harvesting techniques covered included sedimentation, vacuum filtration, pressure filtration, cross flow filtration, disc stack centrifugation, decanter centrifuge, dispersed air flotation, surfactant aided dispersed air flotation, dissolved air flotation, fluidic oscillation, inorganic flocculation, organic flocculation, auto-flocculation, bio-flocculation electrolytic coagulation, electrolytic flocculation and electrolytic flotation. Several criteria were used for evaluation of microalgae harvesting techniques: (a) dewatering efficiency, (b) cost, (c) toxicity, (d) suitability for large scale use, (e) time, (f) species specificity, (g) reusability of media and (h) maintenance. The results of this section give justification for the selection of a harvesting technique for further experimental analysis in later chapters.

3.3 Methodology

3.3.1 Selection of Criteria

Eight criteria (Table 3.1) were used for the evaluation of microalgae harvesting techniques: (a) dewatering efficiency, (b) cost, (c) toxicity, (d) suitability for large scale use, (e) time, (f) species specificity, (g) reusability of media and (h) maintenance. These criteria were selected from the literature review presented in Chapter 2 on microalgae harvesting methods. The comparative analysis was performed using these criteria to determine the most efficient, cost effective and environmentally friendly dewatering technique for a wide array of microalgae species that would be suitable for large scale application.

3.3.2 Assigning Score to Each Criterion

Each of the selected criteria was assigned a maximum score from 7 to 15 which was determined by the importance of the criterion (Table 3.1). Higher values were given to the criteria that were deemed most important for development of an efficient and economic large scale dewatering method for microalgae. Lower values were given to criteria that

Table 3.1. Criteria used for the comparative analysis of different harvesting techniques.

Criteria	Importance	Description
Dewatering Efficiency	15	The system should be able to effectively concentrate and remove high percentage of the cells from their surrounding liquid media
Cost	15	The operational costs of the process should be low in order to reduce the total processing costs associated with microalgae recovery
Toxicity and health and environmental impact	15	The method should be non-toxic so that the retrieved algae biomass may be processed for a number of value added products including ones for human consumption It should also be environmentally friendly in order to reduce the amount of toxic wastes produced
Suitability for Large Scale Use	15	The method should effective in handling large volumes for industrial production
Time	15	The rate of harvest should be quick to ensure the sustainability purposes
Species Specificity	10	The method should not be species or strain specific
Reusability of Media	8	The media should be recycled for reuse in order to minimize costs
Maintenance	7	Costs for maintaining the method should be low

were deemed necessary for determining a suitable method but were considered less important. These values were then used to determine the effectiveness of each harvesting method.

3.4 Results and Discussion

Using the criteria presented in Table 3.1, each harvesting technique was assessed and assigned a score. The results are presented in Tables 3.2-3.18, where a description is also provided for each criteria to explain the given score. The sum of the scores obtained for each method are presented in Table 3.19.

The results indicate that of the 17 methods used for microalgae harvesting evaluated in this study, five had scores of 80/100 or above and are, therefore, deemed suitable for harvesting a wide array of microalgae species at the industrial scale. These methods are disc stack centrifugation (87/100), cross flow filtration (84/100), decanter centrifugation (82/100), organic flocculation (80/100), and surfactant aided flotation (83/100). The three physical harvesting methods (disk stack centrifugation, cross flow filtration and decanter centrifugation) were considered suitable for large scale harvesting of microalgae because they are highly effective in removing microalgae biomass from the liquid medium, non-toxic and rapid and the medium can be reused. The two chemical treatments (organic flocculation and surfactant aided flotation) were suitable for microalgae harvesting on a large scale because they can be used with a wide range of microalgae species, energy requirement for operation is low, high dewatering efficiency can be achieved, and chemicals are easily degraded or removed from the medium, and thus can be recycled.

Although any of these five techniques is deemed suitable for harvesting of microalgae and can be used alone, a combination of methods can also be used to further enhance the recovery efficiency and improve the economics. Like that seen with surfactant aided dispersed air flotation. The use of surfactants or flocculants as an initial harvesting step to concentrate the algae suspension allows for effective removal of algae biomass from large liquid media. The costs associated with energy intensive centrifugation or filtration techniques can be further reduced by using these methods as secondary techniques since less volumes of microalgae suspension will be required to undergo the secondary treatment.

Table 3.2. Evaluation of sedimentation.

Criteria	Description	Score
Dewatering Efficiency (15)	Settlement is based on density and since microalgae density is similar to that of water media, efficiency is low without the use of flocculants	5
Cost (15)	Minimum energy costs are required for this technique as gravitational forces are cost free	15
Toxicity and health and environmental impact (15)	This method is nontoxic to the cells, since it works by gravitational forces	15
Suitability for Large Scale (15)	Unsuitable for large scale use because of the long periods required for the process and it only works for microalgae cells with higher densities	5
Time (15)	Long periods of time are required to achieve settlement of microalgae cells through gravitational forces	2
Species Specificity (10)	Highly dependent on the type of species used. Species should have a higher density than that of water	4
Reusability of Media (8)	Method does not introduce any chemicals or alter the composition of the species/media	8
Maintenance (7)	No maintenance costs are required	7
Total (100)		**61**

Table 3.3. Evaluation of vacuum filtration.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective recovery of microalgae cells Depends on filter size and the size of microalgae cells	13
Cost (15)	Costs associated with pump and replacement of filters	9
Toxicity and health and environmental impact (15)	Cell composition remains intact and toxic chemicals are not required	15
Suitability for Large Scale (15)	Large pump and large filters are required for large scale	10
Time (15)	Rapid cell recovery	12
Species Specificity (10)	Dependent on the microalgae cell size	6
Reusability of Media (8)	Liquid media can be recycled	8
Maintenance (7)	Frequent filter replacement as a result of clogging	2
Total (100)		**75**

Table 3.4. Evaluation of pressure filtration.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective in dewatering the microalgae. Suspended solids in the filtrate are low	13
Cost (15)	Costs associated with pump to create pressure and with filter replacements	9
Toxicity and health and environmental impact (15)	This method is non-toxic and cell composition is not altered	15
Suitability for Large Scale (15)	Suitable for large volumes but requires large filters and large pump	10
Time (15)	Relatively rapid cell recovery	12
Species Specificity (10)	Dependent on the size of the species	5
Reusability of Media (8)	Filtrate can be recycled and reused again for microalgae growth	8
Maintenance (7)	Costs associated with filter replacement	2
Total (100)		**74**

Table 3.5. Evaluation of cross flow filtration.

Criteria	Description	Score
Dewatering Efficiency (15)	Complete removal of microalgae cells from the media	15
Cost (15)	Costs associated with pump and membrane	12
Toxicity and health and environmental impact (15)	This method is non-toxic	15
Suitability for Large Scale (15)	Suitable for large volumes of microalgae Smaller microalgae result in membrane clogging	10
Time (15)	Rapid cell recovery	12
Species Specificity (10)	Wide range of cell sizes can be used	8
Reusability of Media (8)	Filtrate can be recycled and reused again for microalgae growth	8
Maintenance (7)	Costs associated with filter replacement	4
Total (100)		**84**

Table 3.6. Evaluation of disc stack centrifuge.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective separation of solid particles from liquid suspensions	13
Cost (15)	Large amounts of energy are required for operation	8
Toxicity and health and environmental impact (15)	The use of toxic materials is not required Cell composition is not altered	15
Suitability for Large Scale (15)	Suitable for large volumes of microalgae	12
Time (15)	Rapid	15
Species Specificity (10)	No dependence on the type of species	10
Reusability of Media (8)	Supernatant can be easily recovered and recycled	8
Maintenance (7)	Not much maintenance is required	6
Total (100)		**87**

Table 3.7. Evaluation of decanter centrifuge.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective separation of solid particles from liquid suspensions Solid concentrates are much more dense than those recovered using disc type	15
Cost (15)	More energy is required for operation compared to disc type	6
Toxicity and health and environmental impact (15)	The use of toxic materials is not required Cell composition is not altered	15
Suitability for Large Scale (15)	Suitable for large volumes of microalgae	12
Time (15)	Rapid	15
Species Specificity (10)	Suitable for larger species only	5
Reusability of Media (8)	Supernatant can be easily recovered and recycled	8
Maintenance (7)	Not much maintenance is required	6
Total (100)		**82**

Table 3.8. Evaluation of dispersed air flotation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effectiveness depends on the likelihood of the cells coming into contact with the air bubbles in order to float to the surface Cell may rupture	12
Cost (15)	High costs are required for high speed agitation in order to produce the bubbles. Additional surfactants increase the costs	10
Toxicity and health and environmental impact (15)	The use of toxic materials is not required	12
Suitability for Large Scale (15)	Large volumes of microalgae can be used	13
Time (15)	The time required is dependent on the rate of agitation	10
Species Specificity (10)	Species should have high tolerance to avoid rupturing	5
Reusability of Media (8)	Media may be recycled for further use	8
Maintenance (7)	Not much maintenance is required	7
Total (100)		**77**

Table 3.9. Evaluation of dissolved air flotation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective with the use of additional flocculants	10
Cost (15)	Operational costs are high, large amounts of energy would be required and the cost of flocculants for effective recovery is also high	9
Toxicity and health and environmental impact (15)	Inorganic flocculants are toxic	8
Suitability for Large Scale (15)	Large volumes of microalgae can be harvested	13
Time (15)	Dependent on the likelihood of the cells interacting with air bubble	10
Species Specificity (10)	Wide range of species can be used but species ability to adhere onto gas bubble is key	8
Reusability of Media (8)	Chemicals are not used and the medium can be recycled after it is saturated with air	5
Maintenance (7)	Not much maintenance is required	7
Total (100)		**70**

Table 3.10. Evaluation of fluidic oscillation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective with the use of additional flocculants	9
Cost (15)	Large amounts of energy would be required, but operational costs are much lower than those of dispersed and dissolved air techniques	10
Toxicity and health and environmental impact (15)	Coagulants are required for improving recovery effectiveness	10
Suitability for Large Scale (15)	Large volumes of microalgae can be harvested,	13
Time (15)	Dependent on the likelihood of the cells interacting the with air bubble	10
Species Specificity (10)	Can be used on a wide range of species but species ability to adhere onto gas bubble is key	8
Reusability of Media (8)	Recycled after it is saturated with air	6
Maintenance (7)	Not much maintenance is required	7
Total (100)		**73**

Table 3.11. Evaluation of inorganic flocculation.

Criteria	Description	Score
Dewatering Efficiency (15)	Cell concentration in liquid is low and depends on the position of the flocculant on the cell	10
Cost (15)	Large amounts of flocculants are required Does not require high amounts of energy	11
Toxicity and health and environmental impact (15)	Flocculating agents are toxic and not suitable for food additive and pharmaceutical products	5
Suitability for Large Scale (15)	Large volumes of microalgae suspensions can be used	15
Time (15)	Relatively fast	10
Species Specificity (10)	Wide range of species can be used but the process is dependent on the type of species used and how well the flocculant attaches to the cells	5
Reusability of Media (8)	The pH of the media left after harvest of microalgae is low which is not suitable for some microalgae species	2
Maintenance (7)	No maintenance required	7
Total (100)		**65**

Table 3.12. Evaluation of organic flocculation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effectiveness is dependent on the position of the flocculant on the cell and the cell surface charge	11
Cost (15)	Expenses are associated with cost of flocculant Less amounts are required when compared to inorganic agents	11
Toxicity and health and environmental impact (15)	Organic compounds are non-toxic and can be used in the formation of edible and cosmetic by-products	15
Suitability for Large Scale (15)	Large volumes of microalgae suspensions can be used	15
Time (15)	Relatively fast	10
Species Specificity (10)	Process is dependent on the type of species used and how well the flocculant agent attaches to the cells	6
Reusability of Media (8)	Organic agents are non-toxic and the media can be recycled Changes in pH of media occur with flocculant addition and can affect the microalgae species	5
Maintenance (7)	No maintenance required	7
Total (100)		**80**

Table 3.13. Evaluation of surfactant aided dispersed air flotation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effectiveness of recovery is improved by surfactant addition, increasing bubble particle interaction	12
Cost (15)	The use of a diffuser for bubble generation reduces operational costs. Surfactant purchase is necessary	11
Toxicity and health and environmental impact (15)	Dispersed air flotation is a technique used to recover surfactants from wastewater, therefore little surfactant remains in the majority of the liquid after use	12
Suitability for Large Scale (15)	Large volumes of microalgae can be used	13
Time (15)	The time is dependent on the bubble particle interaction, which is improved by the addition of surfactants	12
Species Specificity (10)	Various species can be used	8
Reusability of Media (8)	Media may be recycled for further use	8
Maintenance (7)	Not much maintenance is required	7
Total (100)		**83**

Table 3.14. Evaluation of auto-flocculation.

Criteria	Description	Score
Dewatering Efficiency (15)	Flocculation is induced by pH change and separation depends on the response of the species to the environment	11
Cost (15)	Cost of chemicals purchased is relatively reasonable	12
Toxicity and health and environmental impact (15)	Chemicals used to induce flocculation can be toxic	8
Suitability for Large Scale (15)	Large volumes of microalgae suspension can be used Prolonged periods of time for sufficient settling is not suitable for large scale use	8
Time (15)	Long periods are required for the settling of the cells	8
Species Specificity (10)	Dependent on the response of the cell to the pH altered environment Is not suitable for all species	4
Reusability of Media (8)	The pH of the media is altered to induce the flocculation making the media unsuitable for reuse	2
Maintenance (7)	No maintenance required	7
Total (100)		**60**

Table 3.15. Evaluation of bio-flocculation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effectiveness is dependent on the linkage of the bio-flocculant to the cells in order to increase their density	11
Cost (15)	Costs are associated with purchasing the microorganisms and the maintenance of the culture	10
Toxicity and health and environmental impact (15)	Bio-flocculant species are non-toxic	15
Suitability for Large Scale (15)	Effective on large volumes of microalgae species	15
Time (15)	Based on the ability of the cells to link onto the microorganisms in order be more dense and improve settling time	10
Species Specificity (10)	Wide array of species can be used	8
Reusability of Media (8)	Microorganisms can be harvested and the medium can be recycled	3
Maintenance (7)	Maintenance of microorganism culture is required	4
Total (100)		**76**

Table 3.16. Evaluation of electrolytic coagulation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective concentration of cells in liquid suspension	13
Cost (15)	High electrical power is required	11
Toxicity and health and environmental impact (15)	The addition of toxic chemicals is not required Cell composition can be altered	12
Suitability for Large Scale (15)	Large volumes require large power inputs which deem this method unsuitable for large scale	7
Time (15)	Rapid	15
Species Specificity (10)	Dependent on the charge of the species Conductivity of the water (marine water requires less energy)	3
Reusability of Media(8)	Microalgae can be harvested and the media can be recycled	5
Maintenance (7)	Effectiveness of electrode is reduced with continued use Frequent replacement of electrode maybe necessary	3
Total (100)		**69**

Table 3.17. Evaluation of electrolytic flocculation.

Criteria	Description	Score
Dewatering Efficiency (15)	Effective in cumulating the cells together with one another	13
Cost (15)	Energy is required for rapid accumulation of the cells	11
Toxicity and health and environmental impact (15)	The addition of toxic chemicals is not required Cell composition can be altered	12
Suitability for Large Scale (15)	Unsuitable for large scale production because of energy requirement and the alteration of cell composition	8
Time (15)	Rapid	15
Species Specificity (10)	Dependent on the charge neutralization of the cell Conductivity of the water (marine water requires less energy)	5
Reusability of Media (8)	Media can be reused Cell composition can be altered	4
Maintenance (7)	Effectiveness of electrode is reduced with continued use Frequent replacement of electrode maybe necessary	3
Total (100)		**71**

Table 3.18. Evaluation of electrolytic flotation.

Criteria	**Description**	**Score**
Dewatering Efficiency (15)	Effective in accumulating the cells together with one another Effectiveness is based on the likelihood that the cell comes in contact with the bubble in order to float to the surface	13
Cost (15)	High costs are associated with power supply	11
Toxicity and health and environmental impact (15)	The addition of toxic chemicals is not required Cell composition can be altered	12
Suitability for Large Scale (15)	Unsuitable for large scale production because of the energy required and the alteration of cell composition	8
Time (15)	Dependent on the likelihood that the cell adheres to a bubbles in order to float to the surface	10
Species Specificity (10)	Dependent on the charge neutralization of the cell Conductivity of the water (marine water requires less energy)	4
Reusability of Media (8)	Media can be reused	4
Maintenance (7)	Effectiveness of electrode is reduced with continued use Frequent replacement of electrode maybe necessary	3
Total (100)		**65**

Table 3.19. Comparative analysis for microalgae harvesting techniques.

Criteria	Physical									Chemical			AF	BF	Electrophoresis		
	S	VF	PF	CFF	DSC	DC	DAF	DVF	FO	IF	OF	SD			EC	EFC	EFT
Dewatering Efficiency (15)	5	13	13	15	13	15	12	10	9	10	11	12	11	11	13	13	13
Cost (15)	15	9	9	12	8	6	10	9	10	11	11	11	12	10	11	11	11
Toxicity and health and environmental impact (15)	15	15	15	15	15	15	12	8	10	5	15	12	8	15	12	12	12
Suitability for large scale (15)	5	10	10	10	12	12	13	13	13	15	15	13	8	15	7	8	8
Time (15)	2	12	12	12	15	15	10	10	10	10	10	12	8	10	15	15	10
Species specificity (10)	4	6	5	8	10	5	5	8	8	5	6	8	4	8	3	5	4
Reusability of Media (8)	8	8	8	8	8	8	8	5	6	2	5	8	2	3	5	4	4
Maintenance (7)	7	2	2	4	6	6	7	7	7	7	7	7	7	4	3	3	3
Total (100)	**61**	**75**	**74**	**84**	**87**	**82**	**77**	**70**	**73**	**65**	**80**	**83**	**60**	**76**	**69**	**71**	**65**

S: Sedimentation; VF: Vacuum filtration; PF: Pressure filtration; CFF: Cross flow filtration; DSC: Disc stack centrifugation; DC: Decanter centrifugation; DAF: Dispersed air flotation; DVF: Dissolved air flotation; FO: Fluidic oscillation; IF: Inorganic flocculation; OF: Organic flocculation; SD: Surfactant aided dispersed air flotation; AF: Auto-flocculation; BF: Bio-flocculation; EC: Electrolytic coagulation; EFC: Electrolytic flocculation; EFT: Electrolytic flotation

It is recommended that the use of centrifugation or filtration microalgae harvesting techniques be coupled (as secondary techniques) with organic flocculation or surfactant flotation (as an initial dewatering step) in order to improve the economics of the overall process.

The other 12 harvesting methods were deemed unsuitable for harvesting microalgae at the industrial scale because they did not meet the evaluation criteria (suitability for dewatering a wide array of microalgae species, suitability for large volumes, low operation costs and low maintenance). The other six physical methods were not as effective in removing the algae biomass, required long time, were not suitable for large scale, required high maintenance and were not effective for a wide array of microalgae. Autoflocculation techniques are unsuitable for large scale use because the chemicals used for pH change are toxic, are species specific, require long time and recycling of the medium requires additional costly treatment. The bio-flocculation technique depends on the desirable end product, since the microorganisms used for flocculation are harvested with the cells, the flocculating cultures must be adjusted for viable growth and the process is costly. Electrophoresis methods were deemed unsuitable for large scale microalgae harvesting because of difficulty in scaling up and the disruption of the cells can affect the quality and yield of the desired end product.

3.5 CONCLUSIONS

The major obstacle for using microalgae biomass on an industrial-scale for production of value added products is the dewatering step which accounts for 20-30% of the total costs associated with the process. A comparative analyses of 17 harvesting techniques was based on the effectiveness, cost, toxicity, processing time, species specificity, maintenance and suitability for operating on large scale. The harvesting techniques that scored the highest were disc stack centrifugation (87/100), cross flow filtration (84/100), decanter centrifugation (82/100), organic flocculation (80/100), and surfactant aided dispersed air flotation (83/100). These techniques were deemed suitable for large scale use because of their effective dewatering ability, low operational costs, suitability for numerous species, rapidness, require minimal maintenance and being environmentally friendly. There is

potential for improving the economics of the overall process by coupling different harvesting techniques.

FOREWORD TO CHAPTER 4

As discussed in Chapter 3, there are multiple methods that have been determined as most suitable for large scale microalgae harvesting operations: disc stack centrifugation, cross flow filtration, decanter centrifugation, organic flocculation and surfactant aided dispersed air flotation. However, the degree to which the biomass is concentrated varies amongst the techniques identified, as well as the characteristics and required concentration of additives needed (i.e. surfactants or flocculants), which would impact further processing. Therefore, consideration needs also to be made for downstream processes such as pre-extraction treatment and product extraction.

The purpose of Chapter 4 is to provide an overview of pre-extraction treatments and the influence of various cell disruption techniques on different species of microalgae. The chapter goes beyond what was presented in the literature review of this thesis by using the composition of the microalgae cell wall as a basis for comparing the performance of different cell disruption techniques. The work in this section was used to identify a suitable cell wall disruption treatment for *Chlorella saccharophila* to investigate experimentally in this thesis.

CHAPTER 4 OVERVIEW OF MICROALGAE DISRUPTION TECHNIQUES FOR PRODUCT RECOVERY- INFULUENCE OF CELL WALL COMPOSITION

Parts of this chapter have been published in the *Journal of Applied Phycology* and are being reproduced here with permission from the publisher (Appendix A). The following is the full citation of the article used.

Alhattab, M., Kermanshahi-Pour, A., Brooks, M. S. (2018). Microalgae disruption techniques for product recovery-influence of cell wall composition. *Journal of Applied Phycology,* 1-28.

4.1 ABSTRACT

Microalgae are one of the most promising feedstocks for the production of commodity and value added products. However, the use of microalgae as a feedstock is hampered by the process economics and sustainability. Overall sustainability can be improved by employing energy efficient cell disruption and recovery processes to maximize the extraction of desired compounds from microalgal biomass. Most often, extraction processes are conducted with solvents using untreated, chemically treated or mechanically treated cells. However, microalgal cell walls are sometimes structurally robust, complex and chemically diverse, and as such high energy inputs or large quantities of chemicals are required to extract products from within the cell. Various chemical, biological and physical techniques have been employed to disrupt the cell walls from a variety of microalgae species, these include the use of surfactants, autoclave, microwave, sonication, beadmilling, enzymatic hydrolysis, high pressure homogenization and steam treatments. Although the cell wall structure is important for product recovery, it is often not considered when selecting the most appropriate disruption method. In this study, the cell wall structure of selected microalgae species and the effectiveness of various cell wall disruption techniques on product recovery are reviewed. It was concluded that future research must focus on developing an understanding of the relationship between cell wall disruption mechanisms, cell wall composition and structure, as well as optimizing the energy consumption of the disruption technique. This approach would enable the design of innovative cell wall disruption techniques for an enhanced product recovery.

4.2 INTRODUCTION

The increasing demand for pharmaceuticals, food, bioactive compounds and bioenergy sources, has intensified research into microalgae, as a viable renewable source for meeting these needs (Jha et al., 2017). Microalgae are a promising feedstock due to their rapid growth rate, compared to other crops, ability to grow in various environments, their diverse biochemical composition (Papazi et al. 2012), and limited competition with edible crops (Demirbas, 2011). Microalgae are considered a good source for the production of a variety of chemicals such as carotenoids, fatty acids (i.e. omega-3, docosahexaenoic acid (DHA)), proteins and phycobiliproteins (Kermanshahi-Pour et al., 2013). Microalgae species are considered as potential feedstocks for a wide array of renewable chemicals and have been the subject of extensive investigations for the production of value-added chemicals (Foley et al., 2011; Borowitzka, 2013). Microalgal species including *Dunaliella salina*, *Chlorella* and *Scenedesmus* have been studied for β-carotene, biofuels, pigments, and omega-3 fatty acids recovery (Chen, 1996; Wiltshire et al., 2000; Hejazi et al., 2004; Lee et al., 2010) and the species *Porphyridium cruentum* for the extraction of B-phycoerythin (Jubeau et al., 2013). However, at present, only a few products are commercially produced from microalgae. These include astaxanthin from *Haematococcus pluvials* (Shah et al., 2016), β-carotene from *Dunaliella salina* (Spolaore et al., 2006), DHA from *Crypthecodinium cohnii* (Wynn et al., 2010). *Chlorella* is also produced in Japan for human consumption, in view of its health benefits (Plaza et al., 2012; Stramarkou et al., 2017).

Research into microalgae derived biofuels has grown in recent years, given the increasing awareness related to climate change, coupled with the diminishing supply of fossil fuels (Milano et al., 2016). However, production of biofuel from microalgae in commercial scale is challenged by high energy consumption and production cost (Borowitzka, 2013). Production of value-added chemicals in combination with biofuel is considered as an approach to improve the economics of microalgal biofuel production (Borowitzka, 2013).

One of the challenges facing the broad application of microalgal biomass as feedstock, is the efficient recovery of the intracellular compounds from biomass (Gerken et al., 2013). Most often, solvents are employed for the extraction of intracellular products (e.g. hexane,

butanol, or ionic liquids) from untreated, chemically treated or mechanically treated biomass (Pienkos and Darzins, 2009; Shen et al., 2009; Brennan and Owende, 2010; Lee et al., 2010; Kim et al., 2012). However, high energy inputs or large quantities of chemicals are required to extract the compounds from within the cell due to recalcitrance, complexity and diversity of microalgal cell wall (Gerken et al., 2013). A simulated life cycle assessment performed by Brentner et al. (2011) showed that the lipid extraction processes, consisting of drying, pressing the biomass and the hexane solvent extraction, requires 23 MJ kg^{-1} of energy. Similarly, the review performed by De Boer et al. (2012) on the life cycle assessment of microalgae biodiesel production, demonstrated an energy consumption totaling 22 MJ kg^{-1}, for biomass recovery, drying, disruption, *in situ* transesterification and solvent recycling. More recently, pretreated biomass have been subjected to supercritical CO_2 (SC-CO_2) extraction for the recovery of lipids, carotenoids and chlorophylls from *Scenedesmus* (Macías-Sánchez et al., 2009; Soh and Zimmerman, 2011), *Haematococcus* (Krichnavaruk et al., 2008; Fujii, 2012), *Tetraselmis chi* (Grierson et al., 2012), *Dunaliella salina, Synechococcus* sp., *Nannochloropsis* (Macias-Sanchez et al., 2008; Bjornsson et al., 2012) and *Chlorella vulgaris* (Safi et al., 2014). This technique is much less energy intensive compared to conventional solvent extraction. Additionally, in the life cycle assessment performed by Brentner et al. (2011), they indicated that the extraction of lipids directly from flocculated biomass requires an energy consumption of 4.9 MJ kg^{-1}, which requires 4.5 to 4.7 times less energy than drying the biomass followed by conventional solvent extraction or direct transesterification.

Microalgae are diverse with respect to their cell wall and cell covering (Domozych et al., 2012). Some species lack the cell wall (e.g., *Dunaliella spp* (Grima et al., 2013)) or cell covering such as the diatom *Rhizosolenia*, which possess silica frustules (Zhu et al., 1997; Shipe et al., 1999). While others possess a cellulosic cell wall (e.g., *Chlorella sorokiniana* (Takeda, 1991)) or one that consists of complex polysaccharides (e.g., *Tetraselmis suecica* (Becker et al., 1998)). Furthermore, species such as *Haematococcus*, only develop a cell wall in the aplanospore stage (Hagen et al., 2002). Some dinoflagellates (i.e. *Alexandrium catenella*) and euglenoids (i.e. *Euglena*) have proteinaceous coverings (Nakano et al., 1987; Wang et al., 2011; O'Neill et al., 2015). Microalgae extracellular coverings vary widely due to their diverse lineages (Domozych et al., 2012). Extracellular coverings may

consist of multiple layers of scales or complex structural fibril cell walls (Domozych, 2011). In some cases these coverings act as barriers that must be overcome in order to make the recovery of intracellular components efficient and cost effective (Grima et al., 2013; Gerken et al., 2013). In order to determine effective practices for maximizing product recovery, it is important to characterize the extracellular coating of the microalgae species of interest (Domozych et al., 2012; Grima et al., 2013). There are several cell disruption techniques that are commonly practiced, including mechanical treatments such as cavitation, bead milling, high pressure homogenization (HPH) and the use of chemical and biological agents, such as surfactants and enzymatic treatments. Although several studies have been conducted to examine the effectiveness of different pretreatment techniques (Pernet and Tremblay, 2003; Lee et al., 2010; Prommuak et al., 2012; Postma et al., 2015), the influence of the microalgal extracellular structure and composition is typically not considered. Thus, the aim of this review is to critically examine the literature related disruption techniques and cell wall structure to highlight the knowledge gaps that currently exist.

4.3 Overview of Microalgae Cell Wall Composition and Structure

The resilient cell wall structure of some microalgae species act as barriers to the industrial exploitation of microalgae (Burczyk et al., 2014). The composition of the cell wall is species dependent and may be used as an identification marker for its taxonomy (Takeda, 1993). An overview of the various microalgae cell wall structures, and composition, as well as the cell size and shape, is given in Table 4.1.

Algaenan, which is a highly resistant outer layer of several microalgal species, is hydrocarbonaceous, non-hydrolyzable biopolymer, also known as sporopollenin (Burczyk and Dworzanski, 1988; Gerken et al., 2013). It is comprised of C30-40 mono- or di-unsaturated ω-hydroxy fatty acids (Blokker et al., 1998) that are linked to one another by chemical bridges of various types, such as ether, ester and glycosidic bonds (Burczyk et al., 2014). The presence of algaenan in the microalgae cell wall, reduces the susceptibility of the microalgae to degradation through disruption techniques such as mechanical, enzymatic, and chemical hydrolysis (Burczyk et al., 2014). Algaenan is present in species belonging to the Chlorophyceae and Trebouxiophyceae classes of the Chlorophyte group

Table 4.1. Overview of the diversity of microalgae cell wall structure and biochemical composition based on taxonomic class.

Microalgae Species	Taxonomic Class	Cell Shape/ Size	Cell Wall Structure	Cell Wall Composition	Product of Interest
Phaeodactylum tricornutum	Bacillariophyceae	Fusiform, Triradiate, Cruciform 8-25µm[1]	Fusiform & triradiate: organic frustule with silica bands in gridle region [2] Oval: Siliceous frustule on one side of the cell, surrounded by an organic wall[2]	Organic cell wall has up to 10 silica bands [2] Silica	Lipid [3,4]
Scenedesmus sp./ *Tetradesmus*	Chlorophyceae	Bean shape 10-12µm[5]	Non-hydrolyzable algaenan structure [3,6]	Crystalline glycoprotein, algaenan[7]	FAME [8,9]
Graesiella emersonii	Chlorophyceae	Spherical 4-8 µm[10]	Algaenan tri-laminar structure[6]	Algaenan [6]	Carotenoid [11], Lipid[12]
Chlorococcum sp.	Chlorophyceae	Ovoid/ spherical <10 µm[13]	Fibrillar cell wall; crystalline glycoprotein [8]	Cellulose [14]	Lipid [15]
Neochloris oleoabundans	Chlorophyceae	Spherical 3-3.5 µm[16]	Cellulose [17,18]	Extracellular polysaccharides and protein [18]	Lipid[19]
Tetraselmis suecica	Chlorodendrophyceae	Oval 10-25 x 7-20 µm[20]	Scales [21]	Extracellular polysaccharides [22]	α-Tocopherol, β-Carotene, Gallic acid [23]
Tetraselmis striata	Chlorodendrophyceae	Oval 10-25 x 7-20 µm[20]	Scales [22]	Extracellular polysaccharides [22]	Proteins [25]
Nannochloropsis	Eustigmatophyceae	Round 2-4 µm[19]	Cellulose and algaenan structure [26,27]	Glucose, cellulose, mannans, rhamnose, fucose, galactose, galacturonic acid [26]	Lipids [3,28]
Chaetoceros ceratosporus	Mediophyceae	Oval cylinder 5x8µm[29]	Siliceous frustules [30]	Silica [30]	Lipid [31]
Porphyridium	Porphyridiophyceae	Round 1.5-5 µm[32]	Polysaccharide capsule[33]	Glucose, galactose, xylose, glucuronic acid, methyl-glucuronic acid [33]	B-Phycoerythrin[3 4]
Botryococcus sp	Trebouxiophyceae	Oval 3-5 µm[35]	Complex extracellular matrix[35] Polysaccharide colony sheath; retaining wall; drape; secreted hydrocarbons Individual cell: Algaenan[35]	Colony sheath: Arabinose galactose polysaccharides[35] Hydrocarbonaceous non-hydrolyzable biopolymer (algaenan) [35]	Lipid [9,15]

Chlorella minutissima	Trebouxiophyceae	Spherical 2-4 µm[36]	Algaenan tri-laminar structure [27]	Algaenan [27]	Lipids, pigments, carotenoids [37]
Chlorella vulgaris	Trebouxiophyceae	Spherical 3-4 µm[38]	Lacks tri-laminar structure [8]	Extracellular polysaccharides [8], Rhamose, galactose, xylose [39]	Lipid[9,40,41] Protein, Carotenoids, pigments[42]
Chlorella sorokiniana	Trebouxiophyceae	Spherical 2-4 µm[40]	Glucosamine-rigid wall [43]	Cellulose, glucose mannose, glucosamine, β-galactofuranan, algaenan [44-46]	Lipid [3,15]
Chlorella zofingiensis	Trebouxiophyceae	Spherical 2-4 µm[40]	Glucosamine-rigid wall[43,47]	Cellulose, glucose, xylose[43,48,49]	Astaxanthin[50]
Chloroidium saccharophilum	Trebouxiophyceae	Ovoid 7 x 5 µm[51]	Outer non tri-laminar layer and an inner microfribrillar layer [44]	Glucose-mannose [43], rhamose, galactose [39]	Lipids [52], carotenoids[53]
Chloroidium ellipsoideum	Trebouxiophyceae	Ellipsoidal 6-16 x 5-15 µm[51]	Algaenan tri-laminar structure[44]	Algaenan[44]	Lipid[54], Carotenoids[55,56]
Staurastrum	Zygnematophyceae	Cylindrical with arms 25-50 µm[57]	Porous with spinules or warts extending through mucilaginous layer[58]	-	Pigments [52]

[1](He et al., 2014); [2](Borowitzka and Volcani, 1978); (Johnsen, 1991); (Grima et al., 1996); [3](Lorente et al., 2015); [4](Ryckebosch et al., 2012); [5](Shen et al., 2009); [6](Allard et al., 2002); [7](Voigt et al., 2014); [8](Corre et al., 1996); [9](Lee et al., 2010); [10](Mezhoud et al., 2014); [11](Malis et al., 1993); [12](Allard and Templier, 2001); [13](Peterfi et al., 1988); [14](Miller, 1978); [15](Rakesh et al., 2015); [16](Hajar et al., 2017); [17](Chantanachat and Bold, 1962); [18](Wang et al., 2015); [19](Wang et al., 2014); [20](Kawaroe et al., 2016); [21](Domozych et al., 1981); [22](Becker et al., 1998; Safi et al., 2014); [23](Mendez et al., 2014); [25](Gödel et al., 2000); [26](Scholz et al., 2014); [27](Allard and Templier, 2000); [28](Ryckebosch et al., 2014); [29](Olenina et al., 2006); [30](Nagao et al., 2007); [31](Pernet and Tremblay, 2003); [32](Morineau-Thomas et al., 2002); [33](Heaney-Kieras and Chapman, 1976); [34](Jubeau et al., 2013); [35](Weiss et al., 2012); (Metzgar et al., 2008); (Kodner et al., 2008); [36](Bhatnagar et al., 2010); [37](Ordog et al., 2012); [38](Gour et al., 2016); [39](Blumreisinger et al., 1983); [40](Azaman et al., 2017); [41](Doucha and Lívanský, 2008); [42](Kose and Oncel, 2015); [43](Takeda, 1991); [44](Yamada and Sakaguchi, 1982); [45](Cordeiro et al., 2005); [46](Simpson et al., 2003); [47](Rodrigues and Bon, 2011); [48](Kloareg and Quatrano, 1988); [49](Mackie and Presten, 1974); [50](Liu et al., 2014); [51](Darienko et al., 2010); [52](Herrera-Valencia et al., 2011); [53](Singh et al., 2013); [54](Zhang et al., 2014); [55](Cha et al., 2008); [56](Koo et al., 2012); [57](Barbosa et al., 2014); [58](Andersen et al., 1987)

(Kodner et al., 2009), such as *Scenedesmus*, Scenedesmaceae, *Tetraedron*, Chlorococcaceae, *Chlorella*, Chlorellaceae (Atkinson et al., 1972; Goth et al., 1988; Allard et al. 2002), and *Botryocococcus,* Trebouxiophyceae (Templier et al., 1992; Metzger et al., 2008). As can be seen in Table 4.1, the cell wall of some species of *Chlorella* (e.g., *Chlorella minutissima*, *Chlorella zofingiensis, Chlorella homosphaera*) (Malis et al., 1993; Allard and Templier, 2000; Ordog et al., 2012) and *Cloroidium* (e.g., *Chloroidium ellipsoideum*), consist of algaenan (Allard and Templier, 2000; Allard and Templier, 2001; Rodrigues and Bon, 2011; Domozych et al., 2012). Algaenan may also be as a component in cell walls that consist of a tri-laminar structure, i.e. *Scenedesmus* (Thompson, 1996). However, this is not to suggest that algaenan-producing organisms have a tri-laminar cell wall structure (Gerken et al., 2013; Burczyk et al., 2014), and that a tri-laminar structure does not necessarily indicate the presence of algaenan (Allard et al., 2002).

As can be seen in Table 4.1, species of the same taxonomic class or even genus can have varying cell wall structures and compositions. For instance, Burczyk and Hesse (1981) and Takeda (1991) noted that algaenan was not present in the extracellular matrix of *Chloroidium saccharophilum,* Trebouxiophyceae (synonymous with *Chlorella saccharophila* (Darienko et al., 2010; Guiry and Guiry, 2018)) and Yamada and Sakaguchi (1982) also reported that *Chloroidum saccharophilum* did not show evidence of secondary carotenoids, indicating that algaenan was absent from the outer cell wall. They observed that this species possessed a cell wall composed of two primary layers, an outer non tri-laminar layer and an inner microfribrillar layer (Yamada and Sakaguchi, 1982). However, the algaenan structure was present in *Chloroidium ellipsoideum* (synonymous with *Chlorella ellipsoidea* (Darienko et al., 2010; Guiry and Guiry, 2018)), which also belong to the class of Trebouxiophyceae.

Microalgae with a cellulosic cell wall structure, such as *Chlorella zofingiensis*, typically consist of cellulose contents that make up to 70 % of the total dry cell wall weight (Kloareg and Quatrano, 1988; Takeda, 1991; Latala et al., 2009) and can contain sugars other than glucose, such as xylose (Mackie and Preston, 1974). The high glucose and mannose content contribute to the rigidity of the cell wall (Rodrigues and Bon, 2011) and

the function of these polysaccharides found in the microalgal cell wall is to support the structure of the cell (He et al., 2016).

Microorganisms with complex sugars forming the cell wall, such as *T. suecica* and *T. striata* are predominantly made up of galactose, xylose, rhamonse, mannose and arabinose (Schwenzfeier et al., 2011; Safi et al., 2014). Such compounds form a rigid barrier that hinders the extraction of intracellular products (Doucha and Livansky, 2008).

The wide diversity in the cell wall structure and composition seen amongst different microalgae species, emphasizes the need to characterize the cell wall in order to understand the effects of various treatment types on the biomass. This is crucial in the systematic optimization of the recovery of intracellular value added products from microalgae, which in turn would improve the economics of the process.

4.4 Effect of Cell Wall Disruption on Product Recovery for Various Microalgae Species

Insight into the cell wall structure is important in the selection of an appropriate cell wall disruption technique (Middelberg, 1995). There are numerous methods that have been employed to disrupt the microalgae cell wall to enhance intracellular product recovery. These have been the subject of recent review articles (Alhattab et al., 2015; Byreddy et al., 2015; Günerken et al., 2015; Kim et al., 2016), where the details on the principles underlying the techniques are given. In the current section, various microalgae species are discussed in terms of their cell wall structure, shape and size (Table 4.1). Additionally, the results from the various chemical, biological, physical and mechanical cell wall disruption methods (Table 4.2) are presented with the goal of providing insight into the role of cell wall structure and composition in the performance and efficacy of cell wall disruption techniques.

4.4.1 *Scenedesmus/ Tetradesmus*

Scenedesmus and *Tetradesmus* species both belong to the class of Chlorophyceae. The cells comprise of proteins, carbohydrates, lipids and nucleic acid (Demirbas, 2011), which can be utilized for the synthesis of a number of value-added products related to the biofuels (Gour et al., 2016), pharmaceutical, cosmetics and food industries (Abd El Baky et al.,

Table 4.2. Effects of chemical, biological and physical disruption techniques on the recovery of intracellular biomolecules for various microalgae species.

***Microalgae Species/* Taxonomic class**	**Cell Disruption Method/ Conditions**	**Growth Stage**	**Method of Extraction**	**Product Recovery**	**Ref.**
Scenedesmus sp. Chlorophyceae	None (0.5 g L^{-1} DCW)	Late linear growth phase	Chloroform: Methanol (1:1)	Lipid 2%	1
	Autoclave (125°C and 1.5 MPa, 5 min, 0.5 g L^{-1} DCW)			Lipid 5%	
	Bead-beating (BD 0.1mm, AS 2800 rpm, 5 min, 0.5 g L^{-1} DCW)			Lipid 9%	
	Microwave (100°C and 2450 MHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 11%	
	Sonication (10 kHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 6%	
Botryococcus sp. Trebouxiophceae	None (0.5 g L^{-1} DCW)	Stationary		Lipid 8%	
	Autoclave (125°C and 1.5 MPa, 5 min, 5 g L^{-1} DCW)			Lipid 12%	
	Bead-beating (BD 0.1mm, AS 2800 rpm, 5 min, 0.5 g L^{-1} DCW)			Lipid 28%	
	Microwave (100°C and 2450 MHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 29%	
	Sonication (10 kHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 9%	
C. vulgaris Trebouxiophceae	None (0.5 g L^{-1} DCW)	Late linear growth phase		Lipid 4%	
	Autoclave (125°C and 1.5 MPa, 5 min, 0.5 g L^{-1} DCW)			Lipid 10%	
	Bead-beating (BD 0.1mm, AS 2800 rpm, 5 min, 0.5 g L^{-1} DCW)			Lipid 8%	
	Microwave (100°C and 2450 MHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 10%	
	Sonication (10 kHz, 5 min, 0.5 g L^{-1} DCW)			Lipid 6%	
Scenedesmus sp. Chlorophyceae	Microwave (100 °C, 1000 W, 10 mins, 50 g L^{-1} DCW)	Stationary	Chloroform: Ethanol (1:1)	Lipid 28 %	2
	Sonication (15 kHz, 2 mins, 50 g L^{-1} DCW)			Lipid 19 %	
Scenedesmus sp. Chlorophyceae	None	Stationary	Isopropanol	5 % FAME	3
	SDS^- (SC 50 mM, DB)			52 % FAME	
	NLS^- (SC 50 mM, DB)			18 % FAME	
	Tween-20* (SC 50 mM, DB)			4 % FAME	
	Triton-100* (SC 50 mM, DB)			18 % FAME	
	3-DMAPS$^{\varphi}$ (SC 50 mM, DB)			8 % FAME	
	3-DAPS$^{\varphi}$ (SC 50 mM, DB)			72 % FAME	
	$MTAB^+$ (SC 50 mM, DB)			54 % FAME	
	3-DAPS$^{\varphi}$ (SC 1-50 mM, WB)	Lag phase	Hexane: Isopropanol (1:1)	5-9 % FAME	
		Exponential		0-17 % FAME	
		Stationary		1-26 % FAME	

	$MTAB^+$ (SC 1-50 mM, WB)	Lag phase		2-14 % FAME	
		Exponential		1-22 % FAME	
		Stationary		2-30 % FAME	
	SDS^- (SC 1-50 mM, WB)	Lag phase		8-22 % FAME	
		Exponential		2-18 % FAME	
		Stationary		1-9 % FAME	
Scenedesmus sp. Chlorophyceae	None (1 g, 6.8 % DCW)	Exponential	Hexane	4 % Lipid	4
	Cellulase (6.8% DCW, 10 mg mL^{-1}, 37°C, 30 min)			15.5 % Lipid	
	Lysozyme(6.8%DCW, 10 mg mL^{-1}, 37°C, 30 min)			16.6 % Lipid	
	Freeze dried (1 g, 6.8 % DCW, -54°C, 6h)			13 % Lipid	
Scenedesmus sp Chlorophyceae.	None (2 g DCW)	Stationary	Bligh and Dyer (Chloroform: methanol (3:1))	Lipid 2 % (dw)	5
	Sonication (100 W, 30 mins, 2 g DCW)			Lipid 6 % (dw)	
Tetradesmus dimorphus Chlorophyceae	None (beadbeater for 5 mins, 40 g L^{-1})	-	Bligh and Dyer (Chloroform: methanol (2:1))	FAME 100%	6
	None (1 h, 40 g L^{-1})		$_{SC}CO_2$ (6000 PSI, 100°C)	FAME 100%	
			$_{SC}CO_2$ (4800 PSI, 80°C)	FAME 43%	
	Sonication (50/60 Hz, 5 mins, 40 g L^{-1})		$_{SC}CO_2$ (4800 PSI, 80°C)	FAME 73%	
	Microwave (100°C, 5 mins, 40 g L^{-1})		$_{SC}CO_2$ (4800 PSI, 80°C)	FAME 80%	
	Beadbeating (1 mm borosilicate glass balls, 5 mins, 40 g L^{-1})		$_{SC}CO_2$ (4800 PSI, 80°C)	FAME 20%	
	Freeze dried (-80°C, 48 h, 40 g L^{-1})		$_{SC}CO_2$ (4800 PSI, 80°C)	FAME 60%	
Tetradesmus obliquus Chlorophyceae	None (1 mins)	-	P- 1-3 ml of nanograde acetone; FA-Solvent: Dichloromethane: methanol (2:1)	P 100 mg mg^{-1} (dw); FA 17 µg mg^{-1} (dw)	7
Staurastrum Zygnematophyceae				P 200 mg mg^{-1} (dw); FA 21 µg mg^{-1} (dw)	
Tetradesmus obliquus Chlorophyceae	Sonication (35 kHz, 80 W, 90 mins)		P- 1-3 ml of nanograde acetone; FA- Dichloromethane: methanol (2:1)	P 2200 mg mg^{-1} (dw); FA 50 µg mg^{-1} (dw)	
Staurastrum Zygnematophyceae				P 1250 mg mg^{-1} (dw); FA 51 µg mg^{-1} (dw)	
Chlorella sp. Trebouxiophyceae	None (20 g L^{-1} WB)	-	Hexane	Lipid 32 %	8
	Cellulase (28 °C, 1h, 20 g L^{-1} WB)			Lipid 56 %	
Chlorella sp. Trebouxiophyceae	None (DB)	Late linear	Modified Folch method[9]	8.3-11.4 % Lipid (DCW)	10

	CTAB[+] (SC 6.6 mg L^{-1}, DB)			14-17 % Lipid (DCW)	
Chlorella sp. Trebouxiophyceae	None (5 g L^{-1} WB)	-	Modified Bligh and Dyer (Chloroform: methanol (2:1))	Lipid 0.08 g	11
	Sonication (50 Hz, 15 mins)			Lipid 0.21 g	
	Microwave (2450 MHz, 100°C, 5 mins, 5 g L^{-1} WB)			Lipid 0.18 g	
	Autoclaving (121°C with 15 lbs in^{-2}, 5 mins, 5 g L^{-1} WB)			Lipid 0.11 g	
	Bead milling (3500 rpm, 5 mins, 5 g L^{-1} WB)			Lipid 0.15 g	
Chlorella sp. Trebouxiophyceae	None (20 min to 24 h, 3.6 g L^{-1})	Exponential	Bligh and Dyer	Lipid 2-25 %	12
	HPH (880 bar)			Lipid 26 %	
	Autoclave (105°C, 20 min)			Lipid 27 %	
	HPH + Autoclave			Lipid 30 %	
Neochloris oleoabundans Chlorophyceae	None (20 g L^{-1} WB)	Late linear	Hexane phase separation	Lipid 0.55 g L^{-1}	13
	Sonication (25 kHz, 600 W, 30 mins, 20 g L^{-1})			Lipid 4 g L^{-1}	
	HPH (60 MPa, 20 g L^{-1})			Lipid 5 g L^{-1}	
	Enzyme (Papain (1 %), Cellulase (1 %), 5 h, 20 g L^{-1})			Lipid 2.25 g L^{-1}	
	Enzyme + HPH (Papain (1 %, 2 h), Cellulase (1 %, 3 h), HPH at 60 MPa)			Lipid 6.5 g L^{-1}	
	Enzyme + Sonication ((Papain (1 %, 2 h), Cellulase (1 %, 3 h), 600 W, 30 min)			Lipid 5.5 g L^{-1}	
Nannochloropsis oculata Eustigmatophyceae	None (100 g DCW, 90 mins)	Stationary	Folch chloroform: methanol (2:1)	Lipid 7 g	14
	Ultrasound (30 kHz, 360 mins, 100 g DCW)			Lipid 24 g	
Nannochloropsis oculata Eustigmatophyceae	None (0.8 g L^{-1})	Late Linear	Lowry	Protein 15 mg g^{-1}	15
	HPH (75 MPa, 1-6 passes, 0.8 g L^{-1})			Protein 30-48 mg g^{-1}	
	HPH (125 MPa, 1-6 passes, 0.8 g L^{-1})			Protein 33-50 mg g^{-1}	
	HPH (75 MPa, 1-6 passes, 0.8 g L^{-1})		Phenol-sulfuric[y]	Sugar 57-60 mg g^{-1}	
	HPH (125 MPa, 1-6 passes, 0.8 g L^{-1})			Sugar 60-61 mg g^{-1}	
	None (0.8 g L^{-1})		Bligh and Dyer	Lipid 29 %	
	HPH (75-230 MPa, 1-6 passes, 0.8 g L^{-1})			Lipid 29-31 %	
	None (0.8 g L^{-1})		Petroleum ether	Lipid 4 %	
	HPH (75-230 MPa, 1-6 passes, 0.8 g L^{-1})			Lipid 8-16 %	
	HPH (125 MPa, 6 passes, 0.16-0.18 g L^{-1})	Exponential -Stationary	Bligh and Dyer	Lipid 31-34 %	
			Lowry	Protein 29-22 mg g^{-1}	
			Phenol-sulfuric	Sugars 43-34 mg g^{-1}	
Nannochloropsis gaditana Eustigmatophyceae	Solvent (60 mins, 5 g DCW)	NA	Bligh Dyer (1:10)	Lipid 8.9%	16
			Folch (1:10)	Lipid 12%	
			Hexane (1:10)	Lipid 0.73%	

			Methanol (1:10)	Lipid 33%	
			Acetone (1:10)	Lipid 1.1%	
	Ultrasound (19.5 kHz, 100 W, 50-60°C, 5-20 mins)		Methanol (1:10)	Lipid 31-38%	
	Microwave (25-35 W, 60-90°C, 10-20 mins)			Lipid 30-40%	
Nannochloropsis Eustigmatophyceae	None (1 g DCW)	Late linear growth phase	Chloroform: methanol (2:1) with 0.3 g SrO, 2 mins	FAME 3 %	17
	Sonication (20 kHz, 40 % amplitude,5 min, 1 g DCW)			FAME 21 %	
	Microwave (2.45 GHz, 5 mins cycle mode, 21 s on and 9 s off, 1 g)			FAME 37 %	
Nannochloropsis sp. Eustigmatophyceae	Solar drying (Thin film <5 mm, 30–34 °C, 8 h)	Late linear-growth phase	Hexane: methanol (3:2)	Lipid 29 %	18
	Freeze drying (Thin film <5 mm, 16 h)			Lipid 30 %	
	Oven drying (Thin film <5 mm, 60 °C, 3 h)			Lipid 31 %	
	Sonication (50 Hz, 30 W, 1 g, 5 min at 1 min interval)			Lipid 22.5 %	
	Sonication (50 Hz, 30 W, 1 g, 5 min at 1 min interval)		Chloroform:Methanol (2:1)_	Lipid 35 %	
Nannochloropsis oculata Eustigmatophyceae	None (2 g DCW)	Stationary	Bligh and Dyer (Chloroform: methanol (1:2))	Lipid 26.4%	19
	Autoclave (121°C, 15lbs, 10-30 mins, 2 g DCW)			Lipid 28-29 %	
	Microwave (100°C, 900W, 2455 MHz, 5-15 mins, 2 g DCW)			Lipid 27-17 %	
	Sonication (24 kHz, 50°C, 5-15 mins, 2 g DCW)			Lipid 28-30 %	
	Enzyme (Cellulase (5mg L^{-1}), 37°C, pH 5.5, 8-12 h, 2 g DCW)			Lipid 29-33 %	
Nannochloropsis gaditana Eustigmatophyceae	None (20 mL WB)	-	Bligh and Dyer (Chloroform: methanol (1:2))	Lipid 9.8 %	20
	Autoclaving (120 °C, 29 lbs in^{-2}, 5 mins, 20 mL WB)			Lipid 10.8 %	
	Ultrasound (37 kHz, AT, 5 mins, 20 mL WB)			Lipid 10.5 %	
	Microwave (2450 MHz, 150 °C, 5 mins, 20 mL WB)			Lipid 11.2 %	
	Steam explosion (120°C &150°C, 5 mins, 20 mL WB)			Lipid 17.9 & 18.2 %	
C. sorokiniana Trebouxiophyceae	None (20 mL WB)			Lipid 11.4 %	
	Autoclaving (120 °C, 29 lbs in^{-2}, 5 mins, 20 mL WB)			Lipid 14.4 %	
	Ultrasound (37 kHz, AT, 5 mins, 20 mL WB)			Lipid 14.1 %	
	Microwave (2450 MHz, 150 °C, 5 mins, 20 mL WB)			Lipid 14.5 %	
	Steam explosion (120°C, 5 mins, 20 mL WB)			Lipid 18.4%	
Phaeodactylum tricornutum Bacillariophyceae	None (20 mL WB)			Lipid 27 %	
	Autoclaving (120 °C, 29 lbs in^{-2}, 5 mins, 20 mL WB)			Lipid 27.2 %	
	Ultrasound (37 kHz, AT, 5 mins, 20 mL WB)			Lipid 28 %	
	Microwave (2450 MHz, 150 °C, 5 mins, 20 mL WB)			Lipid 27.1 %	

	Steam explosion (120°C, 5 mins, 20 mL WB)			Lipid 29 %	
Phaeodactylum tricornutum Bacillariophyceae	None (100 mg DCW)	Stationary	Chloroform: methanol (1:1)	Lipid 37 %	21
	Freeze drying			Lipid 36 %	
	Sonication (15 mins, 100 mg DCW)			Lipid 38 %	
	Bead beating (60 Hz, 1 min, 100 mg DCW)			Lipid 39 %	
Chlorococcum sp. Chlorophyceae	None (20 g L^{-1} DCW)	Late linear-stationary growth phase	Bligh and Dyer	Lipid 12-13 %	22
	Microwave (100°C, 2450 MHz, 2-6 mins, 20 g L^{-1} DCW)			Lipid 18-24 %	
	Autoclaving (121°C, 1.5 MPa, 15 mins, 20 g L^{-1} DCW)			Lipid 15-25 %	
Botryococcus sp. Trebouxiophyceae	None (20 g L^{-1} DCW)			Lipid 34-44 %	
	Microwave (100°C, 2450 MHz, 2-6 mins, 20 g L^{-1} DCW)			Lipid 22-48 %	
	Autoclaving (121°C, 1.5 MPa, 15 mins, 20 g L^{-1} DCW)			Lipid 38-48 %	
C. sorokiniana Trebouxiophyceae	None (20 g L^{-1} DCW)			Lipid 21-29 %	
	Microwave (100°C, 2450 MHz, 2-6 mins, 20 g L^{-1} DCW)			Lipid 11-35%	
	Autoclaving (121°C, 1.5 MPa, 15 mins, 20 g L^{-1} DCW)			Lipid 15-32 %	
C. sorokiniana Trebouxiophyceae	Soxhlet (25 g L^{-1} DCW)	Exponential	Chloroform: methanol (1:1)	Lipid 12.8 %	23
	Microwave (100°C, 2450 MHz, 5 mins, 25 g L^{-1} DCW)			Lipid 0.9 %	
	Bead-bating (2800 rpm, 5 mins, 25 g L^{-1} DCW)			Lipid 6.6 %	
	Sonication (10 kHz, 10 mins, 25 g L^{-1} DCW)			Lipid 4.4 %	
	Autoclave (121°C, 15 mins, 25 g L^{-1} DCW)			Lipid 0.6 %	
C. vulgaris Trebouxiophyceae	None	Exponential growth phase	Lowry Method	Protein 1%	24
				Carotenoids 0μg/mL	
				Chloro 0 μg/mL	
	Bead Mill (BD 1-1.6 mm, 2500 rpm, 1-30 mins)			Protein 2-50%	
				Caro 0-60 μg/mL	
				Chloro 0-260 μg/mL	
	Ultrasonication (20 kHz, 5-30 mins at 5s on and 15 s rest)			Protein 4-9%	
				Caro 0.5-2 μg/mL	
				Chloro 2.1-10 μg/mL	
	HPH (39160 PSI, 2% DCW, 5-40 min)			Protein 31-35 %	
				Caro 4-4 μg/mL	
				Chloro 16.5-18 μg/mL	
C. vulgaris Trebouxiophyceae	None (5-120 mins, B:S 1:100)	Exponential	Chloroform: methanol (2:1)	Lipid 50-72 %	25
	Microwave (40-50°C, 300 W, 5-30 mins, B:S 1:100)			Lipid 70-72 %	
	Sonication (40 kHz, 40-50°C, 5-30 mins, B:S 1:100)			Lipid 70-80 %	

C. vulgaris Trebouxiophyceae	Bead Milling (AS 6-12 m s^{-1}, 3 min, 25-145 g DCW kg^{-1})	Late linear growth phase	Chemical: SDS lysing matrix E	Protein yield 42 to 32 %	26
C. vulgaris Trebouxiophyceae	None (2.68 g L^{-1})	Late linear-early stationary growth phase	Modified Bligh and Dyer (Chloroform: methanol (1:1))	Lipid 2.5 %	27
	Grinding (ceramic mortar and pestle, 10 mL of liquid nitrogen for1 min, 500 mL WB; Quartz sand for 10 mins 0.1 g DCW; Oven dried 8 h, Quartz sand, 2.68 g L^{-1})			Lipid 5-29%	
	Sonication (600 W, 20 mins at 5 and 30 s intervals, 2.68 g L^{-1})			Lipid 15 %	
	Bead Milling (AS 1500 rpm, 20 mins, 2.68 g L^{-1})			Lipid 10 %	
	Microwave (100°C, 2450 MHz, 5 mins, 2.68 g L^{-1})			Lipid 18 %	
	Cellulase (55°C, 10 h, 2.68 g L^{-1} DCW)			Lipid 24 %	
	Lysozyme (55°C, 10 h, 2.68 g L^{-1} DCW)			Lipid 22 %	
	Snailase (37°C, 2 h, 2.68 g L^{-1} DCW)			Lipid 7 %	
C. vulgaris Trebouxiophyceae	None (DB)	Late linear	Hexane: Methanol (7:3)	30 mg lipid g^{-1}	28
	$SDBS^-$ (SC 0-2.86 mM, DB)			>30 mg lipid g^{-1}	
	Sulfuric acid (conc. 0.5-2%, DB)			201-225 mg lipid g^{-1}	
C. vulgaris Trebouxiophyceae	None (Ultrasonication, WB)	-	Solvent: Chloroform: methanol (2:1)	2.72 mg FAME	29
	$CTAB^+$ (SC 0.15 g L^{-1}, WB)			6.34 mg FAME	
C. vulgaris Trebouxiophyceae	Ultrasonication+ Cellulase (Conc. 0.5-8%, 55°C, pH 4.8, 18% DCW)	-	Centrifuged for phase separation	Lipid 10-16.5 %	30
	Ultrasonication+ Snailase (Conc. 0.5-8%, 37°C, pH 5.8, 18% DCW)			Lipid 19-35 %	
	Ultrasonication+ Neutral protease (Conc. 0.5-8%, 50°C, pH 7.0, 18% DCW)			Lipid 6-12 %	
	Ultrasonication+ Alkaline protease (Conc. 0.5-8%, 55°C, pH 8.5, 18% DCW)			Lipid 5.3-9 %	
	Ultrasonication+ Trypin (Conc. 0.5-8%, 37°C, pH 8.0, 18 % DCW)			Lipid 19-34 %	
C. vulgaris Trebouxiophyceae	Pancreatin (Conc. 1-20 %, pH 7.5, 45°C, 1 h, 1 g DCW)	Late linear	Lowry Method	Protein 500-1090 mg L^{-1} (1-10 %), 925 mg L^{-1} (20 %)	31
C. vulgaris Trebouxiophyceae	None	-	Chloroform: methanol (2:1)	Lipid 5.5 %	32
	Cellulase (2 %), β-glucosidases (1 %), pH 4.8, Temp. 50°C			Lipid 9 %	
C. vulgaris Trebouxiophyceae	None	Late linear	Chloroform: methanol (2:1)	Lipid 34 %	33
	AES-Bt (pH 7, 24 h, 25°C, 10 g/L WB)			Lipid 44 %	

C. vulgaris Trebouxiophyceae	None	Late linear	Chloroform: methanol (2:1)	Lipid 34 %	34
	Sonication (400 W, 25 min, 40 mg biomass)			Lipid 35%	
	Bead milling (speed 1/30 s, 25 min, 40 mg biomass)			Lipid 40%	
C. vulgaris Trebouxiophyceae	None (6 g)	Exponential	$scCO_2$ (8700 PSI, ,60°C, 30 g min^{-1}, 150 min)	Lipid 9 % (DCW)	35
				Chlorophyll 11 %	
				Carotenoids 11.5 %	
			$scCO_2$ (8700 PSI, ,60°C, 30 g min^{-1}, 150 min) + 5 % ethanol	Lipid 12.5 %	
				Chlorophyll 47 %	
				Carotenoids 38 %	
	Bead milling (2500 rpm, 1 h, 7.7 % solids)		$scCO_2$ (8700 PSI, ,60°C, 30 g min^{-1}, 150 min)	Lipid 10 %	
				Chlorophyll 17 %	
				Carotenoids 15 %	
Chloroidium ellipsoideum Trebouxiophyceae	None (1 g, Ultrasound assisted, 2 h)	-	Methanol: ethyl acetate (1:1)	Zeaxanthin 0.61 mg g^{-1}	38
	Pressurized liquid extraction (1500 PSI, 125°C, 20 mins)		Hexane	Zeaxanthin 1 mg g^{-1}	
	Pressurized liquid extraction (1500 PSI, 125°C, 20 mins)		Ethanol	Zeaxanthin 4.2 mg g^{-1}	
	Pressurized liquid extraction (1500 PSI, 125°C, 20 mins)		Isopropanol	Zeaxanthin 4 mg g^{-1}	
Tetraselmis suecica Trebouxiophyceae	Triton X-114* (SC 0.1 to 5 %, DB)	Stationary	Sodium Tartrate salt $+H_2O$	100 % Gallic acid	36
				80 % α-Tocopherol	
				55 % β-Carotene	
	Tween 20* (SC 0.1 to 5 %, DB)			100 % Gallic acid	
				60 % α-Tocopherol	
				50 % β-Carotene	
	Triton X-114* (DB)	Stationary	-	5.1 mg g^{-1} Gallic acid	
				174.6 µg g^{-1} α-Tocopherol	
				1.3 mg g^{-1} β-Carotene	
	Ultrasound (45 min, DB)			3.2 mg g^{-1} Gallic acid	
				47.3 µg g^{-1} α-Tocopherol	
				0.04 mg g^{-1} β-Carotene	
Chaetoceros ceratosporus Mediophyceae	Sonication (50 W, 5°C, 30 mins)	Exponential	Dichloromethane: methanol (2:1)	TAG 1.5 to 3 µg 10^{-6} cells	37
	Grinding (glass pestle)			TAG 1.5 to 3.5 µg 10^{-6} cells	

	Sonication+ Grinding (30 mins + grinding)	TAG 1.5 to 3.75 µg 10^{-6} cells

Ref: Reference; DCW: dry cell weight; BD: bead diameter; P: pigments; FA: fatty acids; conc: concentration; temp: temperature; AT: ambient temperature; FAME: Fatty acid methyl ester; B:S: Biomass:solvent; Chloro: chlorophyll; Caro: carotenoids; HPH: high pressure homogenization; SDS: Sodium dodecyl sulfate; DB: dried biomass; SC: surfactant concentration; NLS: N-Lauroylsarcosine sodium salt; 3-DMAPS: 3-(N,N-dimethylmyristylammonio)propanesulfonate; 3-DAPS: 3-(Decyldimethylammonio) propanesulfonate inner salt; MTAB: Myristyltrimethylammonium bromide; WB: wet biomass; conc.: concentration; SDBS: sodium dodecyl benzene sulfonate; CTAB: Cetyltrimethylammonium bromide; $^{-}$Anionic; $^{+}$cationic; *non-ionic; $^{\varphi}$Zwitterionic;

[1](Lee et al., 2010); [2](Guldhe et al., 2014); [3](Lai et al., 2016); [4](Taher et al., 2014); [5](Ranjan et al., 2010); [6](Soh and Zimmerman, 2011); [7](Wiltshire et al., 2000); [8](Fu et al., 2010); [9](Floch et al., 1957); [10](Coward et al., 2014); [11](Prabakaran and Ravindran, 2011); [12](Spiden et al., 2015); [13](Wang et al., 2015); [14](Converti et al., 2009); [15](Shene et al., 2016); [16](Bermudez-Menendez et al., 2014); [17](Koberg et al., 2011); [18](Balasubramanian et al., 2013); [19](Surendhiran and Vijay, 2014); [20](Lorente et al., 2015); [21](Ryckebosch et al., 2012); [22](Rakesh et al., 2015); [23](Yu et al., 2015); [24](Safi et al., 2015); [25](Prommuak et al., 2012); [26](Postma et al., 2015); [27](Zheng et al., 2011); [28](Park et al., 2014); [29](Huang and Kim, 2013); [30](Liang et al., 2012); [31](Kose and Oncel, 2015); [32](Cho et al., 2013); [33](Bai et al., 2015); [34](Bai et al., 2015); [35](Safi et al., 2014); [36](Ulloa et al., 2012); [37](Pernet and Tremblay, 2003); [38](Koo et al., 2012)

2012). *Scenedesmus* and *Tetradesmus* species have cell walls consisting of two main layers, an inner and outer layer (Burczyk and Dworzanski, 1988; Derenne et al., 1992). As indicated in Table 4.1, the inner layer is mainly comprised of polysaccharides, such as cellulose and the outer layer being the tri-laminar structure made up of algaenan, which acts as a barrier to the recovery of intracellular components (Allard et al., 2002; Kodner et al., 2009; Burczyk et al., 2014; Obeid et al., 2014; Domozych, 2016). Glucosamine biopolymers and glycoproteins are also components that are present in the rigid walls of these species (Burczyk et al., 1999). These species are bean shaped and vary in size from 10 to 12 µm (Shen et al., 2009).

Disruption Techniques

The application of surfactants for protein separation from cells has been well established (Arachea et al., 2012). Surfactants have also found their way as agents for microalgae cell disruption, where lipid recovery is typically the goal (Corre et al., 1996; Huang and Kim, 2013; Coward et al., 2014; Lai et al., 2016). In the study by Lai et al. (2016), Table 4.2, the effect of varying surfactant types, concentration, and microalgae growth phase on cell wall disruption for *Scenedesmus,* and other species, was investigated. They found that the Fatty Acid Methyl Ester (FAME) recovery of *Scenedesmus* biomass improved by 10-16 folds using 3-decyldimethylammonio propanesulfonate (3-DAPS), myristyltrimethylammonium bromide (MTAB), and sodium dodecyl sulfate (SDS), compared to extraction from untreated cells under similar conditions. Generally, increasing the surfactant concentration resulted in higher FAME recoveries for all surfactant systems, and prolonged growth (lag to stationary phase) resulted in greater FAME yields up to 72% using 3-DAPS system (Table 4.2). It was also noted that surfactants without a charge (non-ionic), i.e. Tween 20 (Table 4.2) were less effective in improving FAME recoveries using the solvent isopropanol (Lai et al., 2016). However, the SDS resulted in the highest FAME yields during the high growth phase, but decreased with prolonged culture growth, for all concentrations tested, which may be attributed to the change in chemical composition of the cell wall, with prolonged growth, that would alter the charge specific interactions (Gerken et al., 2013; Lai et al., 2016).

The effectiveness of various mechanical, thermal, and electromagnetic radiation techniques on the non-hydrolyzable algaenan cell wall structure of *Scenedesmus* and *Tetradesmus*, are also demonstrated in Table 4.2. Therein, the study of Lee et al. (2010) compares the use of beadmilling, sonication, autoclaving and microwave as pre-treatment methods on solvent extracted lipid recovery, and found that the electromagnetic radiation and beadmilling resulted in improvements by factors of 5.5 and 4.5, respectively, compared to untreated biomass. Meanwhile, autoclaving and sonication treatments had a lower impact on product recovery with increases by factors of 2.5 and 3, respectively. Similarly, sonication of *Scenedesmus* sp. also improved lipid recoveries by a factor of 3 as noted by Wiltshire et al. (2000) and Ranjan et al. (2010), compared to untreated biomass. It was determined by Guldhe et al. (2014), that sonication of *Scenedesmus* sp. biomass resulted in lipid yields that were 1.5 times less than those recovered from microwave treated biomass, Table 4.2. In contrast however, sonication of *Tetradesmus obliquus* (synonym with *Scenedesmus obliquus* (Wynne and Hallan, 2015; Guiry and Guiry, 2018)) biomass enhanced pigment recovery by a factor of 22 (Wiltshire et al., 2000), when compared to untreated biomass. Furthermore, the disruption of *Tetradesmus oblique* biomass was assessed using high and low frequency focused sonication by Wang et al. (2015), and it was determined that effective disruption occurred using low frequency at exposers of 1-5 min, and using high frequency, but only at exposures of 5 min. The variation was attributed to the greater power exerted using low frequency, which is consistent with the need of prolonged exposures to achieve significant disruption using high frequency sonication. These findings suggest that for algaenan-containing species and for by-products derived from algal lipids, more vigorous techniques are necessary for lipid extraction such as microwave radiation and beadmilling. Sonication techniques, although less effective in lipid recovery, were significantly effective in improving pigment recovery (Wiltshire et al., 2000). *Scenedesmus* biomass harvested in the stationary phase resulted in greater lipid content compared with cells recovered during the late linear growth phase (Lee et al., 2010; Guldhe et al., 2014), the change in which may be attributed to chemical composition of the cell wall (Gerken et al., 2013; Lai et al., 2016), and the diversion of intercellular pathway from cell division (linear growth phase) to storage compounds in the form of lipids (stationary phase) (Hu et al., 2008).

A comparison of the FAME recovery from *Tetradesmus dimorphus* (previously known as *Scenedesmus dimorphus* (Wynne and Hallan, 2015; Guiry and Guiry, 2018)) using conventional solvent extraction and SC-CO_2, was performed with and without pre-treatment (Soh and Zimmerman, 2011). It was observed by these researchers that no difference in FAME recovery of treated and non-treated biomass, when the SC-CO_2 was performed at optimum operational parameters (41.4 MPa, 100°C). However, at lower SC-CO_2 operating parameters, and without pretreatment, lower recoveries were achieved. Following this, the use of pre-treatment disruption techniques as a means for improving SC-CO_2 recoveries at lower pressure of 33.1 MPa and lower temperature of 80°C was studied, and it was found that some techniques worked better than others. Microwave radiation pre-treatment, resulted in the highest recoveries (of 80 %), as was observed in other studies where solvent extraction methods were employed (Lee et al., 2010; Ranjan et al., 2010). Similarly, sonication and freeze drying treatments achieved FAME recoveries of 73 and 60 %, respectively, which almost doubled the recovery yields obtained using conventional solvent extraction of beadmilled biomass (Soh and Zimmerman, 2011). However, bead milling treatment in this study, resulted in lower yields compared to untreated biomass using SC-CO_2 extraction.

4.4.2 *Botryococcus*

The extracellular matrix of *Botryococcus* species, belonging to the class of Trebouxiophyceae, is quite complex, Table 4.1. The cells are oval ranging from 3-5 µm in size (Weiss et al., 2012). They exist in colonies with each cell possessing an individual algaenan cell wall, encompassed by hydrocarbons secreted from the cell that cross link to form a network of hydrocarbons with other colonial cells, whereby this network of cells and hydrocarbons are further housed by a retaining wall with a polysaccharide colony sheath (Weiss et al., 2012; Uno et al., 2015). Nonetheless the composition of the cell wall of each individual cell, resembles that of *Scenedesmus* and *Tetradesmus* sp. in that it consists of the non-hydrolyzable algaenan structure (Simpson et al., 2003; Metzger et al., 2008; Kodner et al., 2009) composed of β-1,4- and/or β-1, 3-glucan (Weiss et al,. 2012). Raman spectrometry and electron microscopy analysis performed by Largeau et al. (1980) also indicated that this species accumulates hydrocarbons that are in contact with the

trilaminar layer composed of a compound that is sporopollenin-like. It was determined from several studies (Mendes et al., 1994; Lovejoy et al., 2013; Moheimani et al., 2014; Griehl et al., 2015), that the recovery of hydrocarbons from within the colony sheath, can be performed using non-destructive techniques. However, these techniques were found ineffective after extended periods of time (beyond 70 days) and low external hydrocarbon productivity was achieved of 11.6 mg L^{-1} day^{-1} compared to the total oil productivity of 24.1 mg L^{-1} day^{-1} using *B. braunii* in the study of Moheimani et al. (2014). The low productivity achieved may be attributed to the barrier formed by the colony sheath which has been found to require pre-treatment by several authors (Lee et al., 2010; Kita et al., 2010; Tsutsumi et al., 2018).

4.3.2.1 Disruption Techniques

The mechanical and electromagnetic treatments of *Botryococcus* using bead-beating and microwave radiation improved conventional solvent recoveries of lipids (Table 4.2) by factors of 3.5 and 3.6 times, respectively, as observed by Lee et al. (2010). The use of autoclaving and sonication was also tested on this species, by the same authors, and it was found that these techniques were less effective, improving the lipid yields by only 4 and 1 %, respectively. The extent of product improvement by the various techniques is similar to those seen in the case of *Scenedesmus* (mentioned above), which possess a cell wall composition of the same nature as that of *Botryococcus*. The slight variation, however, maybe attributed to the extra colony barrier formed by *Botryococcus*, and/or the variation in size. Similarly, it was found by Rakesh et al. (2015) that the use of microwave radiation improved lipid yields by a factor of 1.2 in fully grown cultures. However, this was comparable to the results achieved using autoclaving, which resulted in an increase in lipid yield by a factor of 1.3. The differences in total lipid improvements using microwave radiation by the two various studies (Lee et al., 2010; Rakesh et al., 2015), may be attributed to the variation in operating parameters, such as biomass concentration. This would also explain the greater improvements observed for autoclaving in the latter study, compared to those achieved by Lee et al. (2010), where a greater treatment time of 15 mins was used by Rakesh et al. (2015), compared to 5 mins used by Lee et al. (2010). An increase in culture growth from 7 to 14 days did not significantly vary lipid recoveries (Rakesh et al., 2015).

4.4.3 *Chlorella/ Chloroidium*

The microalgae genera, *Chlorella* and *Chloroidium,* stem from the same Trebouxiophyceae class, however, vary in family classification (Guiry and Guiry, 2018). *Chlorella* belongs to the Chlorellaceae family and *Chloroidium* belongs to the Trebouxiophyceae family. Despite this, the cell wall structure and composition of species found across these two genera are similar. Various extracellular wall structures have been reported for species within these genera (Table 4.1). Generally, *Chlorella* species possess a fibrillary cell wall or glycoprotein structures. The cell wall of these species consists of glucose as the major component and can include mannose, glucosamine, algaenan and/or β-galactofuranan (Table 4.1). Other species also possess the tri-laminar structure made up of algaenan (such as *Chlorella minutissima*). Similarly, both types of cell wall structures have been observed for species belonging to *Chloroidium* genera. The cell shape and size of *Chlorella* species are spherical and range from 2-4 μm in size (Bhatnagar et al., 2010; Gour et al., 2016; Azaman et al., 2017). In contrast, those of *Chloroidium* can be ovoid or ellipsoidal with sizes of 7x5 μm and 6-16 x 5-15 μm, respectively (Darienko et al., 2010). Value added products from these species vary widely from biofuels to supplements, pharmaceutical products, and aquaculture feeds (Chen, 1996).

In particular, *Chlorella vulgaris* has been heavily researched as a candidate for use in biofuel production. This microorganism has a polysaccharide extracellular cell wall structure that lacks the tri-laminar matrix (Corre et al., 1996; Henderson et al., 2008). The biochemical composition of the cell wall (Table 4.1) is mainly comprised of neutral sugars, which have been reported to make up 24-74 % of the cell wall matrix (Blumreisinger et al., 1983). These compounds form a rigid cell wall where disruption is strongly recommended to avoid underestimation of protein content as stated by Safi et al. (2013). Various techniques have been employed for cell wall disruption on several *Chlorella* and *Chloroidium* species (Table 4.2).

4.3.3.1 Disruption Techniques

The use of the anionic surfactant, SDBS, was ineffective in improving lipid extraction (>30 mg g^{-1} cell) from *Chlorella vulgaris* species (Park et al., 2014), although, the addition of sulfuric acid (0.5-2 %) improved lipid recovery by factors of 6.7-7.5, Table 4.2.

Furthermore, the disruption of *Chlorella* was investigated by Huang and Kim (2013) and Coward et al. (2014) using the cationic surfactant CTAB, which led to lipid recoveries that more than doubled those achieved from untreated biomass. It was determined that the surfactant exposure time is an important factor that can improve the lipid yields for this species from 93.4 to 100 % using a CTAB surfactant system (Huang and Kim, 2013).

The use of surfactants for cell wall degradation is most suited for organisms lacking the algaenan (tri-laminar structure) as noted by Corre et al. (1996) and Burczyk et al. (2014). However, the effectiveness of this technique is dependent on the surfactant concentration, which must be high enough to form surfactant-complex micelles with the cell wall (Corre et al., 1996; Lai et al., 2016). In a study by le Maire et al. (2000), the susceptibility of *C. vulgaris* and *Graesiella emersonii* (previously known as *Chlorella emersonii* (Nozaki et al., 1995; Borowitzka, 2016)) to detergents was compared, and it was found that *C. vulgaris* was readily degraded due to the absence of a tri-laminar cell wall structure, compared to *Graesiella emersonii*, where it is present. The use of surfactant treatment processes results in the presence of surfactants in the recovered products, which should be considered when using this technique. However, it is unlikely that the increase in lipid recovery is due to the chemical itself, since it was noted by Chatsungnoen and Chisti (2016) that the addition of chemical flocculants did not impact the recovery of lipids from various microalgae species (*Chlorella vulgaris*, *Nannochloropsis salina* and *Neochloris* sp).

The use of surfactants as a cell disruption technique offers an added advantage since it can also aid in the recovery of microalgae cells by flotation (Corre et al., 1996; Coward et al., 2014), and this should be given consideration when designing an effective bioproduct recovery process. Flotation techniques can recover both the surfactants and microalgae biomass, which would limit the environmental discharge and increase the concentration of surfactant in the recovered biomass. Moreover, the energy input requirement is low, and the cationic surfactant, CTAB has been used as a food grade chemical in the extraction process of pigments for animal/human consumption (Coward et al., 2014). Therefore, the presence of surfactants in the biomass does not limit the types of value added products that can be marketed to food, cosmetics and pharmaceutical industries (Thimmaraju et al.,

2003). The coupling of downstream processing techniques has been noted as an effective means for improving microalgae biodiesel economics (Kim et al., 2016).

Enzymatic cell disruption is another treatment that has also been investigated for *Chlorella* sp. The effectiveness of this process is governed by the type of enzyme used, which depends on the composition of the cell wall. The lipid recoveries from *Chlorella vulgaris* using modified Bligh and Dyer, lysozyme, cellulase, and snailase were 2.5, 22, 24 and 7%, respectively, as reported by Zheng et al. (2011), Table 4.2. However, Liang et al. (2012) obtained lipid recovery values of 9, 12, 16.5 and 35 %, for *Chlorella vulgaris*, using alkaline protease, neutral protease, cellulase and snailase, respectively. The differences may be attributed to the higher enzyme concentrations and initial sonication step used by Liang et al. (2012). The snailase enzyme is a cocktail that includes a mixture of cellulase, hemicellulase, and pectinase enzymes that can hydrolyze the compounds within the cell wall of *C. vulgaris* (Liang et al., 2012). While, snailase alone may not have been effective in disruption of the cell wall, ultrasonication resulted in better accessibility. In the study by Zheng et al. (2011), the cellulase enzymatic treatment of *Chlorella* sp. improved lipid recovery by 24 %, when compared to untreated biomass. Fu et al. (2010) reported that protoplast isolation from *Heterochlorella luteoviridis* (synonym with *Chlorella protothecoides* (Neustupa et al., 2009; Guiry and Guiry, 2018)), Trebouxiaceae, was greater using snailase compared to cellulase alone, due to the complex cell wall composition and mixture of enzymes present in snailase enzyme with activity toward the cell wall components. The greatest recoveries were achieved using a combination of snailase and cellulase, emphasizing the importance of hydrolysis of cellulose to improve the product recovery.

More recently, *Chlorella vulgaris* cell disruption was investigated using the active extracellular substance produced by the bacterium *Bacillus thuringiensis* ITRI-G1 (AES-Bt), Table 4.2 (Bai et al., 2015; Bai et al., 2017). It was noted by Bai et al. (2015) that treatment using this substance increased oil recoveries in untreated biomass from 34.2 to 44.3 %, and that recycling of the material four times resulted in a decrease in product recovery by 8 %. They also compared the AES-Bt treatment with physical disruption techniques (sonication and bead-beating) and noted no significant variation in lipid extract with the use of this substance compared to bead-beating (40 % recovery), however, there

was a significant improvement in recovery when compared to sonication treatment which resulted in 35 % recovery.

Cell disruption for *Chlorella* species (Table 4.2) was also achieved using autoclaving, bead-beating, microwave and sonication techniques, however, the degree of degradation varied amongst them (Lee et al., 2010). Both autoclaving and microwave radiation resulted in lipid improvements by a factor of 2.5, while bead-beating and sonication resulted in increases by factors of 2 and 1.5, respectively (Lee et al., 2010). The effect of cell wall degradation using microwave radiation and autoclaving was also investigated by Rakesh et al. (2015) on *Chlorella sorokiniana* and it was found that microwave treatment improved lipid recoveries by 6 % in 6 mins, while 15 mins of autoclaving improved yields by 3 %. Postma et al. (2015) reported that the effectiveness of cell disintegration using bead milling on *Chlorella* species, was dependent on the concentration of biomass placed into the unit, the agitator speed, exposure time and the feed rate. The highest percentage of disintegration (99 %), determined as the fraction of intact cells remaining after treatment, resulted using lower biomass concentrations (107 to 158 g L^{-1}) and feed rates (3-40 kg h^{-1}), higher agitator speeds (10-14 m s^{-1}) and prolonged treatment time (30-90 min) (Doucha and Lívanský, 2008).

Additionally, when using bead milling for disruption of *Chlorella vulgaris* species, the protein recovery was highest (42 %) at lower biomass concentrations over the tested range of 25-145 g dry weight kg^{-1} (Postma et al., 2015). In comparison with ultrasonication, Safi et al. (2015) noted that 9 % recovery was achieved after 30 minutes of ultrasonication, while 30 minutes of beadmilling (2 % dcw) resulted in protein recoveries close to 50 % (Table 4.2). Furthermore, the carotenoids content increased from 0 to 2 µg/ml (0-30 min) and 0 to 60 µg/ml (0-30 min) with sonication and beadmilling treatments, respectively, and the chlorophyll content was also significantly greater using beadmilling (260 µg/ml, 30 min) compared to sonication (10 µg/ml, 30 min). Comparing these to HPH, as little as 5 min treatment resulted in 32 %, 4 µg/ml and 18 µg/ml protein, carotenoids and chlorophyll, respectively, and a further increase in treatment did not improve recoveries much. However, Zheng et al. (2011) found that sonicating *C. vulgaris* biomass for 20 minutes resulted in lipid yields of 15 %, compared to 10 % achieved using bead milling. The

rotational speed used in this study was 1000 rpm less than that used by Safi et al. (2015), which may explain the variation in the results. Furthermore, Zheng et al. (2011) noted that grinding with liquid nitrogen resulted in higher lipid recovery by a factor of 11, amongst which microwave and enzymatic treatment resulted in improvements by factors of 7.2 and 9.6, respectively. Interestingly, Duan et al. (2017) found that the disintegration of *Chlorella pyrenoidosa* (synonym to *Auxenochlorella pyrenoidosa* (Guiry and Guiry, 2018) which possess a cellulose rich cell wall, using sonication (60 min) resulted in biomass disruption of 7.5 %. The biomass concentration used in this study ranged from 0.02-0.03 % (dcw) with an operational power of 35 kHz, which is higher compared to the levels used by other studies, however they attributed this to the rigidity of the cellulose cell wall, and noted that sonication is better suited for hydroxyproline rich glycoprotein cell wall structures (i.e. *Chlamydomonas reinhardtii*) which resulted in 80 % disintegration using 5 min of disintegration.

Similarly, the findings reported by Zheng et al. (2011) suggest that amongst the various disruption techniques employed for *Chlorella* species, sonication improved recoveries by a factor of 2.6, while other techniques including microwave, bead milling and autoclaving resulted in lipid yields that improved by 2.25, 1.9 and 1.4 times, respectively. Again, it is difficult to compare the effectiveness of these techniques, since the disruption time varied from 15 minutes for sonication to 5 minutes for the other techniques. Nonetheless, amongst the various techniques operated for 5 mins, microwave radiation resulted in the greatest improvement followed by beadmilling. However, Prabakaran and Ravindran (2011) found that the greatest lipid recoveries were achieved using sonication treatment as opposed to microwave treatment, which agrees with the work of Prommuak et al. (2012), but differs from those of Lee et al. (2010) as a result of different operational parameters. Limited studies are available on the effects of steam explosion on cell disruption, however, the study of Lorente et al. (2015) explores the steam explosion, autoclaving, sonication and microwave on *Chlorella sorokiniana*, and noted greater lipids of 7, 3, 2.7, and 3.1 %, respectively, compared to untreated solvent extracted biomass. This would suggest that steam explosion is more superior compared with the other techniques, which also seems to be the case when compared using *Nannochlorpsis*. However, as seen previously with other techniques, great variation is noted with operational parameters which maybe the case here,

thus a concise conclusion cannot be made due to limited studies reported. In addition to cell disruption techniques, the method of recovery also plays a role in the amount of extract achieved for a particular product of interest (Cha et al., 2009; Koo et al., 2012; Stramarkou et al., 2017).

It was observed by Stramarkou et al. (2017) that extract yields achieved from ultrasonication of *C. vulgaris* biomass were up to 3 times higher using water as a solvent system compared to ethanol. However, total carotenoid and chlorophyll *a* contents were greatest in ethanol solvent system compared to those recovered using water. The greater recovery of extracts using water is attributed to its polar nature and smaller molecule size allowing it to better penetrate the biomass than ethanol (Stramarkou et al., 2017). Despite this, ethanol was found to be more selective in carotenoids and chlorophyll *a* recovery as a result of higher proportion of water-soluble contents in the biomass (Cha et al., 2009; Stramarkou et al., 2017). Zeaxanthin extraction from the algaenan containing species *Chloroidium ellipsoideum* was also performed by Koo et al. (2012) using pressurized liquid extraction (Table 4.2). They compared the use of hexane, isopropanol and ethanol as the pressurized solvents for recovery, and noted that ethanol resulted in greatest yields, 4.2 mg g^{-1}. The carotenoid recovery was also compared using ultrasound-assisted methanol and ethyl acetate solvent system and a seven time increase in pressurized liquid extraction compared to that of ultrasound, was noted. This variation is attributed to the change in liquid viscosity due to the high temperatures used in pressurized liquid extraction, resulting in improved matrix penetration (Itoh et al., 2008).

4.4.4 *Tetraselmis*

The marine microalgae strain *Tetraselmis*, belonging to the class Chlorodendrophyceae, has gained significant research interest due to its high intracellular polysaccharide content (Prabakaran and Ravindran, 2011). The composition of the *Tetraselmis* cell covering is primarily made up of polysaccharides (Table 4.1) with a biochemical composition of 2-Keto sugars and proteins (Becker et al., 1998; Kermanshahi-pour et al., 2014). Cells of this species take on the oval form and range in size from 10-25 x 7-20 μm (Kawaroe et al., 2016).

4.4.4.1 Disruption Techniques

The recovery of gallic acid, α-tocopherol, β-carotene as well as the degree of cell wall disruption has been investigated for *T. suecica* species, using surfactants and sonication techniques, Table 4.2 (Becker et al., 1998; Ulloa et al., 2012). The use of Triton X-114 and Tween 20 were used as a means of cell disruption by Becker et al. (1998) for recovery of these compounds. No variation in the recovery of gallic acid was noted amongst the different surfactant compounds used, however, tocopherol and carotene recoveries were 20 and 5 %, respectively, higher using Triton X-114. This disruption technique was further compared with sonication and it was found that the surfactant use resulted in gallic acid, tocopherol and carotene contents that were 1.6, 3.7 and 32 folds greater than those using sonicated biomass, respectively. Furthermore, in the study by Ulloa et al. (2012) it was found that cell disruption of *T. suecica* resulted in 100 % disruption using low power sonication (500 W and 1 kWh L^{-1}). The composition of the cell wall of this species is similar to that of *Chlorella*, in that it is composed of polysaccharides, which may explain the effectiveness of surfactants in improving product recoveries for these two genera.

Lipid extraction from finely ground *Tetraselmis chui* biomass was performed using conventional solvent extraction (dichloromethane:methanol), SC-CO_2 and SC-CO_2 assisted with the co-solvent system methanol and ethanol by Grierson et al (2012). Lipid extractions (weight percentage) were highest using the solvent system (14.6 %) and lowest using SC-CO_2 (0.01 %), alone. The addition of the co-solvents ethanol and methanol to SC-CO_2 improved the extract to 3.8 and 4.3 %, respectively. Thus, the method of extraction also plays a role in the amount of product recovered.

Another type of cavitation that is less commonly used, hydrodynamic cavitation, was investigated by Lee et al. (2015) and compared to sonicated biomass of *T. suecica*. It was noted that the crude lipid yields achieved using hydrodynamic cavitation appeared to result in greater lipid recoveries, however, no statistical analysis was performed on the data. The average lipid from sonicated biomass reported was 450 mg g^{-1} biomass, varying from 200 to 700 mg g^{-1}. The average yield for hydrodynamic disruption (treatments of 4 to 16 mins) was 500 mg g^{-1} and ranged from a minimum of 300 and a maximum of 720 mg g^{-1}.

4.4.5 *Nannochloropsis*

Nannochloropsis species belonging to the class of Eustigmatophycease (Natarajan et al., 2014) have been researched as candidates for industrial biofuel production (Scholz et al., 2014). The interest in this species arises from its ability to accumulate high lipid amounts and its capability of growth in various environments (Scholz et al., 2014). Additionally, this microorganism has been used for the recovery of other value added products such as pigments (Scholz et al., 2014). *Nannochloropsis* species have an outer algaenan cell wall coating with an inner cellulose layer (Table 4.1) (Lubian et al., 2000). The cell wall mainly consists of carbohydrates that make up 69-79 % of the cell wall (Scholz et al., 2014), 6.2 % protein, 1.1 % mineral, with the remainder as algaenan (Lubian et al., 2000). *Nannochloropsis* cells are spherical and small in size, ranging from 1 to 2 μm, which makes them difficult to disrupt (Grima et al., 2013; Wang et al., 2014).

4.4.5.1 Disruption Techniques

The cell wall composition of the microalgae species can have a significant influence on the effectiveness of sonication as a disruption technique. For example, *Nannochloropsis* cell walls are comprised of algaenan and the effectiveness of sonication in improving product recovery varied from 10.5-38 % (Scholz et al., 2014). Studies performed on *Nannochloropsis* biomass (Lee et al., 2010; Ranjan et al., 2010) reported an improvement in lipid recoveries by factors that range from 3.4 to 7 using sonication treatment, Table 4.2. By contrast, other studies (Converti et al., 2009; Koberg et al., 2011; Mendez et al., 2014; Lorente et al., 2015; Wang et al., 2015) observed very little to no increase in product recovery. It is likely that variation in the effectiveness is due to the different operational parameters used (i.e. temperature, exposure time, power).

Steam explosion has been an effective cell wall disruption technique for a number of species and similarly it has been reported effective on *Nannochloropsis*, however, the operational parameters greatly impacts the degree of product improvement (Table 4.2). For example, in the study by Lorente et al. (2015), they did not notice any significant differences in lipid yields of *Nannochloropsis gaditana* with increasing steam explosion temperatures over the tested range of 120 to 150°C, however an increase in lipid of 8-9 % was observed when compared to untreated biomass. Nurra et al. (2014) found that steam

explosion of *Nannochloropsis gaditana* at 120 and 180°C improved hexane lipid recovery by 3.6 and 8.8 %, respectively. Studies investigating the optimization of steam explosion suggest that an optimal concentration exists for effective cell disruption and this has been attributed to the amount of cell wall area exposed to the steam treatment, whereby, lower concentrations result in more exposed surfaces (Aguirre and Bassi, 2014).

Pretreatment of *Nannochloropsis* biomass using microwave radiation technique, resulted in a 12 fold increase in FAME content (Koberg et al., 2011) and a 4 fold increase in lipid recovery as noted by Bermudez-Menendez et al. (2014), Table 4.2. However, Surendhiran and Vijay (2014) and Lorente et al. (2015) observed a slight increase, and at times, a decrease in lipid recoveries of microwave treated *Nannochloropsis* biomass, compared to those achieved by conventional Bligh and Dyer solvent extraction. The variation in results obtained by researchers can be explained by the differences in power levels, temperatures and treatment time. Higher temperatures and prolonged exposures can cause lipid degradation. For example, in the case of Surendhiran and Vijay (2014), they observed a decrease in lipid content as the exposure time increased from 5 to 15 min. Autoclaving cell wall disruption technique has also been applied on *Nannochloropsis* biomass, where little to no improvement in lipid recoveries was achieved (Surendhiran and Vijay, 2014).

Nannochloropsis biomass was also subjected to HPH treatment by Yap et al. (2016) and they noted this species to be more resistant to mechanical rapture compared to *Chlorella* sp. Furthermore, the recovery of sugars, proteins and lipids using HPH on *Nannochloropsis oculata* biomass was investigated by Shene et al. (2016), where it was determined that the operational conditions of HPH influenced the protein and sugar recoveries, however the recovery of lipids were dependent on the extraction solvent used. Furthermore, they noted that the culture age affected the content of sugar and protein more drastically then it did on the lipids. Lipid recoveries varied with the type of extraction technique employed. The Bligh and Dyer resulted in a 2 fold increase in lipid recovery compared to petroleum ether.

4.4.6 Other Microalgae

Chlorococcum sp., *Neochloris oleoabundans* and *Haematococcus pluvialis* are other green microalgae species where pretreatment techniques have been investigated to improve extraction of intracellular products, Table 4.2. They all belong to the taxonomic class of Chlorophyceae. *Chlorococcum* sp. have an extracellular cell wall covering made up of fibrillar structure and glycoprotein, Table 4.1. The major component that makes up the structure is cellulose (Surendhiran and Vijay, 2014; Lorente et al., 2015), which is also the major component of the cell wall coating for *Neochloris oleoabundans* (Miller, 1978). *Chlorococcum* sp. and *Neochloris oleoabundans* are spherical in shape and are less than 10 and 3-3.5 μm, respectively (Peterfi et al., 1988; Hajar et al., 2017). *Haematococcus pluvialis*, Haematococcaceae, possesses a cell wall made up of a thick layer of sporopollenin in the aplanospore stage (Mendes-Pinto et al., 2001).

The species *Staurastrum*, belonging to the Demidiaceae family and Charophyta phylum, has a porous cell wall structure with spinules extending through the mucilaginous layer (Andersen et al., 1987). Disruption techniques have been applied on *Staurastrum* for improving pigment recovery processes (Herrera-Valencia et al., 2011). Furthermore, a number of diatoms have found their way in the search for a sustainable energy source, pharmaceutical products, food and feed for humans and animals. Diatoms such as *Phaeodactylum tricornutum* and *Chaetoceros ceratosporus* (synonymous with *Chaetoceros gracilis* (Ostenfeld, 1910; Guiry and Guiry, 2018)) of the Bacillariophyta phylum, possess a siliceous-based rigid cell wall (Wang et al., 2015). The red microalga *Porphyridium cruentum* belonging to the class of Porphyridiophyceae possess a polysaccharide cell enclosure, made of glucose, galactose, xylose, glucuronic acid and methyl-glucuronic acid (Heaney-Kieras and Chapman, 1976; Razaghi et al., 2014). This species has been explored for the recovery of B-phycoerythin (Jubeau et al., 2013).

4.4.6.1 Disruption Techniques

Cell wall disruption of *Chlorococcum* sp. using microwave and autoclave treatments resulted in improved lipid recoveries from solvent extraction techniques of close to 12 % (Rakesh et al., 2015). Here, increasing the culture growth time resulted in greater lipid accumulation, discussed previously for *Scenedesmus*. It was also observed by Yap et al.

(2016) that treatment of *Chlorococcum* sp. using HPH resulted in complete disintegration of the biomass when subjected to pressures of 35-40 MPa. Similarly, pre-treatment of *Neochloris* biomass improved solvent extractions significantly. Sonication of the biomass and enzyme treatment (using papain and cellulose) increased lipid recoveries from 0.55 to 4 and 2.25 g L^{-1}, respectively (Wang et al., 2015). The degree of disruption using sonication of *Neochloris* species was shown to be a function of both time and power. Wang et al. (2015) showed that disruption degree increased from 18 to 30 % with sonication treatment time (10-30) using a power of 600 W, however, further treatment up to 50 min only improved disruption to 32 %. The increase in sonication power from 400 to 800 W improved disruption from 17 to 38 % using 30 min treatment. The study further showed that biomass concentration over the tested range of 4 to 20 g/L did not affect the cell disruption degree using HPH, however, the technique was significantly influenced by the change in pressure (40-80 MPa). A change in pressure from 40 MPa to 60 MPa increased the disruption from 41 to 76 %, and further increases to 80 MPa only improved the disintegration by an additional 6.6 %. The authors noted a positive relationship between lipid yields and disintegration. The recovery of total carotenoids using acetone from *Haematococcus pluvialis*, was improved by 3.5 and 3.75 times using autoclaving and mechanical disruption, respectively (Mendes-Pinto et al., 2001). These findings are similar to those reported by other authors using species composed of similar cell wall structures.

The *Staurastrum* species had an increase in pigment recovery by a factor of 6 when sonicated compared to conventional solvent recovery technique. Additionally, sonicated biomass improved the recovery of fatty acids by a factor of 2.4. The effectiveness of various physical cell wall disruption techniques on *Phaeodactylum tricornutum* for the recovery of lipids, for both biofuel and as a source of fatty acids for food and feed was investigated by Andersen et al. (1987) and Lorente et al. (2015). These studies illustrated that autoclaving, sonication, microwave, steam explosion, bead beating and freeze drying resulted in similar lipid recoveries as those achieved using solvent extraction. The study performed by Pernet and Tremblay (2003) using *Chaetoceros ceratosporus* found that triacylglycerols (TAG) recovery improved by 0.16 % using a grinding technique and a further increase of 1.25 % was achieved using the combination of both sonication and

grinding of the biomass. However, these improvements are low and do not justify the added costs of operation.

The recovery of B-Phycoerythrin from *Porphydridium cruentum* using HPH has been performed by Jubeau et al. (2013). It was concluded B-Phycoerythrin content (3.6 mg g^{-1} biomass) was greatest when exposed to pressures of 270 MPa. However, no comparison was made to other extraction techniques for assessment of improvements.

4.5 Effect of Cell Wall Disruption on Product Recovery from Microalgae with Similar Characteristics

In an attempt to gain more insight regarding cell wall structure and the effectiveness of various disruption techniques, the product recovery performance from different studies was compiled and grouped according to similarities in microalgal cell wall structure (Table 4.3), and the energy consumptions were tabulated for these techniques (where reported). The techniques were assessed based on the degree of product improvement and assigned either high or low rating. Disruption techniques that resulted in improvements of 15 % or greater were given a high rating and those with lesser improvements as low. This was determined a suitable improvement value for justifying the added costs of disruption to the downstream processing of microalgae. Given the diversity of cell wall composition and structure of the species within the same taxonomic class, microalgae species were classified according to their cell wall structure/composition to understand the relationship between cell wall and efficacy of a specific cell wall disruption technique, rather than the taxonomic class. For example, several species pertaining to both Chlorophyceae class and Trebouxiphyceae (i.e. *Botruococcus* sp) possess the non-hydrolyzable algaenan structure, cellulose, or a fibrillar cell wall, and algaenan has been widely observed to contribute to recalcitrance of cell wall and act as a barrier to efficient recovery of bioproducts. Therefore to provide insight into the efficacy of disruption techniques for product recovery, the role on cell wall structure, composition and cell size needs to be considered, rather than the taxonomic class.

Enzymatic treatment, as shown in Table 4.3, was deemed effective in the disruption of less rigid microalgae species, those that lack the tri-laminar algaenan structure and are

Table 4.3. Improvement in product recovery for microalgae species according to cell wall structure and composition, and energy consumption noted for the cell disruption technique.

Species	Cell Wall Disruption Technique	Degree of Product Improvement (High/Low)	Improvement in Product Recovery*	E (MJ kg^{-1} dw) (Experimental Conditions)	Scale Tested
Cell Wall Structure: Lacks tri-laminar structure [1-3]/ Extracellular polysaccharides [1-7]					
Chlorella sp.	Autoclaving	Low	6-14 % Lipid [8,9]	1020 (100 mL, 5 g L^{-1}, 5 min, 1.7-1.9 kW[α])[10]	Lab (17-50 L unit)[10]
	Bead Mill	High	33 % Lipid [9] 99 % Disruption [11]	16-504 (100-180 mL, 5-145 g L^{-1}, 0.07-0.248 kWh)[10-12]	Lab/Ind [10-12]
	Enzymatic (Cellulase)	High	24 % Lipid [18]		
	HPH	High	24 % Lipid[13]		
	Microwave	Low -High	-14-47 % Lipid [8,9,14]	420 (100 mL, 5 g L^{-1}, 5 min, 0.7 kW)[10,12]	Lab [10,12]
	Sonication	Low -High	-8.4-62 % Lipid [9,14]		
	Surfactant (CTAB)	Low	5.6-8.7 % Lipid [15]		
		High	57 % FAME[16]		
Chlorella vulgaris	Autoclave	Low	6 % Lipid [10]		
	Bead Mill	Low- High	1-49 % Protein [16]		
		Low	4-7.5 % Lipid [10,17]		
	Enzymatic (Cellulase)	High	21.5 % Lipid [19]		
	Enzymatic (Cellulase)	Low	3.5 % Lipid [20]		
	Enzymatic (Lysozyme)	High	19.5 % Lipid [19]		
	Enzymatic (Snailase)	Low	4.5 % Lipid [19]		
	HPH	High	30 % Protein[21]	1056 (0.5 g, 2.2 kW, 2 passes[^])[21]	Lab[21]
	Microwave	Low- High	-2-20 % Lipid [10,22]	90-540 (1 % solids, 300 W, 5-30 min)[22]	Lab[22]
	Sonication	Low-High	-2-30 % Lipid [22,23]	54 (500 mL, 2.68 g L-1, 600 W, 20 min)[19]	Lab[19]
		Low	3-8 % Protein [16]		
Neochloris oleoabundans	Sonication	High	17 % Lipid[24]	54 (1000 mL, 20 g L^{-1}, 0.6 kW, 30 min)[24]	Lab[24]

	HPH	High	22 % Lipid[24]	48 (1000 mL, 20 g L^{-1}, 4 kW, 4 min)[24]	Lab/Ind[24]
Cell Wall Structure: Non-hydrolyzable algaenan structure [25,26]/ algaenan [27,28], Cellulose [29]					
Scenedesmus sp./ Tetradesmus	Autoclave	Low	3 % Lipid [10]	1020 (100 mL, 5 g L^{-1}, 5 min, 1.7-1.9 kW^{α})[10]	Lab (17-50 L unit)[10]
	Bead Mill	Low	7 % Lipid [10]	504 (100-180 mL, 5-145 g L^{-1}, 0.07-0.248 kWh)[10,12]	Lab/Ind [10,12]
		Low	-23 % FAME [30]		
	Enzymatic (Lysozyme)	Low	12.5 % Lipid[43]		
	Enzymatic (Cellulase)	Low	11.5 % Lipid[43]s		
	Microwave	Low	9 % Lipid [10]	420 (100 mL, 5 g L^{-1}, 5 min, 0.7 kW)[10,]	Lab [10,12]
		High	37 % FAME [30]		
	Sonication	Low	4 % Lipid [10,31]	90 (20 mL, 100 g L^{-1},30min, 0.1 kW)[31]	Lab[31]
		High	30 % FAME [30]	1012 (1 mL, 40 g L^{-1}, 5 min, 0.135 kW)[30]	Lab[30]
		High	95 % Pigment [23]		
		High	66 % Fatty acid [23]		
	Surfactant (3-DAPS)	High	67 % FAME [32]		
	Surfactant (MTAB)	High	49 % FAME [32]		
	Surfactant (Tween-20)	Low	-1 % FAME [32]		
	Surfactant (3-DMAPS)	Low	3 % FAME [32]		
Botryococcus sp	Autoclave	Low	4-14 % Lipid [8,10]	1020 (100 mL, 5 g L^{-1}, 5 min, 1.7-1.9 kW^{α})[10]	Lab (17-50 L unit)[10]
	Bead Mill	High	20 % Lipid [10]	504 (100-180 mL, 5-145 g L^{-1}, 0.07-0.248 kWh)[10,12]	Lab/Ind [10,12]
	Microwave	Low- High	-22-21% Lipid [8,10]	420 (100 mL, 5 g L^{-1}, 5 min, 0.7 kW)[10]	Lab [10,12]
	Sonication	Low	1 % Lipid [10]		
Nannochloropsis	Autoclave	Low	1-2.6 % Lipid [25, 33]		
	Enzymatic (Cellulase)	Low	2.6-6.6 % Lipid [33]		

	Freeze Drying	Low	1 % Lipid [34]	140 (mathematical modeling)[35]	Ind[35]
	HPH	Low	1.5-3.5 % Protein[36]		
		Low	12 % Lipid[36]		
	Microwave	Low	-9.4-7 % Lipid [33,37]	3.2-6 (5 g, 25-35 W, 10-20 min)[37]	Lab[37]
		High	34 % FAME [38]	231 (1 g, 0.77 kW, 5 min)[38]	Lab[38]
	Steam explosion	Low	8.1-8.4 % Lipid [25]		
	Sonication	Low-High	-2-18 % Lipid [33,37,38,39]	6-24 (5 g, 30-100 W, 5-20 min)[34,37]	Lab[34,37]
		High	18 % FAME [38]	180 (1 g, 600 W, 5 min)[38]	Lab[38]
Chlorella sorokiniana	Autoclaving	Low	-12-11 % Lipid [8,14,25]		
	Bead Mill	High	-6.2 % Lipid [14]		
	Microwave	Low	3-6 % Lipid[8,25]		
	Steam Treatment	Low	7 % Lipid[25]		
Cell Wall Structure: Scales [40]/Extracellular polysaccharides [28]					
Tetraselmis suecica	Surfactant (Triton X-114)	High	100 % Gallic acid** 80 % α-Tocopherol** 55 % β-Carotene** [41]		
	Bead Mill	High	27 % Protein[42]	248-300 (200 mL, 120 $g L^{-1}$, 30 min, 3.3-4 kW)[42]	Lab[42]

*Improvement is reported as the gain in product recovery achieved by applying the cell wall disruption technique compared to values reported for the control (non-treated biomass). **Untreated biomass values not reported, values indicated as recovery. [α]Energy usage from Panasonic (2014) and SciCAn (2015)

[1](Coward et al., 2014); [2](Corre et al., 1996); [3](Takeda, 1991); [4](Yamada and Sakaguchi, 1982); [5](Blumreisinger et al., 1983); [6](Rodrigues and Bon, 2011); [7](Cordeiro et al., 2005); [8](Rakesh et al., 2015); [9](Prabakaran and Ravindran, 2011); [10](Lee et al., 2010); [11](Postma et al., 2015); [12](Lee et al., 2012); [13](Spiden et al., 2015); [14](Yu et al., 2015); [15](Coward et al., 2014); [16](Lai et al., 2016); [17](Johansen, 1991); [18](Fu et al., 2010); [19](Zheng et al., 2011); [20](Cho et al., 2013); [21](Safi et al., 2015); [22](Prommuak et al., 2012); [23](Wiltshire et al., 2000); [24](Wang et al., 2015); [25](Lorente et al., 2015); [26](Simpson et al., 2003); [27](Postma et al., 2015); [28](Voigt et al., 2014); [29](Allard and Templier, 2000); [30](Soh and Zimmerman, 2011); [31](Ranjan et al., 2010); [32](Lai et al., 2016); [33](Surendhiran and Vijay, 2014); [34](Balasubramanian et al., 2013); [35](Ratti, 2001); [36](Shene et al., 2016); [37](Bermudez-Menendez et al., 2014); [38](Koberg et al., 2011); [39](Converti et al., 2009); [40](Scholz et al., 2014); [41](Ulloa et al., 2012); [42](Schwenzfeier et al., 2011); [43](Taher et al., 2014)

made up of primarily polysaccharides. Approximately, 19.5-24 % increase in lipid recovery was observed in the case of *Chlorella* sp. and *Chlorella vulgaris* when cellulase and lysozyme were used (Fu et al., 2010; Zheng et al., 2011). Whereas, cellulase and lysozyme treatment of *Scenedesmus* sp. and *Nannochloropsis* species, possessing the algaenan tri-laminar structure were not as effective in improving the lipid yields. These enzymes hydolyze the polysaccharides present in the cell wall, thus effectiveness is dependent on the cell wall composition, Table 4.1 and 4.2. The application of surfactants for microalgae cell wall disruption also resulted in enhancing product recovery for various microalgae species. Those made up of polysaccharides (*Chlorella*), algaenan (*Scenedesmus* sp. and *Tetradesmus*) and scales (*Tetraselmis suecica*) were susceptible to cell wall degradation, however, the effectiveness is dependent on the charge of the cell wall and the surfactant. Surfactant treatment using the cationic (MTAB) resulted in FAME improvement of 49 % in *Scendesmus*, which maybe the most practical option for disruption of these algaenan containing microalgae species. However, the zwitterionic (3-DAPS) surfactant system also resulted in improvements in FAME recovery from *Scenedesmus* sp. species, but the 3-DMAPS (zwitterionic) was not effective using the same species, thus the effectiveness of these compounds on other species of this class cannot be determined, due to little literature available on the subject.

Autoclaving is an energy intensive technique, noted to consume 1020 MJ kg^{-1} (Lee et al., 2010), and was not generally successful in improving product yields in both the case of microalgae species that possess the non-hydrolyzable algaenan and those that lack the trilaminar structure (Lee et al., 2010; Prabakaran and Ravindran, 2011; Rakesh et al., 2015), Table 4.2. In addition, freeze drying of microalgae biomass has been calculated to consume 140 MJ kg^{-1} energy (Ratti, 2001), and as little as 1 % lipid improvement has been noted for *Nannochloropsis* (Balasubramanian et al., 2013), Table 4.3, and *Phaeodactylum tricornutum* (Balasubramanian et al., 2013). These results suggest that microalgae with the siliceous frustule and algaenan cell wall compositions, Table 4.3, are resistant to freeze drying treatment.

Bead milling of *Botryococcus*, on the other hand, improved recoveries of lipids by 20 %, however, 504 MJ kg^{-1} of energy was required which is much higher compared to other

techniques (Lee et al., 2010). However, product improvement using bead milling was less effective in *Scenedesmus* (Lee et al., 2010; Soh and Zimmerman, 2011), which is similar in cell wall structure as *Botryococcus.* As for *Chlorella*, lacking the algaenan trilaminar structure, the product recoveries ranged from low to high (Lee et al., 2010; Lai et al., 2016). Interestingly enough, the siliceous frustules of *Tetraselmis suecica* were disrupted using bead milling and resulted in protein recovery improvements of 27 %. Despite the slight increases achieved using this technique, the energy requirement is much higher than other methods that resulted in greater recoveries, Table 4.3.

The cell wall disruption using HPH, resulted in product improvements in microalgae species lacking the tri-laminar structure. Lipid recoveries were greater compared to untreated biomass by up to 24 % in *Chlorella* sp. and *Neochloris oleoabundans* species (Spiden et al., 2015; Wang et al., 2015), Table 4.3. Additionally, this technique was found to improve protein recovery by 30 % in *Chlorella vulgaris* (Safi et al., 2015). However, the technique proved to be less effective in the treatment of the algaenan possessing species, such as *Nannochloropsis* (Shene et al., 2016). The study of Safi et al. (2015) utilized an energy consumption of 1056 MJ kg^{-1}, but effective improvement of 22 % on *Neochloris oleoabundans* using this technique, was also reported using as little as 48 MJ kg^{-1} by Wang et al. (2015).

Product recoveries were also improved by microwave treatment for both species possessing the non-hydrolyzable algaenan structure and those lacking this structure, nonetheless, the degree of improvement varied widely based on operational parameters used, Table 4.2 and 4.3. Microwave treated *Chlorella* sp. biomass resulted in as much as a 47 % increase in lipid recovery and as high as 34 and 30 % FAME recovery from *Nannochloropsis* and *Tetradesmus*, respectively (Soh and Zimmerman, 2011; Koberg et al., 2011). The energy consumption for microwave treatment ranged from 3.6-540 MJ kg^{-1} microalgae biomass, where greater recoveries were found with increasing energy (Koberg et al., 2011; Prommuak et al., 2012; Bermudez-Menendez et al., 2014).

Sonication and steam explosion treatments were also employed as disruption techniques. Steam explosion treatment on the algaenan cell wall structures of *Nannochloropsis* and *Chlorella sorokiniana* resulted in low product recovery. However,

sonication resulted in greater improvements in product recovery from *Chlorella*, *Neochloris oleoabundans, Scenedesmus* and *Nannochloropsis*, but only to a low degree in *Botryococcus* sp., Table 4.3, which are species that consist of both algaenan and algaenan-lacking cell wall compositions. The energy consumption of sonication treatments ranged from 6-1012 MJ kg^{-1}, based on operational parameters (Soh and Zimmerman, 2011; Balasubramanian et al., 2013; Bermudez-Menendez et al., 2014). In comparison to sonication, microwave treatment resulted in high product improvements for all three types of species, whereas sonication did not greatly improve lipid yields in both *Scenedesmus* sp., *Botryococcus,* respectively. In the study of Bermudez-Menedez et al. (2014) they found that sonication levels of 24 MJ kg^{-1} resulted in a lipid improvement in 5 % using *Nannochlorpsis*, while microwave radiation improved yields to 7 % using 6 MJ kg^{-1}. However, microwave disruption of *Nannochloropsis* was also tested at higher energy amounts (231 MJ kg^{-1}) than sonication (180 MJ kg^{-1}) and resulted in a FAME improvement of 1.8 times that of sonication (Koberg et al., 2011). It was reported by Grima et al. (2013) that sonication is more suited for less resistant cell wall structures, and added that sonication treatment is high in cost and is less practical at industrial level.

In some cases, however, these disruption techniques resulted in decreased lipid yields as shown in Table 4.3 with negative values for improvement in product recovery. This may be attributed to the type of extraction method used to compare the untreated biomass (i.e. Soxhlet vs. conventional solvent extraction). Furthermore, care should be taken to avoid product degradation when employing certain disruption techniques such as electromagnetic radiation (microwave) and the mechanical techniques (sonication and bead milling).

With regards to the energy consumption of the various disruption techniques, autoclaving requires much greater energy, of the order of 1020 MJ kg^{-1}, with product improvements of only 6 %. It should be noted, however, that the calculation of energy is based on the parameters used and the operational power of the unit itself, and that these values do not necessarily imply that they are the lowest attainable values, which is an observation also noted by Lee et al. (2012). This explains the great variation in energy seen for the same technique (i.e. HPH ranged from 48 to 1056 MJ kg^{-1} (Safi et al., 2015; Wang et al., 2015)). Despite this, the data gives an idea of the magnitude of energy

requirement evaluated at similar levels and conditions. Bead milling requires lower energy (16-504 MJ kg^{-1}), than autoclaving, and great disruption efficiencies have been observed using energies of less than 55 MJ kg^{-1}. Assuming the higher disruption efficiencies achieved can result in the lipid improvements of up to 33 % noted in Table 4.3, then this technique maybe suited for species lacking the tri-laminar algaenan structure. Similarly, other techniques such as microwave radiation, sonication, HPH, and surfactant treatment may also be employed for similar reasons. The lower energy consumption range of these techniques ranges from 48-90 MJ kg^{-1}, with lipid improvements ranging from 17-22 % for *Neochloris*, *C. vulgaris* species ranging in size from 2-5 μm (Gour et al., 2016; Hajar et al., 2017). Surfactant treatment does not require high operational expenses but costs associated with purchase are of the order of \$ 0.37 g^{-1} CAD (Sigma-Aldrich), where improvements of up to 57 % FAME have been reported using concentrations of 16 g L^{-1} (Lai et al., 2016).

There are only a few studies where different microalgae species are subjected to the same cell disruption techniques and operating conditions. One example is the study by Rakesh et al. (2015), on the species, *Chlorella*, *Botryococcus* and *Chlorococcum* sp. where they confirmed the structure of the cell wall had a significant impact on the efficiency of the disruption technique used. They also noted that other factors such as the growth conditions, age of the culture and cell size may also impact the effectiveness of the technique. It has been well documented in the literature that the cell shape and size are directly linked with the nutrients supplied for cultivation (Duc et al., 2013; Yap et al., 2016; Hajar et al., 2017; Azaman et al., 2017). However, Yap et al. (2016) subjected *Nannochlorpsis*, *Chlorococcum* sp. and *Chlorella* sp. biomass to both nitrogen replete and deplete conditions, and noted that despite the greater cell size and cell wall thickness achieved in nitrogen deplete conditions for all tested species, their susceptibility to HPH was not affected. It should be noted that HPH is considered an intensive disruption technique, and in the study of Yap et al. (2016) they tested HPH on *Chlorococcum*, which possesses a fibrillary cell wall structure, and it was observed that the cell wall was completely broken using lower operational pressures of 35-40 MPa, while *Chlorella* and *Nannochlorpsis* required 120 MPa for achieving 80 and 62 % cell disruption, respectively. Thus, it could be concluded that the slight increase in cell size and wall thickness due to cultivation changes was not significant enough to affect the degree of disruption. It is

difficult to confirm this, since limited studies are available, comparing the effects of disruption techniques on product recovery from species, grown in various conditions. Moreover, the finding of Yap et al. (2016) indicates that the different degree of disruption seen for *Nannochlorpsis* and *Chlorella* sp. must be attributed to the their algaenan and polysaccharide cell wall structure, respectively, since these two species are round with similar dimensions ranging from 2-5 μm, Table 4.1.

Therefore, we conclude that there is the need for systematic research in which different microalgae species are subjected to the same cell wall disruption technique in order to develop an understanding on the extent of effectiveness of the technique in response to varying cell wall structure. Additionally, the effect of varying operating conditions on cell wall disruption must be investigated to provide insight into a critical comparison of the techniques with respect to their effectiveness, scalability and the extent of energy and material consumption.

4.6 Limitations and Future Challenges

In this review, the composition of the microalgae cell wall (or extracellular covering) was used as a basis for comparing the performance of different cell disruption techniques, Table 4.3. However, there are other factors that are important to consider, such as cell size and the thickness of the cell walls. Both of which would affect the mechanical properties of the cell and the resistance to breakage. The age of the cell and stage of growth may also be important, however there is seldom consistency across studies, as shown by the different stages in growth phase noted in Table 4.2. It would be desirable to choose the cell disruption technique with the highest product yield for microalgae by referring to specific cell wall composition/structure; however, the data presented in Table 4.2 does not support existing a conclusive trend within a broad range of species. Indeed, for the same disruption technique used on a particular microalgae species, there can be a wide range of values for improved product recovery (from low to high), which may be attributed to the different operating conditions used in the studies such that non-optimal results are reported. This is also demonstrated in Table 4.3, through the large range of energy consumption values reported for a single technique, as a result of little to no optimization performed on minimizing the energy consumption. Furthermore, as can be seen in Table 4.3, the

improvement in the recovery varies amongst the different studies, making comparisons even more difficult. These limitations make comparison of the literature challenging and indicates that further research is required into developing standard procedures for determining the efficacy of the disruption techniques, biomass characterization and reporting the details of the operating conditions.

Although product recovery has been used as a measure of how well the disruption technique has performed in Table 4.3, there are limitations in using this as the main parameter for assessment. For products that degrade with high heat or pressure, techniques such as autoclaving may not be suitable. In addition, there are associated advantages and disadvantages that should be considered in the selection of the most appropriate disruption method for product recovery. Effective sonication disruption treatments are energy intensive (Fu et al., 2010; Zheng et al., 2011; Cho et al., 2013; Surendhiran and Vijay, 2014), and possess scale up difficulties (Guldhe et al., 2014; Mendez et al., 2014; Wang et al., 2014). Bead mill treatments are also difficult to scale up, lipids are susceptible to degradation and require further processing to remove undesired products as well as beads (Luque-Garcıa and De Castro, 2003; Lee and Shah, 2012). Microwave radiation is simple, rapid, can be scaled up and the energy requirement can range from 90 to 540 MJ kg^{-1}, however, high temperatures may result in lipid oxidation (Lee et al., 2010; Halim et al., 2012; Kim et al., 2013; Munir et al., 2013; Bermudez Menendez et al., 2014; Günerken et al., 2015). Surfactant treatment of microalgae reduces operational costs and capital costs, is suitable for large scale operation and surfactants such as CTAB have been used in the food industry. Nonetheless, high concentrations of surfactants can be harmful to aquatic life, and the method can require long durations of time. However, surfactants can also be employed as an agent for recovery of microalgae cells from dilute liquid suspensions (Lee et al., 2010; Halim et al., 2012; Bermudez Menendez et al., 2014) in dispersed air flotation (Coward et al., 2014), which would work as a form of water treatment to remove both the cells and surfactant from the liquid suspension. This is an example of potentially improving the economics of downstream microalgae processing, by combining two or more processes (i.e. dewatering and extraction pre-treatment), as discussed by Kim et al. (2016), is an approach that should be further pursued in future research to make microalgae processing more attractive.

4.7 CONCLUSIONS

There are numerous microalgae cell wall disruption techniques that can be used for improving product recovery. However, as these methods add to the costs of the overall process, there must be an increase in product recovery to compensate for the added costs. The effectiveness of the technique is dependent on the composition and structure of the cell wall, which varies from different taxonomy classes and with different families within the same class. In this study similar product recoveries were obtained for species from different classes with similar extra cellular wall compositions (i.e. *Scenedesmus* sp., *Botryococcus* sp. and *Nannochloropsis*), depending on the disruption technique used. In general, it is difficult to compare the results from different studies and it is recommended that research into optimizing disruption conditions be conducted so that better comparisons can be made. Additionally, there is knowledge gap on how cell wall thickness and composition vary depending on the microalgal growth condition and stage of growth. Therefore, future research should be directed toward building a strong database that include the growth phase and condition of microalgae species, their corresponding cell wall composition, thickness and structure. Visualization techniques such as transmission electron microscopy (TEM) should be employed to provide insight into the structure of the cell wall of various microalgae species at different growth stages and the impact of various cell wall disruption techniques on the cell wall structure. This database would facilitate the selection of the optimum cell wall disruption technique for product recovery from specific microalgae species and also enable the design of innovative cell wall disruption techniques for an enhanced product recovery.

FOREWORD TO CHAPTER 5

From the information presented in Chapter 4, it is clear that it is difficult to determine the best cell disruption techniques to use for microalgae with particular cell wall structures, due to the wide range of parameters used in the literature. The complexity is further exacerbated by the variation in cell size and shape that exists within a particular microalgae species during different cultivation conditions. One pretreatment technique that has been used for various microalgae species is the application of surfactant. Although there are limited studies available, it appears that the surfactant may act as a cell disruption agent for species lacking the algaenan cell wall structure, such as *Chlorella saccharophila*. This is interesting since the use of surfactants is required when harvesting microalgae by dispersed air flotation, which is a harvesting method that was judged to be suitable for large scale processes in Chapter 3. Thus, there is potential for surfactants to work as a pre-extraction technique to aid product recovery, in addition to its role in harvesting by dispersed air flotation. The use of the same technique to serve both pretreatment and harvesting processes has great potential for improving the economics of microalgae-derived biodiesel. In order to test the effectiveness of surfactant aided dispersed air flotation as both a pretreatment and harvesting process, product extraction must be performed. Therefore, in Chapter 5, a comparative analysis is performed to determine a suitable microalgae lipid extraction technique for large scale processes, and this will be used for the experimental studies described later in the thesis. Evaluation of the extraction method is based on criteria that include performance, environmental impact, suitability for scale up, time and cost.

CHAPTER 5 COMPARATIVE ANALYSIS OF MICROALGAE OIL EXTRACTION TECHNIQUES

Parts of this chapter have been published in the Journal of *Fundamentals of Renewable Energy and Applications* and are being reproduced here with permission from the publisher (Appendix A). The following is the full citation of the article used.

Alhattab, M., & Ghaly, A. (2015). Microalgae oil extraction pre-treatment methods: critical review and comparative analysis. *J Fundam Renewable Energy Appl*, *5*, 172.

5.1 ABSTRACT

The commercialization of microalgae derived products, is hindered by the high downstream processing costs associated. One of these steps includes the product recovery, which if improved can have a positive influence on the process economics. Thus, this study was carried out to evaluate the various microalgae oil extraction techniques to determine an economically efficient extraction method for large scale processing of the microalgae biomass for the production of biodiesel. The oil extraction methods investigated included solvent, Soxhlet, accelerated solvent extraction, supercritical carbon dioxide (SC-CO_2), and expeller/press. The effectiveness of these methods was evaluated based on the following criteria: oil extraction efficiency, cost, toxicity, suitability for large scale, time, pre-treatment requirement, reusability, and maintenance. The results indicated that of the techniques reviewed, SC-CO_2 (78/100) and the expeller/press (77/100) scored the highest values based on the selected criteria, but failed to score a value of 80/100 or greater. A pre-extraction treatment could improve these values by lowering the operational time and improving product yields. However, of the two techniques, SC-CO_2 extraction has additional advantages in that no further processing is required to separate the extract from the solvent or biomass debris.

5.2 INTRODUCTION

The aim of this study was to review the current methods used for microalgae oil extraction and perform a comparative analysis to determine the most economically efficient extraction method for large scale processing of microalgae biomass for the production of

biodiesel as an alternative to fossil fuel. The oil extraction methods included solvent, Soxhlet, accelerated solvent extraction, supercritical carbon dioxide (SC-CO_2) and expeller/press extractions. These methods can be used alone or in combination with pre-treatment methods such as microwave radiation, bead mill, ultrasound, freeze drying, autoclave and enzymatic techniques. The effectiveness of the extraction methods were evaluated based on the following criteria: (a) oil extraction efficiency, (b) cost, (c) toxicity, (d) suitability for large scale use, (e) time, (f) pre-treatment requirement, (g) reusability, and (h) maintenance.

5.3 METHODOLOGY

5.3.1 Selection of Criteria

Eight criteria (Table 5.1) were used for the evaluation of microalgae oil extraction techniques: (a) oil extraction efficiency, (b) cost, (c) toxicity (d) suitability for large scale use, (e) time, (f) pre-treatment requirement, (g) reusability and (h) maintenance. These criteria were selected based on the information reported in the literature about these microalgae extraction methods. The comparative analysis was performed using these criteria to determine the most efficient, cost effective and environmentally friendly oil extraction technique(s) for a wide array of microalgae species and also suitable for large scale application.

5.3.2 Assigning a Score to Each Criterion

Each of the selected criteria was assigned a score from 7 to 15 which was determined by the degree of importance of the criterion (Table 5.1). Higher values were given to the criteria that were deemed most important for development of an efficient and economic large scale oil extraction technique for microalgae biomass. Lower values were given to criteria that were deemed necessary for determining a suitable method but were considered less important. These values were then used to determine the effectiveness of each of the investigated pre-treatment method on microalgae biomass.

Table 5.1. Criteria used for the comparative analysis of different oil extraction techniques.

Criteria	Importance	Description
Oil Extraction Efficiency	15	The system should be able to effectively recover high percentages of lipids from the cells
Cost	15	The operational costs of the process should be low in order to reduce the total processing costs associated with microalgae oil recovery
Toxicity and health and environmental impact	15	The method should be non-toxic so that the retrieved algae biomass maybe processed for a number of value added products including ones for human consumption It should also be environmentally friendly in order to reduce the amount of toxic wastes produced
Suitability for Large Scale Use	15	The method should be effective in handling large volumes for industrial production
Time	15	The rate of oil recovery should be quick to ensure the sustainability purposes
Initial Pre-treatment requirement	10	The pre-treatment method should reduce the costs associated with the oil extraction process while minimizing the cost of pre-treatment methods in order to reduce the overall oil extraction costs associated with the process
Reusability	8	The oil extraction method should be reusable in order to reduce equipment costs
Maintenance	7	Costs for maintaining the method should be low

5.4 RESULTS AND DISCUSSION

An assessment based on the eight criteria were evaluated for each method and presented in Tables 5.2-5.6. A description of the resulting score for each criteria is also given in the tables. The sum of the scores obtained for each oil extraction technique are presented in Table 5.7.

The results indicate that of the five chemical and mechanical oil extraction methods evaluated in this study, none scored a value of 80/100 or greater, deeming them unsuitable for large scale microalgae oil extraction. However, one chemical and one mechanical method scored a value greater than 75/100 making them somewhat suitable for microalgae oil extraction. These techniques were SC-CO_2 and expeller press. SC-CO_2 extraction is relatively suitable for oil extraction from microalgae because of its high recovery efficiency, nontoxicity, suitability for large scale use, rapidness, reusability and relatively low maintenance requirements. The mechanical oil extraction method expeller press is relatively cost effective, nontoxic, suitable for large scale use and reusable, however, it suffers from low recovery efficiencies, prolonged periods of time and high maintenance requirements. The low recoveries from this technique indicate that an initial pre-treatment step may be required. SC-CO_2 extraction has additional advantages over other extraction methods in that no further processing is required to separate the extract from the solvent or biomass debris.

Although SC-CO_2 extraction stands out in comparison with other techniques based on the evaluation criteria selected here, assessment using other criteria may not necessarily result in similar findings. This study is limited in that it focuses on criteria that prioritize environmental aspects and reduces downstream processes as means for improving the economics of microalgae biotechnology. However, assessment with an emphasis on energy consumption and net energy gain from the recovered product, may result in different values that may not support the use of SC-CO_2 extraction technology. Therefore, life cycle assessments need to be performed to validate the feasibility of using this technique by determining the costs of operation, and comparing them to the net energy value after extraction. Nevertheless, the results found here highlight the features of SC-CO_2 as an effective, scalable technique that offers reduced processing after extraction, as

Table 5.2. Evaluation of solvent extraction.

Criteria	Description	Score
Oil Extraction Efficiency (15)	High oil extraction recoveries can be achieved, but the method is limited to the solvent and lipid contact as well as the diffusion ability of the solvent	12
Cost (15)	Costs are associated with purchasing of the chemicals and the recovery/purification of the solvent/lipid mixture	10
Toxicity and health and environmental impact (15)	Chemicals can be toxic and residues may remain in the biomass, making it unsuitable for processing into certain value added products	5
Suitability for Large Scale Use (15)	Method can be easily scaled up for industrial scale, however handling of large quantities of hazardous chemicals can be difficult	13
Time (15)	Extraction process is rapid	15
Initial Pre-treatment requirement (10)	Pre-treatment of microalgae biomass is not necessary, however the efficiency of the oil recovery can be improved pre-treatment methods that enhance the solvent to lipid contact	4
Reusability (8)	Distillation of solvent/lipid mixture allows for reuse, however the efficiency of reuse depends on the purity of the solvent	6
Maintenance (7)	Maintenance is required to ensure handling of the chemicals is safe and no leakage into the environment	5
Total (100)		**70**

Table 5.3. Evaluation of Soxhlet solvent extraction.

Criteria	Description	Score
Oil Extraction Efficiency (15)	Continued recycling of the solvent ensures high extraction efficiency Lipid decomposition can occur as a result of heating	12
Cost (15)	High costs as a result of prolonged heating, costs associated with purchasing of chemicals, but less quantities are required compared to solvent extractions processes	8
Toxicity and health and environmental impact (15)	Toxic chemicals are used and residues can persist in biomass which can enter into the environment	7
Suitability for Large Scale Use (15)	Difficulty with up-scaling of the apparatus	3
Time (15)	Prolonged durations of time required	3
Initial Pre-treatment requirement (10)	Pre-treatment not required	6
Reusability (8)	Distillation of solvent/lipid mixture allows for reuse, however the efficiency of reuse depends on the purity of the solvent	7
Maintenance (7)	Maintenance required for glassware cleaning	6
Total (100)		**53**

Table 5.4. Evaluation of accelerated solvent extraction.

Criteria	Description	Score
Oil Extraction Efficiency (15)	High oil extraction efficiencies can be achieved	14
Cost (15)	Costs are associated with elevated temperature and pressure operation as well as costs associated with solvent purchase	5
Toxicity and health and environmental impact (15)	No solvent residues are left in the extract	15
Suitability for Large Scale Use (15)	The intensive energy requirement make it unsuitable for large scale use	3
Time (15)	Rapid extraction method	15
Initial Pre-treatment requirement (10)	Biomass needs to be dried before use, thus adding heating costs to the overall process Solar heating can overcome these costs	6
Reusability (8)	Reuse can be achieved by altering the temperature and pressure	8
Maintenance (7)	Minimal maintenance associated with pump and gas line flushing	6
Total (100)		**64**

Table 5.5. Evaluation of supercritical carbon dioxide extraction.

Criteria	Description	Score
Oil Extraction Efficiency (15)	High oil extractions can be achieved that are solvent free	13
Cost (15)	Costs are associated with temperature and pressure operation and high start-up costs	5
Toxicity and health and environmental impact (15)	No solvent residues are left in the extract	15
Suitability for Large Scale Use (15)	Moderately suitable for industrial scale	12
Time (15)	Relatively rapid extraction time	12
Initial Pre-treatment requirement (10)	Sensitivity to moisture content, thus biomass needs to be dried Drying costs can be minimized with solar drying	7
Reusability (8)	Method can be reused	8
Maintenance (7)	Minimal maintenance associated with pump and gas line flushing	6
Total (100)		**78**

Table 5.6. Evaluation of expeller/press.

Criteria	Description	Score
Oil Extraction Efficiency (15)	Oil recoveries are relatively sufficient	10
Cost (15)	High costs associated with high energy consumption	10
Toxicity and health and environmental impact (15)	No solvent requirement	15
Suitability for Large Scale Use (15)	Technique can be easily scaled up for industrial use	15
Time (15)	Prolonged periods of time required for extraction	10
Initial Pre-treatment requirement (10)	Pre-treatment of microalgae biomass not required	6
Reusability (8)	Technique can be reused	8
Maintenance (7)	High maintenance costs associated	3
Total (100)		**77**

Table 5.7. Comparative analysis of oil extraction methods.

Criteria	Chemical				Mechanical
	SE	**SSE**	**ASE**	**SC-CO_2**	**EP**
Oil Extraction Efficiency (15)	12	12	14	14	10
Cost (15)	10	8	5	5	10
Toxicity and health and environmental impact (15)	5	7	15	15	15
Suitability for Large Scale Use (15)	13	3	3	12	15
Time (15)	15	3	15	12	10
Initial Pre-treatment requirement (10)	4	6	6	6	6
Reusability (8)	6	6	8	8	8
Maintenance (7)	5	7	6	6	3
Total (100)	**70**	**53**	**64**	**78**	**77**

SE = Solvent Extraction
SSE = Soxhlet Solvent Extraction
ASE = Accelerated Solvent Extraction
SC-CO_2 = Supercritical CO_2
EP = Expeller/Press

the extract recovered and the de-oiled biomass remain solvent free and offer an alternate stream for revenue through protein extraction and other value added product recoveries, as discussed by Taher et al. (2014).

Despite the low scores attained by the oil extraction methods evaluated here, SC-CO_2 extraction scored 78/100 (Table 5.7), which is close to the score of 80/100 used in Chapter 3 to select suitable harvesting methods. The low scores are the result of the long extraction time required by some methods, which is coupled with greater energy consumption and diffusion gradient for the recovery of microalgae lipids. However, such limitations may be overcome by the implementation of a pre-extraction treatment for the biomass, as outlined in Chapter 4. Cell wall destruction using a pre-extraction treatment would likely assist in the release of intracellular lipids, increasing the solvent to lipid exposure in chemical extraction processes, which would lower the treatment time and the associated costs. However, consideration must also be given to the energy, maintenance requirements and costs of the additional pre-extraction step.

5.5 CONCLUSIONS

The use of microalgae biomass for the production of biodiesel and other value added products is limited by the high processing costs. A comparative analysis of current oil extraction methods for microalgae was conducted to determine the most economically efficient extraction method for large scale processing of microalgae-derived biodiesel. Supercritical carbon dioxide extraction was the top scoring technique (78/100), although the scores were relatively low overall. A pre-extraction step may further improve these extraction processes by lowering the operational time necessary for these techniques and improving the product recovery, although consideration should be given to any additional costs that would be incurred.

FOREWORD TO CHAPTER 6

Based on the findings in Chapters 3-5, the following technologies were chosen for experimental investigation in the remaining chapters of this thesis: surfactant-aided dispersed air flotation (as both a harvesting process and cell disruption pretreatment prior to oil extraction) and supercritical CO_2 extraction. To date, no studies have evaluated the impact of surfactant cell wall disruption of microalgae on supercritical CO_2 extraction. The first part of this experimental work is presented in Chapter 6, which focuses on the use of surfactant aided dispersed air flotation as a harvesting method for recovering microalgae. Optimum operating parameters for simultaneously maximizing both the recovery and enrichment ratio are determined from response surface methodology. These optimized parameters are then used in Chapters 7 and 8 for harvesting biomass prior to SC-CO_2 lipid extraction experiments.

CHAPTER 6 OPTIMIZATION OF MICROALGAE DEWATERING BY SURFACTANT-AIDED DISPERSED AIR FLOTATION

6.1 ABSTRACT

Surfactant-aided dispersed air flotation was investigated for dewatering microalgae cultures of *Chlorella saccharophila*. Response Surface Methodology was used to optimize recovery and enrichment ratio, and it was determined that only CTAB (cetyl trimethylammonium bromide) concentration and flow rate were significant for the recovery, while all the tested variables were significant for the enrichment ratio. When both responses were optimized simultaneously, a recovery of 91.1 % ± 1.62 % and enrichment ratio of 17.9 ± 6.19 was obtained for a CTAB concentration of 100 mg/L, column height of 795 mm, pH of 10 and flow rate of 57.9 mL/min.

6.2 INTRODUCTION

Diminishing fossil fuel supplies and associated environmental green-house effects (Lee et al. 2010), have led to increased interest in alternative environmentally friendly fuel sources. Biodiesel is biodegradable, renewable, and has been associated with lower carbon monoxide, and carbon dioxide emissions (Lee et al., 2010; Valente et al., 2010). It is regarded as the most promising sustainable alternative to current fuels (Cao et al., 2013) and can be produced from a number of feedstocks (Demirbas and Demirbas, 2011). However, many of these are terrestrial crops that are a source of food for human consumption, making microalgae biomass an ideal candidate since it is not considered a food source (Rodolfi et al., 2010; Demirbas, 2011). Furthermore, microalgae feed can generate oil yields that are 7-23 times greater than those generated from terrestrial plants per unit area (Chisti, 2007; Demirbas, 2011).

The use of microalgae as a sustainable feedstock for biodiesel production has been limited by high processing costs (Cao et al., 2013). Currently, microalgae-derived value-added products, have found their way into commercial development and deployment, and these include pigments and essential oils that sell for US $300-$7000/kg (Markou and Nerantzis, 2013) and US $80-160/kg (Borowitzka, 2013), respectively. Biodiesel, on the other hand, is a comparatively low-value commodity that is required in high volumes with

low cost, in order to compete with current diesel prices of US $0.78/L in Canada (Natural Resourves Canada, 2017). Microalgae biodiesel processing costs have been estimated to range from US $1.15 to 4.63/L (Davis et al., 2011; Sun et al., 2011; Richardson et al., 2012; Borowitzka, 2013; de Souza Silva et al., 2014) and require significant reduction in order to be an economical replacement for current unsustainable and environmentally hazardous fuels. The high variation in the reported processing costs can be attributed to the various types of cultivation, recovery and extraction equipment used (de Souza Silva, 2014). One of the major challenges in using microalgae as a feedstock is that the microalgae cultures exist in dilute aqueous suspensions with concentrations that range from 0.1 to 5 g/L (Wang et al., 2008; Halim et al., 2012a). However, oil extraction processes are most efficient using concentrated biomass (300-400 g/L), and current dewatering processes can account for more than 30 % of the total expenditures associated with microalgae production, i.e. cultivation, harvesting, lipid extraction and conversion (Brenter et al. 2011; de Boar et al., 2012). Therefore, in order to improve the economics of biodiesel production from microalgae, the dewatering process needs to be made more efficient and economical in terms of operational costs.

Various solid-liquid separation methods can be employed to dewater/concentrate the microalgae culture to concentrations of 10-450 g biomass/L. Suitable techniques include: sedimentation, vacuum filtration, cross flow filtration, pressure filtration, centrifugation, dissolved air flotation, dispersed air flotation, micro-bubble generation, organic and inorganic flocculation, bio-flocculation, auto-flocculation, electrolytic coagulation, electrolytic flocculation and electrolytic flotation; these have been the subject of a previous review (Alhattab et al., 2015). To summarize, sedimentation is effective and less costly for specific strains of microalgae species, but settling time is long and the concentration of biomass is low (Uduman et al., 2010; Alhattab et al., 2015). Filtration techniques are efficient and do not alter the cell form, but drawbacks include membrane clogging and high costs associated with membrane replacement (Tarleton and Wakeman, 2006; Alhattab et al., 2015). Centrifugation results in highly concentrated biomass, but suffers from high operational costs (Milledge and Heaven, 2013; Alhattab et al., 2015). The use of flocculants provides high settling rates and are suitable for a wide range of species, but chemicals can be harmful to the environment (Vandamme et al., 2013). Autoflocculation techniques are

unsuitable for large scale, are species specific, require extended periods of time, use toxic chemicals and recycling poses additional costs (González-Fernández and Ballesteros, 2013). Bio-flocculants are biodegradable, non-toxic and their use depends on the desirable end product, since the microorganisms used for flocculation will be harvested with the cells. Additionally, this treatment is limited by its selectivity and suffers from high costs (Shen et al., 2009). Electrophoresis techniques are versatile, energy efficient, environmentally compatible and cost effective, but they suffer from fouling and alteration in cell composition (Mollah et al., 2004).

Flotation techniques have also been employed for dewatering microalgae. There are two main types of flotation systems: dissolved and dispersed air flotation. Dissolved air flotation is an energy intensive (7.6 kWh/m^3) process, due to the pressurization of microbubbles required for transporting suspended particles to the surface of the reactor; and requires additional costs for flocculants that further improve recoveries (Liu et al., 1999; Wiley et al., 2009; Uduman et al., 2010). On the other hand, a similar technique, with significantly lower energy requirements (0.003-0.015 kWh/m^3), is one that uses dispersed air flotation coupled with a foam fractionation column (Wiley et al., 2009; Coward et al., 2013). It works by applying gas to the liquid cell suspension (with added surfactants) to produce foam, which aids in cell recovery from the column (Wiley et al., 2009; Wiley et al., 2011; Coward et al., 2013). The use of surfactants has been shown to improve the recovery of microalgae cells up to 90% (Chen et al., 1998; Phoochinda et al., 2005) by forming electrostatic links between the microalgae cell surface and the gas bubble (Henderson et al., 2008). Thus, surfactant-aided dispersed air flotation is a promising low-energy technique that could be used to improve the economics of microalgae-derived biodiesel production.

Previous studies have been conducted to determine the effects of various variables on the recovery of various species of microalgae cells by dispersed air flotation such as *Tetraselmis* sp. (Garg et al., 2015), *Chlorella* sp. (Coward et al., 2013), *Chasetoceros* spp. (Csordas and Wang, 2004), *Chlorella* sp. (Liu et al., 1999; Phoochinda et al. 2005) and *Scenedesmus quadricauda* (Chen et al., 1998). However, there are a number of research gaps that exist and require further investigation in order to develop dispersed air flotation aided by foam fractionation as a harvesting technique for microalgae on a large scale. To

begin, much of literature only reports on the cell recovery percentage and fails to mention the enrichment ratio or concentration factor, which is an important indicator of the effectiveness of this method for dewatering microalgae suspension. The few reports that have assessed both recovery and enrichment ratio show that different operating parameters are needed to achieve maximum enrichment ratio and recovery. The limited reports and challenges in optimization demonstrate a gap in understanding how to maximize the performance of the overall process.

Additionally, there remains a gap in understanding the mechanism of the overall flotation process as a result of limited data on microalgae cell characterization. Different species of microalgae vary in their cell surface structure and composition; without characterization, it is difficult to determine the relationship that the tested parameters have on the performance indicators (recovery and enrichment ratio) of different species, as well as in understanding the mechanism of the overall process. The harvesting efficiencies are highly dependent on the physiochemical extra cellular properties of microalgae cells (Henderson, Parsons, & Jefferson, 2008; Ozkan & Berberoglu, 2013). Determining the zeta potential (surface charge) would be a good indicator for identifying the effect of parameters on foam separation for different microalgae species, however, in many studies this important characterization parameter is not mentioned. Furthermore, the pH is an important factor of cell surface charge, since it affects the protonation or deprotonation of surface compounds, which impacts the bonds formed between different collector agents and this value has often not been mentioned in studies.

Moreover, there is only limited information on the impact of surfactants on the downstream processing of the recovered microalgae biomass for lipid extraction prior to biodiesel production. The only studies that touched on this subject matter, were those performed by Coward et al. (2014) and Kurniawati et al. (2014), both of which noted increased lipid recoveries, and higher polysaccharide and protein recoveries, respectively. The possibility of using the remaining biomass for the recovery of alternate value added products, such as glucose or carotenoids has not yet been explored. Identifying the influence that this surfactant aided recovery process has on microalgae product quality, is key in improving the economics of microalgae processing and determining the suitability of this dewatering technique for large scale production.

The use of dispersed air flotation surfactant aided technology for microalgae is at its infancy. More studies need to be performed in order to better understand the relationship that varying parameters have on the recovery, concentration factor, and impact of the surfactants on downstream processing.

In the present study, we investigated surfactant-aided dispersed air flotation as a dewatering method for *Chlorella saccharophila* cells. Response Surface Methodology was used to predict the optimal conditions (surfactant concentration, column height, pH and air flow rate) needed to achieve high levels of both cell recovery and enrichment ratio, and the model was experimentally verified.

6.3 Materials and Methods

6.3.1 Cultivation of Microalgae

Chlorella saccharophila obtained from the American Type Culture Collection (ATCC® 30408TM), Manassas, VA, USA, was revived using 5 mL of Bristols solution (Atlas, 2010). Revived cells were transferred to a proteose peptone agar plate that was made up of 1.0 g of proteose peptone (Difco 0120) and 15 g of agar to 1 L of Bristols solution. The agar based culture was grown for 3 d at room temperature, following a photoperiod of 14 h light and 10 h dark prior to use. The culture was maintained by transfer to a fresh proteose peptone agar plate every 14 d. For experimentation the cells were scrapped off the agar base using an inoculating loop and suspended into 25 mL of Bristols liquid media, which was left to grow for 14 d. Next, the 25 mL liquid culture was transferred to 500 mL of Bristols solution and allowed to grow for another 14 d period. The optical cell density of the resultant culture was measured to ensure a standard cell density of 1 x 10^6 cells/mL (22.4 mg/L), and if necessary adjusted using distilled water prior to use. Only cultures that could be diluted down to the standard cell density were used. Once ready, 500 mL of the standard culture was used to inoculate 9.5 L of a modified Fitzgerlad medium, previously described by Al-Hattab and Ghaly (2014). Briefly, this consisted of the addition of 1 mL of stock solutions A, B, C and D (Table 6.1), supplemented with 85.7 mg of NH_4NO_3, 194.3 mg of $(NH_4)_2HPO_4$, 94.3 mg of NH_4SO_4 and 1.3 g of $NaHCO_3$ per litre of distilled water. All chemicals were purchased from

Sigma-Aldrich (Oakville, Ontario, Canada and Thermo Fisher Scientific, Ontario, Canada). Cultures were grown for a period of 10 d in an open pond system, previously described by Al-Hattab and Ghaly (2014), with a capacity of 18 units for culture growth. A photoperiod of 14 h light (430 hectolux) and 10 h dark was used. At the end of the 10 d growth period, cultures were used for foam flotation experiments, where enough biomass was generated for 2 experimental runs (each consisting of 3.5 L). Consecutive cultures were cultivated accordingly in order to supply biomass for conducting 2 foam flotation experiments per day. All experiments were conducted within 2 months. The optical cell density was measured at the end of the 10 d experimental runs and reached an average of $3.12 \times 10^6 \pm 8.07 \times 10^5$ cells/mL (70.0 ± 17.5 mg/L).

6.3.2 Determination of Cell Concentration

Chlorella saccharophila cell concentration was correlated with optical density for rapid quantification of microalgae cell concentration. This was done by taking 10 μL aliquots from the open pond microalgae culture suspension throughout the growth period, recording the cell counts using a hemocytometer (1461EMD, Fisher Scientific, Ontario, Canada) and correlating it with the optical density (Genesys 10S UV-Vis, Thermo Fisher Scientific, Ontario, Canada) measured at 484 nm, every other day. The relationship is depicted in Figure 6.1. This model was used to determine the cell concentration before and after the dispersed air flotation experiments in order to quantify the recovery percentage and enrichment ratio.

6.3.3 Determination of Total Suspended Solids

The percentage of total suspended solids (TSS) was determined according to the Standard Methods for the Examination of Water and Wastewater described by Eaton et al. (1995). Briefly, a known volume of microalgae liquid suspension or slurry was placed into an aluminum crucible of known weight. The weight of the sample and crucible were noted and then placed into a gravity oven (Gravity convection, Fisher Scientific, Thermo Fisher Scientific, Ontario, Canada) operating at 105°C. The sample was left to dry until a constant weight was achieved. The percentage TSS was calculated as follows:

$$TSS = \frac{Weight\ of\ Dry\ Sample}{Weight\ of\ Wet\ Sample}\ x\ 100$$

Table 6.1. Formulation of stock solutions for *Chlorella Saccharophila* production medium (modified Fitzgerlad medium).

Stock Solutions (per 200 mL)	**Composition**
A	24.648 g $MgSO_4 \cdot 7H_2O$
B	1.36 g KH_2PO_4
	8.70 g K_2HPO_4
C	1.392 g $FeSO_4 \cdot 7H_2O$
	1.864 g EDTA tri Na
D	0.620 g H_3BO_3
	0.340 g $MnSO_4 \cdot H_2O$
	0.057 g $ZnSO_4 \cdot 7H_2O$
	0.018 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
	0.027 g $CoCl_2 \cdot 6H_2O$
	0.024 g KBr
	0.017 g KI
	0.023 g $CdCl_2 \cdot 5/2H_2O$
	0.091 g $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$
	0.00004 g $CuSO_4 \cdot 5H_2O$
	0.56 mL 97% H_2SO_4

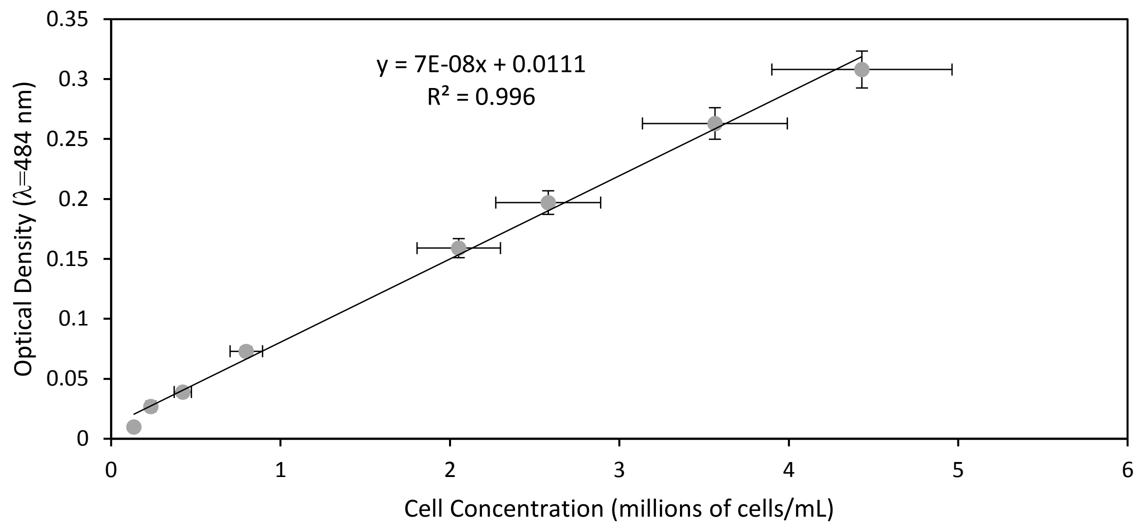


Figure 6.1. Correlation between optical density (λ=484 nm) and cell concentration.

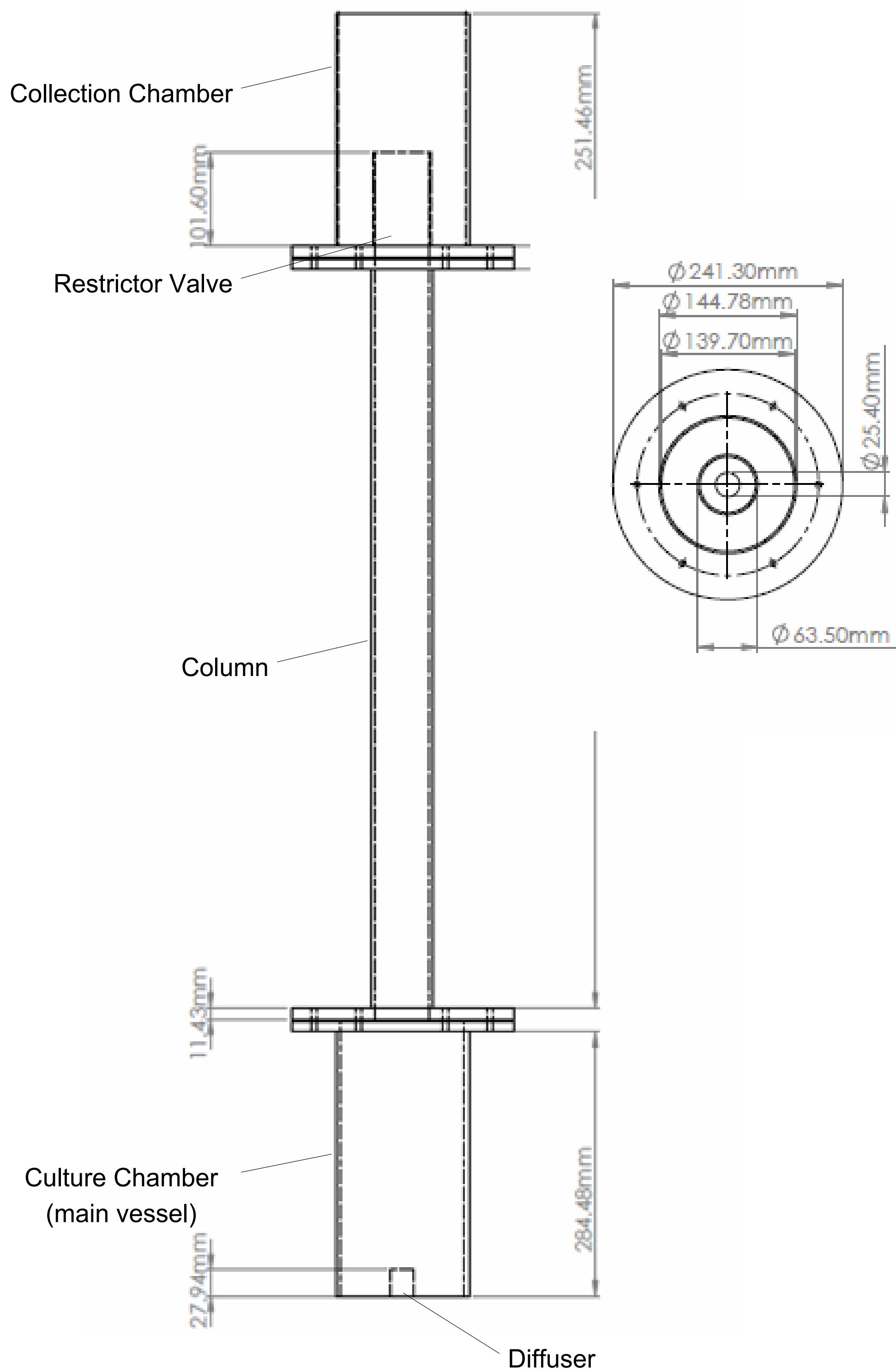


Figure 6.2. Dispersed air flotation harvesting unit with dimensions and cross sectional view.

6.3.4 Dispersed Air Flotation Unit and Components

The recovery of microalgae biomass was performed using a purpose-built dispersed air flotation unit (Figure 6.2). The system consisted of four main components: air supply, diffuser, main vessel (culture and foam collection chambers), and adjustable columns.

6.3.4.1 Air Supply

The central air supply to the laboratory was connected to a mass flow controller (SEC-E50, Horiba Stec, Hudson, New Hampshire) which was powered and operated by a CAPT 4000- 4 channel power supply (Canada Analytical Process and Technologies, Ottawa, Ontario). The outlet end of the flow meter was connected to a 1/8" PVC elbow fitting that was attached to the bottom of the vessel and connected to the diffuser.

6.3.4.2 Diffuser

A cylindrical Fisherbrand Gas Diffusing Stone (ME46944C, Fisher Scientific, Ottawa, Ontario), made of porous fused crystalline alumina grains, was anchored to the interior of the 1/8" PVC elbow fitting, and was used to provide the microbubbles for flotation. The diffuser had a diameter of 22.2 mm, with an average pore size of 60 μm, and particle retention size of 25 μm. The stone was attached to the PVC elbow fitting with a cadmium-plated nipple that was 4.8 mm in diameter.

6.3.4.3 Main Vessel

For each flotation experiment, the cell suspension was loaded into a cylindrical vessel (culture chamber), which was made out of Plexiglas with an inner diameter of 145 mm, outer diameter of 153 mm, and a height of 250 mm. The diffuser was located at the center of the bottom of the vessel and the top of the vessel was attached to a column, which allowed foam to travel upwards to the collection chamber. The collection chamber was similar in dimensions to the main vessel, however, it also had a hollow restrictor (63.5 mm in diameter and 102 mm in height) at its center.

6.3.4.4 Columns

Three columns were used for experiments; these were 312, 554 and 795 mm in height. The outer diameter and inner diameter of the columns were 38 mm and 25.5 mm,

respectively, and the wall thickness of the columns was 6.35 mm. The columns were made from acrylic (8486K531, McMaster-Carr, Atlanta, Georgia).

6.3.5 Experimental Procedure

A 3.5 L volume of the *Chlorella saccharophila* suspension obtained from open pond cultivation was adjusted to the required pH using 0.5 M NaOH or 0.5 M HCl. The cationic surfactant, cetyl trimethylammonium bromide (CTAB), was then added to the suspension and stirred at 400 rpm using a Thermix Stirring Hot Plate (Model 310T, Fisher Scientific, Ottawa, Ontario) for 5 minutes to ensure complete mixing of CTAB in solution. The suspension was added to the culture chamber (main vessel) of the foam flotation unit (Figure 6.2), where the appropriately-sized column was connected. Once in the chamber, the required air flow rate was programed into the control unit. Each batch run was terminated after 45 mins, and the collapsed foamate was collected from the collection chamber. The cell concentration for the collected foamate and the liquid suspension remaining in the culture chamber, were obtained from optical density measurements. In some cases, it was necessary to dilute the collapsed foam by a factor of up to 10, to be within the range of the calibration curve (Figure 6.1). Additionally, the volume of the liquid suspension remaining in the culture chamber was recorded for determination of recovery and enrichment ratio (Chan et al., 2007; Mukhopadhyay et al., 2010; Burghoff, 2012; Garg et al., 2015).

6.3.6 Experimental Design and Data Analysis

6.3.6.1 Box-Behnken Design of Experiments

A Box Behnken design (Minitab® version 17.3.1 Software, Minitab Inc., State College, PA, USA) consisting of 27 runs (3 center point replicates) was used in this study. The optimal conditions for harvesting *Chlorella saccharophila* microalgae using surfactant-aided dispersed air flotation were predicted using Response Surface methodology, where CTAB was used as the surfactant. The selection of the variables and their levels was based on previous findings in literature (Chen et al., 1998; Phoochinda et al., 2005; Burghoff, 2012; Coward et al., 2013; Garg et al., 2014; Garg et al., 2015), and preliminary experimental work. The four variables investigated were: CTAB concentration, pH, air flow rate and column height (Table 6.2). The surfactant concentrations (100 to 375 mg/L)

were selected to include the critical micelle concentration (CMC) range of 335 to 364 mg/L (Zhang et al., 2009). As the charge on the microalgae extracellular wall changes with pH (Phoochinda and White, 2003), the pH range of 4 to 10 was chosen so that the surface charge would vary. The minimum air flow rate of 42 mL/min was the lowest flow achievable with the apparatus, and the maximum of 105 mL/min was selected to give an acceptable performance for dewatering, where preliminary tests (using shortest column height) indicated that at this flow rate, the cells would be concentrated in 30 % of the original volume of the liquid suspension. At greater air flow rates, more liquid was carried to the collection chamber of the apparatus, which corresponds to lower cell concentrations.

6.3.6.2 Data Analysis

Recovery (*R*) and enrichment ratio (*ER*) were used as response variables to determine the effectiveness of surfactant-aided dispersed air flotation. *R* is expressed as the percentage of cells recovered in the foamate compared to the initial culture, Equation (4). Whereas *ER* indicates the degree to which the cells are concentrated (Equation 5). The design matrix and the experimental results for *R* and *ER* are shown in Table 6.3.

$$R = \frac{(\text{Cell concentration (cells/mL)})_{foamate}\ x\ Volume_{foamate}}{(\text{Cell concentration (cells/mL)})_{initial\ culture}\ x\ Volume_{initial}}\ x\ 100 \quad (4)$$

$$ER = \frac{(\text{Cell concentration (cells/mL)})_{foamate}}{(\text{Cell concentration (cells/mL)})_{intial\ feed}} \quad (5)$$

The results for each response were assessed separately and fitted to a second-degree polynomial equation (Equation 6) using Minitab, in order to determine the variable(s) levels for maximizing *R* and *ER*.

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (6)$$

Where y is the response, β_o, β_i, β_{ii} and β_{ij} represent the regression coefficients for the intercept, linearity of the input factor X_i, the quadratic interaction of X_i, and the interaction of X_i and X_j, respectively. The terms X_i and X_j are the interaction coefficients between the factors (Coward et al., 2013). The equation is used to express the relationship between the response variable and the independent variables shown in Table 6.2.

Table 6.2. Dispersed air flotation aided foam fractionation variables and levels.

Variable	**Abbreviation**	**Low**	**High**
CTAB concentration (mg/L)	Conc.	100	375
Flow rate (ml/min)	FR	42	105
pH	pH	4	10
Column height (mm)	Column	312	795

A model for each response variable was determined separately, after which both responses were optimized simultaneously using the desirability function (Derringer and Suich, 1980) in Minitab. Once the optimal parameters were identified, they were validated by comparing the predicted and experimental responses at the optimal conditions.

6.4 RESULTS AND DISCUSSION

In this study, the *R* and *ER* ranged from 76 to 97.5 % and 3 to 18, respectively (Table 6.3). Analysis of variance (ANOVA) was performed for each response variable separately, using the full quadratic model and assessed using a level of significance of α=0.05. A backward stepwise elimination process was then performed to identify the most significant terms that could explain the effects of microalgae recovery using foam flotation (Coward et al., 2013). In this technique, factors with coefficients that have higher *p*-values (using α=0.1) are selectively removed from the model, unless required for hierarchical purposes (Coward et al., 2013). The ANOVA results for the reduced model obtained for the recovery of *Chlorella saccharophila* cells using foam aided dispersed air flotation (Table 6.4) indicates that this model is highly significant (*p*-value <0.0001). The significant factors (*p*-value <0.0001) for the recovery of *C. saccharophila* are concentration and flow rate, meaning these two variables best explain the observed experimental recovery values. Additionally, the lack-of –fit for the model is not significant (*p*-value = 0.455), indicating a good model (Noordin et al., 2004). The reduced model that best predicts the recovery of *C. saccharophila* by foam flotation is given by:

$$R = 84.54 - 0.04464\ Conc. + 0.1753\ FR \qquad (7)$$

Figure 6.3 shows a contour plot illustrating the relationship of the significant variables on the recovery, *R*, where greater recovery values are correlated with higher air flow rates and lower CTAB surfactant concentrations. The model correlation coefficient (R^2) resulted in a value of 63 %, an *adjusted* R^2 of 60 %, and *predicted* R^2 of 50 %, which suggests that 37 % of the variability cannot be explained by this linear regression model. This may be due to higher order interactions that the model does not account for. According to (Allen, 2010), *adjusted* R^2 values for Box Behnken designs should be in excess of 50 % so as to

Table 6.3. Box-Behnken design matrix for harvesting microalgae with recovery (R) and enrichment ratio (ER) from experiments.

Exp. No.	CTAB Conc. (mg/L)	pH	FR (mL/min)	Column Height (mm)	R (%)	ER	$-1/ER$
1	375.0	7	105	554	82.2	3.20	-0.31
2	237.5	10	73.5	312	91.8	5.54	-0.18
3	375.0	7	73.5	312	76.2	4.10	-0.24
4	237.5	7	105	312	93.6	3.68	-0.27
5	237.5	7	42	312	76.6	8.13	-0.12
6*	237.5	7	73.5	554	89.0	6.23	-0.16
7	100.0	10	73.5	554	95.7	14.56	-0.07
8*	237.5	7	73.5	554	82.9	4.46	-0.22
9	375.0	10	73.5	554	82.8	3.41	-0.29
10	237.5	7	42	795	76.8	17.91	-0.06
11	100.0	7	73.5	312	94.4	7.34	-0.14
12	375.0	7	42	554	85.5	8.55	-0.12
13	100.0	4	73.5	554	90.1	6.85	-0.15
14	237.5	10	42	554	79.6	11.15	-0.09
15	237.5	10	73.5	795	85.0	5.41	-0.18
16	375.0	7	73.5	795	78.1	4.56	-0.22
17	100.0	7	105	554	97.4	6.48	-0.15
18	237.5	10	105	554	83.6	3.61	-0.28
19	100.0	7	73.5	795	97.6	8.03	-0.12
20	237.5	4	73.5	312	91.6	5.94	-0.17
21	237.5	4	105	554	97.1	4.30	-0.23
22*	237.5	7	73.5	554	82.5	5.07	-0.20
23	237.5	7	105	795	97.4	4.60	-0.22
24	375.0	4	73.5	554	80.1	4.67	-0.21
25	237.5	4	42	554	83.0	17.09	-0.06
26	100.0	7	42	554	83.4	14.60	-0.07
27	237.5	4	73.5	795	90.3	5.36	-0.19

Exp. No.: Experiment number; *center point replicates; conc.: concentration; FR: flow rate R: recovery; ER: enrichment ratio

Table 6.4. Reduced model ANOVA results for recovery using surfactant-aided dispersed air flotation of *Chlorella saccharophila* according to Box-Behnken design.

Source	Df	SS	MS	F-Value	P-Value
Model	2	817.90	408.95	20.18	<0.0001
Concentration	1	452.13	452.13	22.31	<0.0001
Flow Rate	1	365.77	365.77	18.05	<0.0001
Error	24	486.44	20.27		
Lack-of-fit	22	460.31	20.92	1.60	0.455
Pure Error	2	26.12	13.06		
Total	26	1304.34			

Df: Degrees of freedom; SS: Sum of squares; MS: Mean square

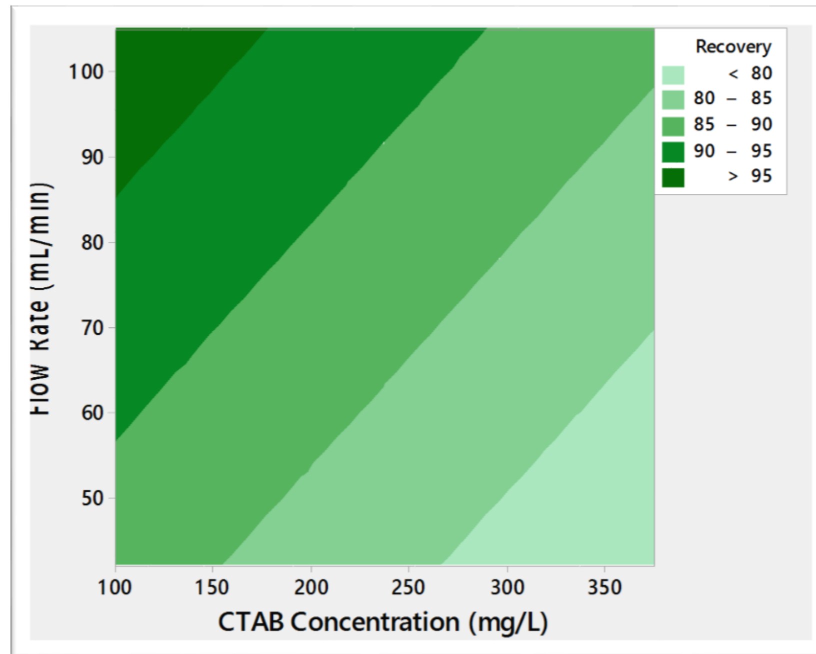


Figure 6.3. Contour plot depicting the relationship between flow rate and CTAB concentration on the recovery of *Chlorella saccharophila* using surfactant aided dispersed air flotation.

indicate that much of the experimental responses obtained, are controlled by the tested factors.

Similarly, a model with *ER* as the response variable was developed after applying a negative reciprocal transformation ($\lambda = -1$) to give -1/*ER*. The transformation was applied to change the distribution of the data such that it is normalized. The negative reciprocal preserves the order among the values such that large observations remain large (Frees, 2010). The full model was highly significant as indicated by the p-value of <0.0001 and non-significant lack-of-fit (*p*-value = 0.823). However, some of the linear, squared and interaction terms were highly insignificant with *p*-values much greater than $\alpha = 0.05$. The R^2, *adjusted* R^2 and *predicted* R^2 for the full model were 95, 88, and 74 %, respectively. A stepwise selection of terms was performed using $\alpha = 0.15$ in Minitab to reduce the model. The ANOVA results suggest a better model with fewer squared and interaction terms (Table 6.5). The reduced *ER* model is represented by Equation (8) with an R^2, *adjusted* R^2 and *predicted* R^2 values of 95, 91 and 88 %, respectively.

$$-1/ER = 0.120 + 0.000705Conc. - 0.0131pH - 0.00643FR + 0.01130Column + 0.001482pH^2 + 0.000029FR^2 - 0.000095Conc.{\bullet}pH - 0.000006Conc.{\bullet}FR + 0.000204\,pH{\bullet}FR \quad (8)$$

The influence of the variables on -1/ER can be seen graphically in Figure 6.4. It can be seen that lower CTAB concentrations and flow rates, and higher pH and greater column heights result in greater values of -1/ER and consequently greater enrichment ratios.

R is a measure of the percentage of cells that have been retrieved compared to that of the original liquid suspension (Equation 4), and column height and pH were not significant in explaining this response variable. *ER*, on the other hand, takes into account the degree of concentration of the cells (Equation 5), which requires the volume of liquid to be known. Although not all linear effects were significant in predicting *ER* such as pH and column height, the interaction with other factors were, thus they were kept in the model for hierarchical purposes (Coward et al., 2013).

The effects of air flow rate were significant for both *R* and *ER* responses. Greater flow rates result in larger bubbles, which provide a greater area for cells to bind (Phoochinda et al., 2005), as well as reducing the foam residence time (Merz et al., 2011). Consequently, in terms of *R*, greater flow led to more rapid processes resulting in higher cell accumulation

Table 6.5. Reduced model ANOVA results for enrichment ratio using surfactant-aided dispersed air flotation of *Chlorella saccharophila* according to Box-Behnken design.

Source	Df	SS	MS	F-Value	P-Value
Model	9	0.120892	0.013432	32.07	<0.0001
Conc.	1	0.041085	0.041085	98.09	<0.0001
pH	1	0.000	0.000	0.000	0.973
FR	1	0.062035	0.062035	148.11	<0.0001
Column	1	0.001531	0.001531	3.66	0.073
pH^2	1	0.001139	0.001139	2.72	0.117
FR^2	1	0.00521	0.00521	12.44	0.003
Conc.*pH	1	0.006129	0.006129	14.63	0.001
Conc.*FR	1	0.003026	0.003026	7.23	0.016
pH*FR	1	0.001486	0.001486	3.55	0.077
Error	17	0.00712	0.000419		
Lack-of-fit	15	0.005095	0.00034	0.34	0.919
Pure Error	2	0.002026	0.001013		
Total	26	0.128012			

Df: Degree of freedom; SS: Sum of squares; MS: Mean square Conc.: surfactant concentration; FR: flow rate

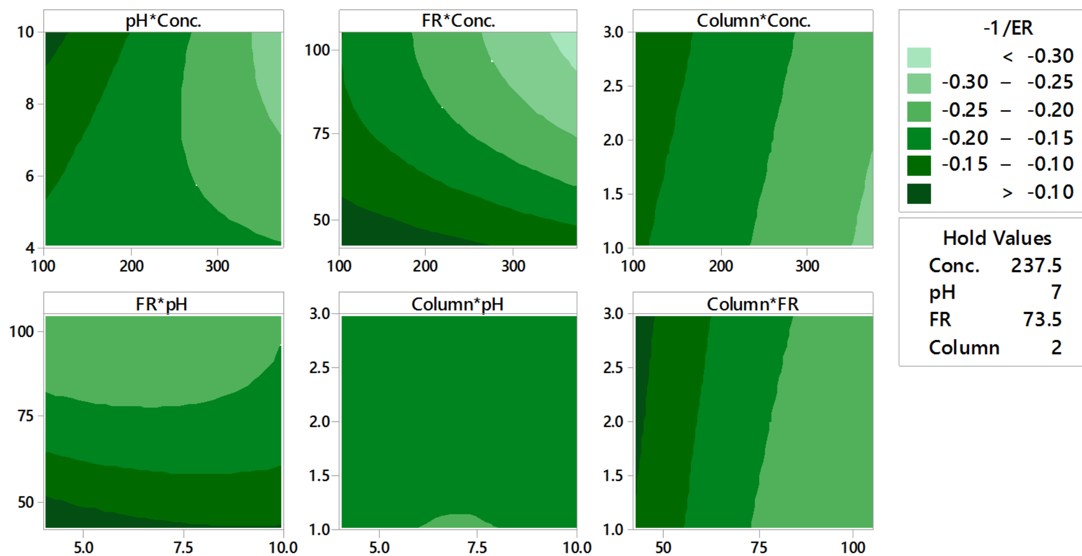


Figure 6.4. Contour plot depicting the relationship between different variables on the transformed enrichment ratio (-1/*ER*) of *Chlorella saccharophila* using surfactant aided dispersed air flotation.

and a higher *R*, however, *ER* was improved with lower flow rates as a result of longer foam residence time and greater drainage of liquid. The influence of flow rate on *R* was positive (Equation 7), however, its impact on *ER* was negative (Equation 8). In this study, the effect of CTAB surfactant concentration was significant for the *R* and *ER* of *Chlorella saccharophila*, using dispersed air flotation. It was observed that lower surfactant concentrations resulted in greater *R* and *ER*. Surfactants aid in the flotation process by electrostatically binding to the microalgae cell surface and improving the cell hydrophobicity, such that the cells attach to the bubbles present in the vessel and are carried up to the collection chamber (Coward et al., 2014). In the studies performed by Chen et al. (1998) and Garg et al. (2014), they demonstrated that increasing CTAB surfactant concentrations resulted in higher recoveries. However, the CTAB surfactant concentrations tested ranged from 0 to 80 mg/L, which are much less than the CMC of 335 to 364 mg/L (Zhang et al., 2009). In this study it was observed that decreasing the concentration from 375 mg/L to 100 mg/L improved *R* and *ER*. Furthermore, the decreased *R* and *ER* observed at greater surfactant concentrations may be attributed to micelle formation of the surfactant (le Maire et al., 2000), disrupting the mechanism of the surfactant-cell binding process. Additionally, greater surfactant concentrations have been noted to result in wetter foams, which decrease the *ER,* as a result of smaller bubble sizes achieved with higher surfactant concentrations that increase the amount of liquid carried between the foam lamellae (Coward et al., 2013).

The amount of liquid carried to the top of the chamber with the foam is governed by the drainage that takes place in the column (Burghoff, 2012). This is reflected in the results from this study, where the column height factor was deemed significant for *ER*, and not *R*. It was observed that greater column heights resulted in higher *ER*. The pH range of 4 to 10 was not a significant factor for *R* and *ER*. However, in the case of *ER*, pH remains in the model due to the significant interaction with surfactant concentration. It is thought that pH alters the surface charge of the cells (Phoochinda et al., 2005) which affects the binding of surfactant to the cells (Coward et al., 2014). The results from the study support this hypothesis as the interaction of pH and surfactant concentration are significant in the model for *ER*, and higher pH and lower CTAB surfactant concentrations resulted in greater *ER*. However, the pH alone did not have a significant effect. A similar observation was noted

by Liu et al. (1999), in that the pH range of 4 to 9 resulted in slight improvement in recoveries from 85 to 92 %. It may be that the pH range explored in this study, did not have a significant effect on the cell surface and therefore little effect on R or ER.

After suitable models were determined for the R (Equation 7) and $-1/ER$ (Equation 8), the desirability function (D) in Minitab was used to predict the conditions that would maximize both responses simultaneously. Using this function, one can vary the weight and importance of each response. In this study, the ER is of key importance for effective dewatering ability, that is, to recover the cells from suspension with as little liquid as possible. An importance value of 3 and a weighted value of 1 was assigned to $-1/ER$, and R was given both weighted and importance values of 1, to signify that ER is more highly desired. For example, having a high R with lower ER would not suggest an effective dewatering technique, since the enrichment value accounts for the volume of the slurry retrieved and indicates the extent to which the cells have been concentrated. Additionally, the range of values achieved for R in this study, were much narrower in comparison to the range of ER values. The highest R value (97.5 %) is 1.3 times greater than the lowest experimental value (76 %) obtained. While the greatest ER value (18) was 6 times greater than the lowest value.

The optimal predicted parameters were CTAB concentration of 100 mg/L, pH of 10, flow rate of 57.9 ml/min, and a column height of 795 mm. From the model, the maximum expected values for ER and R were 17.9 ± 6.19 and 91.1 % ± 1.62 %, respectively. Six experimental runs were then performed to validate the predicted optimum value and the results are shown in Table 6.6, where the average experimental ER was 13.23 ± 0.98 (12.3 to 14.2) and the R was 94.5 % ± 2.7 % (91.8 to 97.2 %). The average experimental ER and R values obtained deviated from the predicated values by 26 and 3.73 %, respectively. These values fall within one standard deviation (95 % confidence interval) of the predicted ER and R.

Studies from the literature on surfactant-aided dispersed flotation have reported that different operating parameters are necessary for achieving either a high enrichment ratio or recovery, with one achieved at the expense of the other. The study performed by Coward et al. (2013) reported a high enrichment ratio of 230 achieved through foam flotation of

Table 6.6. Results of experimental runs performed to validate the predicted optimum values (CTAB concentration of 100 mg/L, pH of 10, flow rate of 57.9 ml/min, and a column height of 795 mm) obtained for maximum recovery and enrichment ratio.

Run No.	***R* (%)**	***ER***
1	97.1	14.16
2	95.1	13.87
3	97.3	14.19
4	93.0	13.02
5	90.1	12.12
6	94.7	12.05
Average ± SD	**94.5 ± 2.71**	**13.24 ± 0.97**
Predicted Values	**91.1 ± 1.62**	**17.9 ± 6.19**

SD: standard deviation

Chlorella sp., however the recovery was only 2.3 %. Conversely, the highest recovery of 23 % was achieved at the expense of enrichment, which was measured at 1. A similar phenomenon was noted using *Tetraselmis* sp. M8 (Garg et al., 2015) and the microalgae *Chasetoceros* sp (Csordas and Wang, 2004). Maximum enrichment ratios in these studies of 10 and 130 resulted in recoveries of 55 and 20 %, respectively. Similarly, the greatest recoveries of 90 to 93 % corresponded with enrichments of 3.6 and 6, respectively, using different operational parameters. However, Mukhopadhyay et al. (2010) noted in their study that the maximum enrichment of whey protein (48.2) could be achieved without jeopardizing the recovery (96 %). The results obtained for the present study followed those noted by Garg et al. (2015), Coward et al. (2013) and Csordas and Wang (2004) in that the highest enrichment ratio of 18 resulted in the lowest recovery of 76 %. However, the greatest recovery of 97.5 % resulted in an enrichment ratio of 8, which is not the lowest one achieved in this study.

The optimal conditions in this investigation using the desirability function, resulted in a *R* of 95 % and *ER* of 13, which are high values for both responses. In fact, the recovery achieved using the optimal conditions is not significantly different from the highest recovery achieved in this study of 97.5 % at an α level of 0.05. The optimum *ER* is within 20 % of the maximum *ER* predicted when considering *ER* as the only response variable. Similarly, optimum *R* is within 10.9 % of the maximum *R* predicated when considering recovery as the only response variable.

The *ER* of 13 that was achieved, corresponds to a biomass slurry with a concentration of 926.8 mg/L compared to an initial value of 70 mg/L. The optimized flotation technique reduced the volume of liquid from 3.5 to 0.25 L, which resulted in a total dry biomass weight of 232 mg (*R* of 94.5%). In this study, the initial concentration of culture was 30 % below the lower concentration noted in literature of 0.1 to 5 g/L (Wang et al., 2008; Halim et al., 2012a). Coward et al. (2013) conducted a similar study on foam flotation of 9.5 L *Chlorella* suspension with an initial concentration of 0.11 g/L, which resulted in a concentration of as great as 230 times. However, this corresponded to a recovered slurry with concentration of 24.7 mg/L recovered in 1 mL volume. Thus, only 24.7 mg of microalgae were recovered from the original suspension containing 1.04 g. Nonetheless, microalgae concentrations of 300-400 g/L are noted for conversion operations which

require concentrations of a thousand folds or greater (Coward et al., 2013). Although the concentration factor of 230 is closer to this value, the recovery of the biomass was low. Uduman et al. (2010) discussed the dewatering of dilute suspensions of microalgae (total suspended solids (TSS) of 0.02 to 0.06 %), where a slurry or paste is obtained with TSS range of 5-25 % and this may occur using a one or two step process prior to product conversions. In this study, the TSS prior to dewatering was 0.022±0.001 %, which is within the range noted in literature for dilute suspensions of microalgae culture (Uduman et al., 2010). After dispersed air flotation, the TSS was 0.30±0.004 % for optimized conditions. This suggests a secondary step should be applied to further concentrate the biomass. Subsequent centrifugation (2000 rpm for 2 mins) of the recovered biomass from the optimized run resulted in TSS of 3.64±0.1 %. Despite the need for a secondary dewatering technique, the use of dispersed air flotation prior to secondary treatment in this study did reduce the working volume by a factor of 14. Centrifugation techniques have been reported to consume 8 kWh/m^3 (Danquah et al., 2009), whereas dispersed air flotation utilizes 0.003-0.015 kWh/m^3 (Wiley et al., 2009; Coward et al., 2013). Therefore, an initial reduction in volume using a technique that utilizes up to 500 times less energy would better improve the economics of dewatering as opposed to applying centrifugation alone.

6.5 Conclusions

Dispersed air flotation with CTAB added as a surfactant was investigated as a dewatering technique for *Chlorella saccharophila,* and optimized using a Box-Behnken experimental design and Response Surface Methodology. The effects of CTAB concentration, column height, pH and flow rate were studied on the R and ER responses. The responses were analyzed separately to determine suitable models and then a desirability function was used to maximize both responses simultaneously. It was determined that R was significantly influenced by the surfactant concentration and flow rate. All the tested factors were included in the model for ER. The optimal processing parameters determined were CTAB concentration of 100 mg/L, flow rate of 57.9 mL/min, and a column height of 795 mm. Experimental validation revealed that R (94.5 %) and ER (13.2) were in agreement with the predicted results within 96 and 74 %, respectively. The TSS increased from 0.022 to 0.30 % by application of this flotation technique. A secondary

dewatering treatment, such as centrifugation is recommended for further concentration of the microalgal slurry. However, this study shows that surfactant aided dispersed air flotation is an effective pretreatment stage that could reduce overall energy consumption and costs.

FOREWORD TO CHAPTER 7

The optimized parameters for maximizing both enrichment ratio and recovery of *Chlorella saccharophila,* using surfactant aided dispersed air flotation, were determined in Chapter 6. In Chapter 7, the focus is on optimizing the SC-CO_2 extraction procedure to maximize the yield of FAMEs, using biomass harvested with surfactant aided dispersed air flotation. In addition, consideration is given to the suitability of these optimized parameters from an industrial processing perspective.

CHAPTER 7 OPTIMIZATION OF FATTY-ACID METHYL ESTERS (FAME) IN LIPIDS EXTRACTED FROM SURFACTANT-EXPOSED *CHLORELLA SACCHAROPHILA* USING SUPERCRITICAL CO_2

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7.1 Abstract

An environmentally sustainable approach for biodiesel production was developed for the recovery of lipids from *Chlorella saccharophila* biomass using supercritical CO_2 (SC-CO_2). Here, the microalgae were harvested by foam-aided dispersed air flotation and simultaneously exposed to surfactant, prior to extraction. Evaluation was based on the total fatty acid methyl esters (T-FAME) and biodiesel dominant FAME (BD-FAME) yields (% dry cell weight (dcw)). Response surface methodology was used to optimize FAME content from lipids extracted by SC-CO_2 and to determine the main and interaction effects of reaction time, temperature, pressure, and biomass moisture content. The optimized parameters for achieving maximum T-FAME (20.4±1.9 % dcw) and BD-FAME (9.8±0.4 % dcw) yields were 90 min, 3500 psi, 73°C and 89 % moisture (*w/w*), and 86 min, 3500 psi, 73°C and 86 % moisture (*w/w*), respectively. Further analysis indicated that good T-FAME and BD-FAME yields (16.5±1.9 and 7.8±0.6 % dcw, respectively) could be achieved when the extraction time was reduced to 30 min and the harvested microalgae was centrifuged then used without further drying, such that the biomass moisture content was 96 % (*w/w*). These conditions may be more favorable from an industrial perspective, as they would eliminate the expenses associated with reducing the biomass moisture content by drying, and potentially reduce the operational costs to one third, since three times the biomass can be treated within the same time span as that proposed for the optimized conditions.

7.2 Introduction

Biodiesel is a renewable fuel source that has the potential to meet the increasing global energy demands for diesel, without the adverse environmental impacts that are associated with fossil fuels (Huang et al., 2012; Milano et al., 2016). Biodiesel can be generated using a number of feedstocks such as palm oil, soybean, corn and coconut, however microalgae biomass can also be used, with the advantage of producing up to nine times greater oil yields per hectare unit compared to other leading crops (Milano et al., 2016). Additionally microalgae are considered a nonfood-based feedstock, are capable of recycling CO_2 (Gebreslassie et al., 2013; Kim et al., 2016), and can be used to generate additional value-added products, such as pigments and fatty acids (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) (Yen et al., 2015).

Currently, only a few microalgae species are used commercially for pigment production (Yen et al., 2015). These include astaxanthin from *Haematococcus pluvialis* (Thana et al. 2008), β-carotene from *Dunaliella salina* (Solana et al., 2014) and lutein from *Scenedesmus* sp. (Yen et al., 2011). Such highly valued products for human nutrition (>$10,000/t USD), can withstand the higher processing costs associated with microalgae, however lower valued commodities such as biodiesel cannot withstand these high expenses (Benemann, 2013). The baseline economics of lipid production from microalgae (achieving a 10 % return) was analyzed by Davis et al. (2011), and it was determined that the cost per gallon is $8.52 and $18.10 USD using open pond and photobioreactors, respectively. At present, the cost of diesel fuel per gallon in Canada is $3.75 USD (Natural Resources Canada, 2018). This emphasizes that a reduction in processing costs is necessary for competing with fuel prices.

Lipid recovery from microalgae biomass remains a challenge in the commercialization of microalgal biodiesel (Li et al., 2014; Günerken et al., 2015). Microalgae oil recovery is most commonly performed using organic solvent techniques such as the Bligh and Dyer method (1959). Other techniques include a combination of solvents or enzyme assisted techniques that are toxic or are energy intensive and inefficient (Li et al., 2014; Lorenzen et al., 2017). Supercritical CO_2 (SC-CO_2) has emerged in recent years and has been

adopted in microalgae oil extraction (Soh and Zimmerman, 2011; Li et al., 2014; Lorenzen et al., 2017).

SC-CO_2 extraction is regarded as an environmentally benign technique for lipid recovery with no adverse impacts on the recovered extracts (Li et al., 2014; Lorenzen et al., 2017; Taher et al., 2014). An additional advantage associated with using SC-CO_2 is that no further processing step is required after extraction, due to its gaseous nature at ambient conditions, leaving only the extract behind (Taher et al., 2014). Effective SC-CO_2 lipid extraction is dependent on a number of variables such as treatment temperature, pressure, moisture content, time and flow rate (Santana et al., 2012; Sarker et al. 2012; Mouahid et al., 2013; Taher et al., 2014). The resultant extract consists primarily of triglycerides, free fatty acids, as well as other minor components such as carotenoids, squalene, and sterols (Sarker et al., 2012). Pre-treatment techniques can be applied to disrupt the integrity of the cell wall, thus further improving the efficiency of the extraction technique and increasing product recovery (Gerken et al., 2013; Li et al., 2014).

In this study, the SC-CO_2 extraction of lipids from *Chlorella saccharophila* was investigated using Response Surface Methodology (RSM), where foam-aided dispersed air flotation was used to harvest the microalgae biomass prior to extraction and also served to expose the cells to surfactant. The optimal SC-CO_2 extraction conditions were evaluated based on the total fatty acid methyl esters (T-FAME) and biodiesel dominant FAME (BD-FAME) yields (% dcw) from the extracted oil using the following parameters: extraction time, temperature, moisture content in biomass, and the reaction pressure.

7.3 Materials and Methods

7.3.1 Microalgae Cultivation

Chlorella saccharophila was purchased from the American Type Culture Collection (ATCC® 30408™), Manassas, VA, USA. The cultivation of the species was performed using a modified Fitzgerlad medium (9.5 L) inoculated with 500 mL of microalgae biomass in an open pond system, as previously described in Al-hattab & Ghaly (2014). The modification made to the Fitzgerlad medium is the supplementation of 85.7 mg/L of ammonium nitrate, 194.3 mg/L of ammonium phosphate, 94.3 mg/L of ammonium sulfate,

and 1.3 g/L of sodium bicarbonate, based on previous work performed (Al-hattab & Ghaly, 2014). Cultures were grown for a period of 10 d at room temperature, following the photoperiod of 14 h light and 10 h dark. A total of 8 cultivation runs were performed to collect sufficient biomass for experimentation. At the end of the 10 d growth period, the cultures typically had a total suspended solids (TSS) concentration of 0.02 % (*w/w*) and were analyzed using a rapid colorimetric sulpho-phospho-vanillin (SPV) technique (Mishra et al. 2014) to ensure consistency of lipid content between production runs (Appendix B). Mishra et al. (2014) had determined that the colorimetric technique gave similar results to conventional Bligh and Dryer extraction and analysis using gas chromatography (GC), as only a small coefficient of variation (<5%) was observed between the two methods. In this study, the biomass obtained from the various cultivation runs averaged a lipid content of 12.1±1.5 % (dcw), using the SPV reaction technique. The cultures were then harvested using foam-aided dispersed air flotation.

7.3.2 Microalgae Harvesting

Foam-aided dispersed air flotation was used for harvesting the microalgae cells from the culture media. It is a promising technique for microalgae recovery as a result of high cell recoveries and good concentration ratios, ease of operation, and its suitability for processing large volumes (Alhattab and Brooks, 2017). The foam-aided dispersed air flotation unit used for harvesting the microalgae and simultaneous surfactant exposure is depicted in Figure 7.1. It consisted of an air supply, diffuser, 4.1 L culture chamber, 3.8 L foam collection chamber and an adjustable column that was set to 795 mm in length. Prior to harvesting, the microalgae culture suspension was adjusted to a pH of 10 with 0.5 M sodium hydroxide. Following this, the surfactant CTAB (cetyltrimethylammonium bromide) was added as a foaming agent to the culture suspension, resulting in a concentration of 100 mg/L, and mixed for 5 min at 400 rpm using a Thermix Stirring Hot Plate (Model 310T, Fisher Scientific, Ottawa, Ontario). Then, the suspension was transferred to the culture chamber of the dispersed air flotation unit (Figure 7.1), and air was introduced into the system at a flow rate of 57.9 mL/min. The harvesting run was terminated after 45 min, and the recovered slurry, consisting of collapsed foam and harvested microalgae, was recovered from the foam collection chamber. The TSS of the recovered slurry was 0.3 % (*w/w*), which was further concentrated to a TSS of 3.6 % (*w/w*)

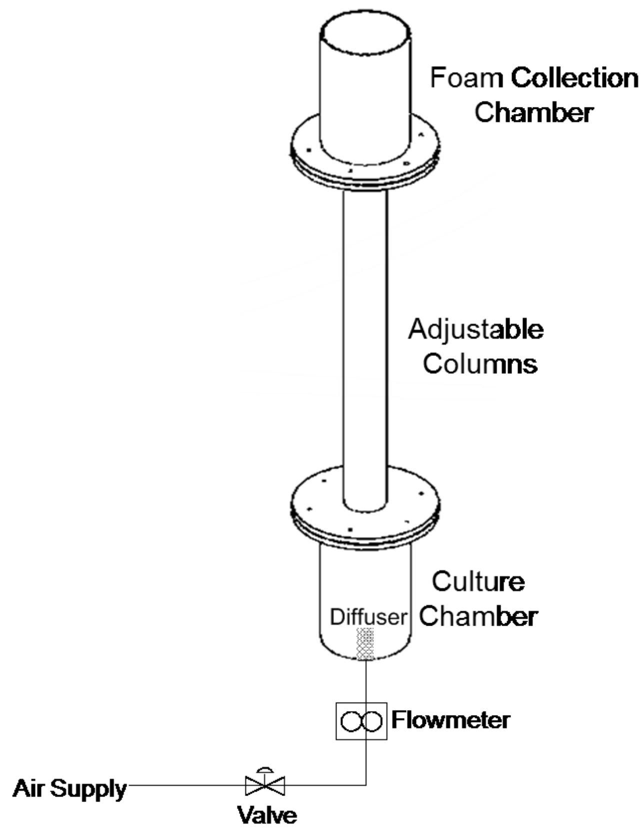

Figure 7.1. Foam aided dispersed air flotation unit.

using centrifugation (Sorvall T1, Thermo Fisher Scientific, Ontario, Canada) operating at 2000 rpm for 2 mins. The supernatant was removed and the biomass pellet was collected and stored at -20°C.

A total of 15 harvesting runs (each with a culture volume of 3.5 L) were performed over two weeks, to ensure that sufficient biomass was available for the SC-CO_2 extraction experiments. The microalgal pellets from all the harvested runs were thawed to room temperature, forming a paste, then mixed together (Thermix Stirring Hot Plate, Model 310T, Fisher Scientific, Ottawa, Ontario) to ensure homogeneity of the samples prior to the extraction experiments. Once mixed, samples were transferred to petri dishes such that each dish contained 2.5 g (3.6 % dcw) of the microalgae sample on pre-weighed filter paper (Grade 415, VWR International, Ontario, Canada). The moisture content for the biomass was adjusted to the desired value using a vacuum oven operating at 45°C and 26 psi, and then the petri dishes were labeled, capped, sealed with parafilm and stored at -20°C until the SC-CO_2 extraction experiments were performed.

7.3.3 Procedure for SC-CO_2 Extraction

SC-CO_2 extractions were conducted with a bench top supercritical fluid extractor (SFT-110, Supercritical Fluid Technologies, Inc., Delaware, USA) using a 5 mL stainless steel (17-4 PH) vessel. Prior to SC-CO_2 extraction, the extraction vessel was cleaned and flushed at 80°C and 7000 psi. Then, a frozen microalgae sample that had been prepared on filter paper (as described previously) was thawed out at room temperature for 5 minutes, and placed inside the extraction vessel. The desired pressure and temperature were set and the extraction was allowed to run for the appropriate run time. Once complete, the run was terminated and the CO_2 supply was shut off. Next, the static/dynamic valve was opened and the restrictor valve was slowly opened such that the flow of expanded CO_2 was maintained at 3 standard litres per minute (SLPM). The collected extract separated out of the gaseous CO_2 and was captured in the collection vial. When the pressure reached 0 psi, the vessel was opened to remove the residual sample and sealed once again to flush with SC-CO_2, to ensure complete elution of product. The extract from the flushing process was also captured in the collection vial and accounted for as part of the collected extract.

7.3.4 Transesterification of Extract

The recovered extract from SC-CO_2 extraction was transesterified to enable later quantification by gas chromatography with a flame ionization detector (GC-FID). The transesterification process was adapted from an existing method (Laurens et al., 2012) for use on biomass quantities of 90 mg (dcw). In this method, the extract was transferred to a 10 mL test tube, where 2.6 mL of chloroform/methanol (2:1, *v/v*) and 3.9 mL of sulfuric acid/methanol (5 %, *v/v*) were added, for solubilizing lipids and simultaneous transesterification. Next, the tube was flushed with N_2, vortexed to ensure complete mixing and placed on a heating block (No. 88871002, Thermo Scientific, Ontario, Canada) set at 85°C for 1 h. Following this, the tubes were left to cool for 15 min at ambient temperature. Once cooled, the samples were transferred to a 50 mL Falcon conical tube, to which 13 mL of deionized water and 13 mL of hexane were added. Again, the samples were flushed with N_2, vortexed and centrifuged at 1,200 *g* for 10 min to achieve phase separation. Using a pasture pipette, the organic top layer was transferred to a 15 mL Falcon conical tube. A small scoop of anhydrous sodium sulfate was added to the tube with the organic layer (containing hexane and FAME) to dry the sample, and left to settle to the bottom of the tube undisturbed for 10 min. A final transfer, to a 10 mL glass test tube, of the hexane and FAME mixture was performed using a pasture pipette. The hexane was evaporated directly from the test tube using a rotary evaporator (HiTEC RE-51, Yamato Scientific America, California, USA) set at 32°C, connected using a 24/40 glass adapter with a threaded vial adapter (CG-1318-40, Chemglass Life Sciences, New Jersey, USA). The remaining sample residue was immersed in known quantity of hexane and quantified using GC-FID.

7.3.5 FAME Quantification Using GC-FID

FAME yields and composition were analyzed by gas chromatography with a flame ionization detector (GC-FID), using an Agilent 7890B chromatograph (Mississauga, Ontario, Canada). Standard curves were prepared for quantification and identification of FAME using Supelco 37 Component FAME Mix (CRM47885, Sigma Aldrich, Oakville, Ontario, Canada). Calibration curves were prepared by diluting the FAME 37 standard by factors of 0, 2, 2.5, 3.33, 5 and 10, using GC grade hexane (97 %, Sigma Aldrich, Oakville, Ontario, Canada). A total volume for each dilution of 100 µL was prepared and placed in

certified glass insets (29435-U, Sigma Aldrich, Oakville, Ontario, Canada) that were inserted into 12x32 mm GC vials (29652-U Sigma Aldrich, Oakville, Ontario, Canada). An injection volume of 1 μL, using a split ratio of 1/50, was loaded onto the DB-23 column (60 m length x 0.25 mm ID, 0.15 μm film, J&W 122-2361, Agilent Technologies Inc., Mississauga, Ontario, Canada) at 250°C with helium carrier gas. The temperature of the column was initially held at 50°C for 1 min. Next, the temperature was raised by 25°C/min to 175°C with a hold time of 4 min. Finally, a ramp in temperature of 4°C/min to 230°C, was performed and this temperature was maintained for 5 min. The temperature of the detector was set to 280°C with hydrogen as the carrier gas flowing at 40 mL/min. The resulting chromatograms were integrated using MSD ChemStation® F.01.03.2357 Software (Agilent Technologies Inc.). A linear relationship between FAME concentration (as stated in the certificate of analysis TraceCERT #AR-1606) and peak area were fitted and used for determining the FAME concentration in the transesterified SC-CO_2 extracts from the microalgae. All GC-FID sample analyses were performed in duplicates. FAME content as a percentage of biomass (dcw) was determined using equation 9.

$$FAME\ (\%\ of\ dcw) = \frac{\frac{Peak\ area\ of\ FAME}{Slope\ of\ FAME_{standard\ curve}} \cdot Hexane\ Dilution\ factor}{Amount\ of\ biomass\ (90\ mg)}\ x\ 100 \qquad (9)$$

7.3.6 Experimental Design and Data Analysis

7.3.6.1 Box-Behnken Design of Experiments

A Box Behnken design consisting of 27 runs (3 center point replicates) was used for the design of experiments and the optimal conditions for lipid extraction from *Chlorella saccharophila* microalgae using SC-CO_2 were predicted using Response Surface Methodology (RSM). Evaluation of the lipid extract was based on maximizing FAME. Minitab® software (version 17.3.1, Minitab Inc., State College, PA, USA) was used for analysis. Selection of the extraction parameters to investigate was based on previous findings in literature (Andrich et al., 2005; Sajilata et al., 2008; Dejoye et al., 2011; Halim et al., 2011; Santana et al., 2012; Crampon et al., 2013; Taher et al., 2014), and preliminary experimental work. The following parameters were studied: biomass moisture content, reaction temperature, extraction pressure and time (Table 7.1). The range of biomass moisture content (15 to 96.4 % *w/w*) were selected to include the moisture content of the

Table 7.1. Supercritical CO_2 extraction parameters and levels used for RSM optimization.

Parameter	**Variable**	**Low**	**High**
Biomass moisture content (%)	*M*	15	96.4
Reaction temperature (°C)	*T*	42	73
Reaction pressure (psi)	*P*	3500	6000
Reaction time (min)	*Ti*	30	90

resulting slurry without drying (upper range) and that typically used for SC-CO_2 extraction (Crampon et al., 2013; Mouahid et al., 2013; Mouahid et al., 2016). The ranges for reaction temperature (42 to 73°C), extraction pressure (3500 to 6000 psi) and time (30 to 90 mins), were chosen based on preliminary work used to identify the lower range necessary to achieve product extract, and the upper range values recommended in the literature as suitable for enhancing lipid recovery from microalgae biomass (Mendes et al., 2003; Andrich et al., 2005; Dejoye et al., 2011; Halim et al., 2011; Santana et al., 2012; Taher et al., 2014). The Box Behnken experimental design matrix is shown in Table 7.2.

7.3.6.2 Data analysis

Total FAME (T-FAME), equation 10, and biodiesel-dominant FAME (BD-FAME) content (Knothe, 2008; Rakesh et al., 2015), equation 11, were used to optimize SC-CO_2 extraction of *Chlorella saccharophila* biomass. The results were separately fitted to a second order polynomial equation using Minitab®, in order to determine the variables and variable coefficients that best explain the measured response. Analysis of the variance (ANOVA) was used to assess the model significance using an α level less than or equal to 0.1. The resulting models were used to predict responses for model validation.

$$T - FAME\ (\%, dcw) = \sum All\ FAME\ (\%, as\ determined\ by\ equation\ 1) \qquad (10)$$

$$BD - FAME\ (\%, dcw) = \sum FAME(C16{:}\,0, C18{:}\,0, C18{:}\,1\ cis, C18{:}\,2\ cis) \qquad (11)$$

7.4 RESULTS AND DISCUSSION

Box Behnken experiments were performed to determine the relationship between the T-FAME and BD-FAME content in lipids and the SC-CO_2 extraction parameters (reaction time, pressure, temperature and biomass moisture content). Table 7.2 summarizes the experimental matrix and both the experimental and model-predicted values obtained from RSM. The experimental results show that the T-FAME and BD-FAME varied from 2.8 to 17.6 % and 0.8 to 8.8 %, respectively, while the lipid yields varied from 2.9 to 18 % (dcw). The large variation in the FAME yields, is attributed to the varying SC-CO_2 operational parameters, which was also reported by other researchers (Santana et al., 2012; Taher et al., 2014). The lower values for both responses were obtained for extractions conducted at 60 min, 4750 psi, 73°C and with a moisture content of 15 % (*w/w*). Conversely, the

Table 7.2. Box-Behnken design matrix for T-FAME and BD-FAME content, as % of dry cell weight, in lipids extracted from *Chlorella saccharophila* using SC-CO_2 obtained from experimental trials and model predicted ones.

Exp. No.	Operational Parameter				T-FAME (% dcw)			BD-FAME (% dcw)			% of T-FAME
	Ti (mins)	*P* (psi)	*T* (°C)	*M* (%)	Exper.	Predicted	|Deviation| (%)	Exper.	Predicted	|Deviation| (%)	
1	60	6000	73.0	56.0	10.7±0.25	9.4±1.2	12.0	5.6±0.10	5.0±0.3	10.4	52.3
2*	60	4750	57.5	56.0	13.7±0.62	13.4±0.6	2.3	7.2±0.41	7.3±0.2	0.9	52.6
3	60	6000	57.5	15.0	5.2±0.37	5.9±0.9	12.5	3.5±0.08	3.4±0.3	3.6	67.3
4*	60	4750	57.5	56.0	13.4±0.22	13.4±0.6	0.1	7.3±0.05	7.3±0.2	0.2	54.5
5	30	6000	57.5	56.0	13.6±0.91	13.8±1.2	1.8	6.7±0.71	6.6±0.3	2.5	49.3
6	30	4750	73.0	56.0	10.3±1.89	12.2±0.9	10.1	5.3±0.97	5.4±0.4	1.5	51.5
7	60	6000	57.5	96.4	11.3±0.96	11.3±0.9	0.8	6.5±0.44	6.7±0.3	3.8	57.5
8	60	4750	73.0	96.4	15.9±0.19	15.1±1.3	5.2	8.2±0.43	8.0±0.4	1.9	51.6
9	60	4750	42.0	15.0	7.1±0.61	7.6±1.3	6.8	4.4±0.07	4.9±0.4	11.1	62.0
10	90	4750	57.5	15.0	6.3±1.12	6.9±0.9	9.7	4.0±0.52	3.6±0.3	10.2	63.5
11	30	3500	57.5	56.0	11.6±0.01	12.2±1.2	5.8	7.0±0.40	7.2±0.3	2.2	60.3
12	90	4750	73.0	56.0	10.4±0.88	13.1±0.9	16.3	6.5±0.20	7.0±0.4	8.6	62.5
13	90	6000	57.5	56.0	13.6±0.05	11.7±1.2	13.9	6.7±0.27	6.5±0.3	3.4	49.3
14	30	4750	42.0	56.0	13.1±0.82	10.3±0.9	14.5	6.9±0.21	6.4±0.4	7.6	52.7
15	60	3500	57.5	96.4	11.0±2.33	12.7±0.9	14.9	6.6±0.66	6.4±0.3	3.1	60.0
16	60	3500	42.0	56.0	9.1±0.66	8.8±1.2	3.6	4.6±0.17	4.9±0.3	4.7	50.5
17	90	3500	57.5	56.0	17.4±0.32	15.9±1.2	8.4	6.9±0.02	7.1±0.3	2.6	39.7
18	30	4750	57.5	96.4	11.4±2.11	11.6±0.9	1.8	5.7±0.41	6.2±0.3	7.3	50.0
19	30	4750	57.5	15.0	6.1±0.09	6.1±0.9	0.5	3.7±0.04	3.7±0.3	1.2	60.7
20	60	3500	73.0	56.0	17.6±0.46	15.9±1.2	9.6	8.8±0.22	8.4±0.3	5.5	50.0
21*	60	4750	57.5	56.0	11.3±0.41	13.4±0.6	18.3	6.6±0.25	7.3±0.2	9.7	58.4
22	60	4750	73.0	15.0	2.8±0.34	2.0±1.3	30.8	0.8±0.04	1.4±0.4	18.2	28.6
23	60	3500	57.5	15.0	8.1±0.96	7.2±0.9	11.3	5.5±0.64	4.9±0.3	10.6	67.9
24	90	4750	57.5	96.4	14.1±2.32	12.4±0.9	11.7	6.1±0.21	6.1±0.3	0.7	43.3
25	60	4750	42.0	96.4	4.9±0.20	5.5±1.3	10.7	3.4±0.19	3.1±0.4	7.6	69.4
26	60	6000	42.0	56.0	12.5±1.72	12.6±1.2	0.7	6.9±1.03	7.0±0.3	1.9	55.2
27	90	4750	42.0	56.0	9.1±1.55	11.1±0.9	11.3	4.6±0.82	4.5±0.4	1.1	50.5

*Model Center points; Exper: experimental values; *Ti*: time; *P*: pressure; *T*: temperature; *M*: moisture.

maximum values for both variables were achieved at 60 min, 3500 psi, 73°C and a moisture content of 56 % (*w/w*).

The reduced RSM model for total FAME content is given in equation 12, where *Ti*, is reaction time, *P* is pressure, *T* is temperature, *M* is biomass moisture content. This was obtained by applying a stepwise selection technique to the full model, and eliminating or retaining variables using an α-level of 0.15.

$$T\text{-}FAME\ (\%) = -52.2 + 0.197\ Ti + 0.00947\ P + 1.199\ T - 0.0029\ M - 0.00728\ T*T - 0.002\ M*M - 0.000039\ Ti*P - 0.000134\ P*T + 0.00601\ T*M \quad (12)$$

The ANOVA results for the reduced T-FAME model (equation 12) for *Chlorella saccharophila* using SC-CO_2, are shown in Table 7.3. The model is highly significant, as indicated by the small *p*-value (<0.0001) and the lack-of-fit of 0.42. The variables of most significance (at α=0.1) are the main and second order effects of biomass moisture content and reaction temperature as well as their interaction, and the interaction between reaction pressure and temperature. Although not all main and interaction effects were significant at α=0.1, such as time and pressure, they were kept in the model for hierarchical purposes. The correlation coefficient of the model, R^2, resulted in a value of 87.0 %, with an adjusted R^2 and a predicted R^2 of 80.1 and 67.0 %, respectively, which indicates that only 13 % of the variability cannot be explained by the linear regression model. The percent error between the experimental and predicted model values are tabulated in Table 7.2. For the most part, the absolute deviation of the predicated T-FAME values from the experimental values observed varied from as little as 0.1 to 30.8 %, with a mean absolute deviation of 8.77 %. The greater deviation values can be explained by the smaller experimental values obtained, where the effect of the variation is more pronounced compared to greater values with the similar deviations. However, the lower mean absolute deviation for T-FAME, suggests that much of the predicted values are in agreement with the experimental values obtained.

The model (Equation 12) was optimized to predict the SC-CO_2 reaction parameters for achieving maximal T-FAME content. The predicted results show that a reaction time of

Table 7.3. ANOVA results for the total FAME content (%) from the reduced RSM model.

Source	Df	SS	MS	F-Value	*p*-Value
Model	9	317.90	35.32	12.64	<0.0001
Ti	1	1.93	1.93	0.69	0.417
P	1	5.42	5.42	1.94	0.182
T	1	11.46	11.46	4.10	0.059
M	1	90.10	90.10	32.23	<0.0001
T^2	1	19.56	19.56	7.00	0.017
M^2	1	109.71	109.71	39.25	<0.0001
$Ti*P$	1	8.36	8.36	2.99	0.102
$P*T$	1	26.91	26.91	9.63	0.006
$T*M$	1	58.35	58.35	20.87	<0.0001
Error	17	47.52	2.80		
Lak-of-fit	15	44.17	2.94	1.75	0.420
Pure Error	2	3.36	1.678		
Total	26	365.42			

Df: Degree of freedom; SS: Sum of squares; MS: Mean square; *Ti*: Time; *P*: Pressure; *T*: Temperature; *M*: Moisture content in biomass

90 min, with a pressure of 3500 psi, a temperature of 73°C and a biomass moisture content of 88.7 % (*w/w*) could result in a FAME content of 20.4 ± 1.7 % (dcw), Table 7.4.

The effects of varying SC-CO_2 extraction parameters for the recovery of FAME, proved significant in this study and others. The increase in reaction time improved the lipid recoveries attained in this study, Figure 7.2, which may be attributed to the time necessary for the diffusion-driven nature of the extraction process to occur, as it depends on the concentration gradient that exists between the biomass and the SC-CO_2 (Halim et al., 2012). The solubility of the lipids is a function of reaction pressure and temperature as it directly impacts the solvent power of the SC-CO_2 (Taylor, 1996). Whereby, the fluid density is increased by higher pressures which results in a greater solvent power (Halim et al., 2012). Increases in reaction temperature result in two features that compete with one another (Halim et al., 2012). Higher temperatures decrease the density of the fluid which lowers the solvent power of the SC-CO_2, while also increasing the volatility of the lipid which improves the lipid mass transfer into the SC-CO_2 (Taylor, 1995; Soares et al., 2007). The effects of reaction parameters on the SC-CO_2 lipid extraction from *Botryococcus braunii,* investigated by Santana et al. (2012), indicated that lipid yields decreased with increasing temperature over the tested range of 50-80°C and increased with pressure (2900 to 3625 psi). In this study, lower pressures of 3500 psi and greater temperatures (73°C) resulted in improved lipid recoveries. The variation may be explained by the crossover phenomenon that has been noted to exist at ~3500 psi (Santana et al., 2012; Taher et al., 2014). At pressures below this point, the CO_2 density decreases with increasing temperatures, however, the solubility of triglycerides increases with the increases in temperature. At higher pressures, the opposite is true in that the density becomes less affected by the temperature than the solute vapour pressure (Halim et al., 2011; Solana et al., 2014; Taher et al., 2014; Esquivel-Hernandez et al., 2016). Thus, at pressures above this point, greater yields are obtained with higher temperature (Solana et al., 2014; Taher et al., 2014). The work of Halim et al. (2011) also showed that the lipid recoveries from *Chlorococcum* sp. did not significantly improve beyond 4350 psi over the tested range of 4350-7250 psi, which is similar to the findings of this study, in that increasing the pressure did not significantly improve the attained yields.

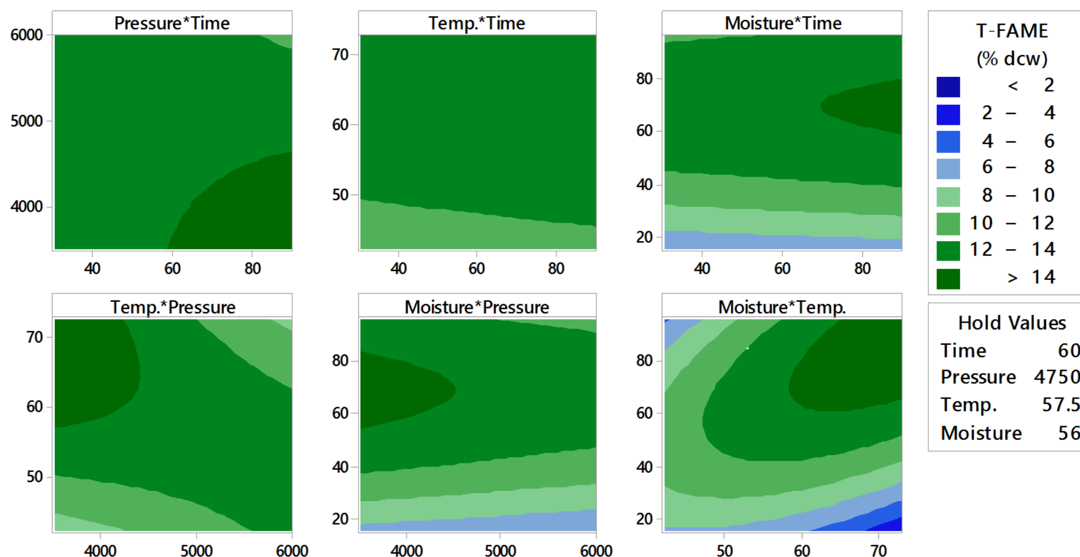

Figure 7.2. Contour plots showing the effects of SC-CO_2 operating parameters (pressure (psi), time (min), temperature (°C), moisture (% *w/w*)) on T-FAME content (% dcw) in lipids extracted from *Chlorella saccharophila* biomass.

Table 7.4. Comparison of optimized and other non-optimized SC-CO_2 extraction parameters for predicted FAME content in lipids from *Chlorella saccharophila*.

Response Variable	Optimized Parameters				Predicted FAME (% dcw)
	Ti (min)	*P* (psi)	*T* (°C)	*M* (% *w/w*)	
Total FAME	90	3500	73	88.7	20.4 ± 1.72
BD-FAME	86.4	3500	73	86.2	9.8 ± 0.49
Other operational parameters					
T-FAME	30	3500	42	96.4	1.7 ± 1.86
	30	3500	73	96.4	16.5 ±1.86
	86	3500	73	96.4	19.9 ±1.80
	86.4	3500	73	86.2	20.1 ±1.63
BD-FAME	30	3500	42	96.4	2.1 ±0.60
	30	3500	73	96.4	7.8 ±0.60
	86.4	3500	73	96.4	9.6 ±0.56
	90	3500	73	88.7	9.7±0.53

Ti: Time; *P*: Pressure; *T*: Temperature; *M*: Moisture content in biomass

Similarly, Dejoye et al. (2011) reported that an increase in temperature from 40 to 70°C at a pressure of 4114 psi improved lipid extract from 1.81 to 3.90 % in *Chlorella vulgaris* biomass. Furthermore, Taher et al. (2014) found that the optimum parameters for SC-CO_2 extraction of *Scenedesmus* sp. were 53°C and 7251 psi over the tested temperature and pressure ranges of 35 to 65°C and 2900 to 7251 psi, respectively. Their work also indicated that the main and interaction effects of temperature and pressure were strongly affecting the lipid yields, as was observed in this study.

In order to practically implement SC-CO_2 extraction technology at an industrial level for the production of biodiesel from microalgae, the technology has to be capable of efficiently extracting lipids from wet biomass, just as it does for dried (Halim et al., 2011; Soh and Zimmerman, 2011). However, the presence of moisture or water in biomass during SC-CO_2 extraction has been most commonly perceived to act as a barrier that impedes the mass transfer of the analyte in the bulk of the fluid (Halim et al., 2011). Interestingly, the results obtained in this study suggest that greater FAME contents are achieved at higher biomass moisture contents, Figure 7.2. There are a few other studies that have shown that the presence of water does not reduce the extraction performance when SC-CO_2 extraction is performed on moist microalgae samples (Pourmortazavi and Hajimirsadeghi, 2007; Halim et al., 2011; Soh and Zimmerman, 2011). For example, in the study performed by Halim et al. (2011), it was observed that wet *Chlorococcum* sp. biomass (30 % solids content) resulted in a lipid yield of 7.1 % (dcw), which was greater than that achieved with dried biomass (5.8 % dcw). It was also noted that the increase occurred as a function of time (1-2 h). In the study by Soh and Zimmerman (2011), the addition of water (from 96 to 98.4 %) was found to have no change on the quantity and profile of FAME obtained from the SC-CO_2 extraction of *Scenedesmus dimorphus*. Furthermore, it was observed both in their study and the present work, that greater temperatures (above 60°C) were required for greater FAME recovery, which may be attributed to the water-CO_2 mixture not achieving the critical state at or below this temperature, as hypothesized by these authors. Taher et al. (2014) found that SC-CO_2 extraction of freeze dried *Scenedesmus* sp. biomass resulted in a lipid yield of 10 % (dcw), while wet biomass (6.8 % solids) resulted in a lipid yield of 4.3 % (dcw). However, they also noted that prior treatment of the wet biomass using lysozyme improved wet SC-CO_2

biomass lipid yields to 12.5 % (dcw), which was greater than that achieved using freeze dried biomass. This is similar to what has been observed in this study, where the presence of surfactant in the biomass (from initial harvesting step), acts as a means for disrupting cell wall biomass. In the literature, surfactants have been successfully used to increase product recovery from different microalgae species including *Chlorella* (Huang and Kim, 2013; Coward et al., 2014) *Scenedesmus* (Lai et al., 2016) and *Tetraselmis suecica* (Ulloa et al., 2012; Huang and Kim, 2013; Coward et al., 2014; Lai et al., 2016). For example, both Coward et al. (2014) and Huang and Kim (2013) observed improvements in the lipid yield of 1.7 and 2.3 times, respectively, for *Chlorella* biomass that had been exposed to CTAB surfactant, in comparison to untreated biomass.

SC-CO_2 technology can also be employed to extract other components from the biomass (Halim et al., 2011; Soh and Zimmerman, 2011). Although not measured, it was qualitatively observed in this study that with biomass moisture contents of 96.4 and 56 % a light green liquid was recovered in the extract. However, in biomass with a moisture content of 15 %, only a clear yellow extract was retrieved. This may be attributed to additional pigment extraction due to the presence of water in the biomass, as water can act as a polar co-solvent during the SC-CO_2 extraction of microalgae biomass (Halim et al., 2011). It was noted by Kitada et al. (2009) that SC-CO_2 extraction assisted with the polar solvent ethanol (7.5 % volume) extracted chlorophyll *a* and chlorophyll *b* (9 mg/g sample) from *Chlorella vulgaris*, however, these were not detected using SC-CO_2 extraction alone under conditions of 4350 psi and 60°C. Furthermore, they observed that under the same conditions, the addition of the ethanol co-solvent improved lutein recovery from 0.5 mg/g to 3 mg/g. Similarly, Guedes et al. (2013) found that the addition of ethanol co-solvent (7.7 % concentration) to the SC-CO_2 extraction of pigments from *Scenedesmus obliquus*, improved the yield of carotenoids, chlorophyll *a*, and chlorophyll *b* from 0.05, 0 and 0 mg/g, respectively, to 0.30, 0.35 and 0.85 mg/g, respectively.

In the present study, optimal parameters were determined for maximizing BD-FAME in lipids extracted using SC-CO_2. This was performed by assessing the response of the SC-CO_2 extraction process in terms of the major FAMEs that are most commonly present in biodiesel, which are palmitic (C16:0), steric (C18:0), oleic (C18:1n9*cis*), and linoleic

acid (C18:2n6*cis*) (Knothe, 2008; Halim et al., 2011; Rakesh et al., 2015). The experimental values obtained for BD-FAME, are depicted in Table 7.2, reported as a percentage of dry cell weight. The reduced model that best describes the observed results, obtained using a backward elimination (α=0.1), is depicted by equation 13 and the ANOVA results are summarized in Table 7.5.

$$BD\text{-}FAME\ (\%) = -13.56 - 0.0476\,Ti + 0.003364\,P + 0.538\,T - 0.0736\,M - 0.000531\,Ti*Ti - 0.004105\,T*T - 0.001146\,M*M + 0.001905\,Ti*T - 0.000072\,P*T + 0.000009\,P*M + 0.003279\,T*M \quad (13)$$

Where Ti, is reaction time, P is pressure, T is temperature and M is biomass moisture content.

The correlation coefficient of the model, R^2, resulted in a value of 95.8 %, with an adjusted R^2 and a predicted R^2 of 92.6 and 81.8 %, respectively. The deviation of the model predicated BD-FAME values and those obtained from experimental runs varied from 0.2 to 18.2 % with a mean absolute average deviation of 5.3 %, indicating that overall the model is a good predictor of the observed values. It can be seen that the larger deviation values are a result of smaller FAME content, where the effect is less pronounced with higher values. The main effects of pressure, temperature and biomass moisture content and the second order effects of time, temperature moisture content are highly significant at an α-level of 0.05. Additionally, the interaction effects of all variables with temperature were highly significant (at α=0.05), and that between pressure and moisture content was significant at an α-level of 0.1. The contour plots illustrating these effects, are presented in Figure 7.3.

Comparing the models obtained for T-FAME and BD-FAME content in lipids extracted by SC-CO_2 from *Chlorella saccharophila*, it can be seen that the effects of the tested operational parameters are more pronounced for the BD-FAME content than that of T-FAME. This can be seen by both the significance of the variables in each model and the correlation coefficient. For example, the main effects of pressure were highly significant in explaining the BD-FAME, but not in explaining the T-FAME content. This can also be seen by the greater R^2 of 95.8 % for the BD-FAME model compared to the 87 % of the T-FAME model, which indicates that only 4.2 % of the variation cannot be explained by the

Table 7.5. Reduced ANOVA results for the BD-FAME content (% dcw) model identified using response surface methodology.

Source	Df	SS	MS	F-Value	*p*-Value
Model	11	73.65	6.70	30.72	<0.0001
Ti	1	0.032	0.03	0.14	0.709
P	1	1.10	1.10	5.04	0.040
T	1	1.60	1.60	7.32	0.016
M	1	17.90	17.90	82.11	<0.0001
Ti^2	1	1.37	1.37	6.28	0.024
T^2	1	5.84	5.84	26.78	<0.0001
M^2	1	22.26	22.26	102.12	<0.0001
*Ti*T*	1	3.14	3.14	14.40	0.002
*P*T*	1	7.69	7.69	35.28	<0.0001
*P*M*	1	0.87	0.87	3.98	0.064
*T*M*	1	17.37	17.37	79.70	<0.0001
Error	15	3.27	0.22		
Lack-of-fit	13	3.02	0.23	1.90	0.397
Pure Error	2	0.24	0.12		
Total	26	76.92			

Df: Degree of freedom; SS: Sum of squares; MS: Mean square; *Ti*: Time; *P*: Pressure; *T*: Temperature; *M*: Moisture content in biomass

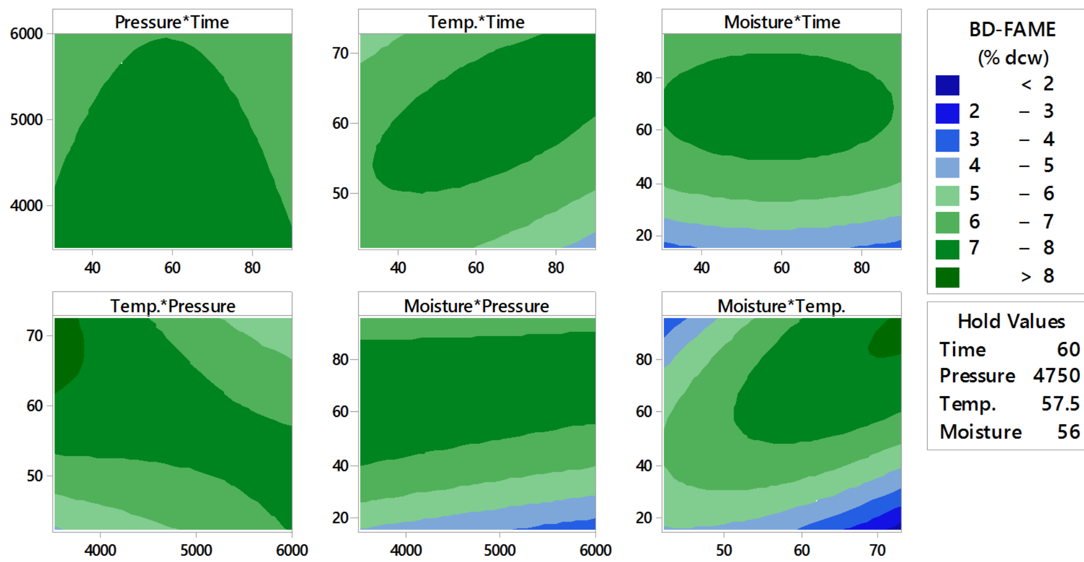

Figure 7.3. Contour plots showing the effects of SC-CO2 operating parameters (pressure (psi), time (min), temperature (°C), moisture (% *w/w*)) on BD-FAME content (% dcw) in lipid extraction from *Chlorella saccharophila*.

BD-FAME model. This deviation may be attributed to the great variation in FAME composition across the tested runs. The quantity of BD-FAME was noticeable in every run and made up anywhere from 28.6 to 69.4 % of the total FAME, Table 7.2. Thus, integration of GC data for quantification was much clearer with prominent peaks. Whereas some of the other FAMEs may have gone unaccounted for as a result of lower quantities making identification difficult.

Furthermore, the interaction of time and temperature was more pronounced for the BD-FAME than T-FAME, as greater content was achieved at higher temperatures and greater reaction times. As stated previously, higher temperatures resulted in improved component volatility (Halim et al., 2011), thus greater FAME contents are achieved with the greater solvating power. Santana et al. (2012) noted that increasing the reaction time from 10 to 150 min resulted in improved lipid recoveries from 3 to 14 % using *Botryococcus braunii*. Char et al. (2011) also reported improved lipid yields (up to 35 %) with increased reaction times up to 3 h.

The optimized T-FAME and BD-FAME yields of 20.4 and 9.8 % (dcw), respectively, achieved in this study (Table 7.4) were comparable to FAME recoveries from other studies in the literature using SC-CO_2 extraction technology. In the work by Taher et al. (2014), they reported that the T-FAME content achieved using SC-CO_2 extraction from wet *Scenedesmus* sp. was 12.5 % (dcw) using enzyme disrupted biomass. The proportion made up by the BD-FAME was 74 % of the T-FAME content, which is close to the upper range determined in this study, Table 7.2. Further, the study by Santana et al. (2012) on *Botryococcus braunii* demonstrated that the lipid extracted (17.6 % dcw) using SC-CO_2, varied with operational parameters, and that the majority of lipids were primarily made up of FAME (98.8 % of total lipids). They also noted that the BD-FAME made up 6.8 % of the dcw, which is closer to the proportions found in this study. The variation in the results may be attributed to the different disruption techniques used, operational parameters and the lipid content of each species, which is influenced by the nutrient availability in the media (Chinnasamy et al., 2010; Isleten-Hosoglu et al., 2012; Taher et al., 2014) and the growth stage of the cell (Zheng et al., 2011; Lai et al., 2016; Yodsuwan et al., 2017). It was observed by Zheng et al. (2011) that increasing the culture age resulted in increased lipid yields in *C. vulgaris*, and they attributed this to the continued assimilation of lipid in

the stationary phase as the substrate for lipid synthesis is CO_2. Similarly, the work of Yodsuwan et al. (2017) also demonstrated an increase in the lipid content of *Phaeodactylum tricornutum* with culture aging, as well as media type.

The optimized parameters for achieving the highest BD-FAME of 9.8 ± 0.49 % (dcw) were predicted at a reaction time of 86.4 min, pressure of 3500 psi, temperature of 73°C and a moisture content of 86.2 % (*w/w*). These parameters vary only slightly from the optimized parameters for T-FAME extraction, Table 7.4, as at the optimized BD-FAME parameters, the T-FAME content of 20.1±1.63, is only 0.3 % less than that achieved at the optimal and falls within one standard deviation of the optimized response for the T-FAME model. When considering optimized operational parameters, the conditions of operation must be justified. For instance, the highest attainable BD-FAME of 9.8 % (dcw) requires the sample to have a moisture content of 86 % (*w/w*), which would require drying to reduce the moisture from the original level of 96.4 % (*w/w*). If this is compared to FAME content in lipids from SC-CO_2 extraction at the optimized conditions but using undried sample (with original moisture content) instead, this results in a predicted BD-FAME content of 9.6 % (dcw), Table 7.4. Thus, one must consider whether the added expense of drying or moisture removal is justifiable from an economic stand point. Similarly, the same argument applies to the recovery of T-FAME, as shown in Table 7.4. The operational parameters of 30 min, 3500 psi, 73°C and 96.4 % (*w/w*) moisture resulted in predicted T-FAME and BD-FAME contents of 16.5 and 7.8 % (dcw), respectively, which are very close to the optimal values. At these parameters one could improve the overall economics of T-FAME recovery, by processing three times the biomass in the same time frame as that proposed by the optimal conditions (run time of 90 min), obtaining three times the T-FAME yields.

7.5 CONCLUSIONS

The SC-CO_2 extraction of lipids from surfactant-exposed *Chlorella saccharophila* biomass was optimized based on FAME content using RSM in order to maximize recoveries. Although lower moisture contents were predicted for maximum recovery of T-FAME and BD-FAME (20.4 and 9.8 % (dcw)), T-FAME and BD-FAME yields of 16.5 and 7.8 % (dcw), could be achieved using undried biomass at a moisture content of 96.4 %

(*w/w*), while the other extraction conditions suitable for industrial processing and high recovery were at 30 min, 3500 psi, and 73°C. There are advantages in being able to process the microalgae without needing to reduce the moisture content by drying, as this can substantially reduce the time and energy required. Such considerations must be made for practical application on a large scale.

FOREWORD TO CHAPTER 8

The purpose of Chapter 8 is to explore the use of surfactant as a combined harvesting aid and cell disruption agent. Here, the effect of surfactant on the extraction of lipids from *Chlorella saccharophila* is investigated by comparing: (1) biomass without surfactant treatment; (2) biomass from dispersed air flotation with no hold time and (3) biomass from dispersed air flotation with 24 h hold time. The previous experimental work in Chapters 6 and 7 was used to select operating conditions for harvesting by surfactant aided dispersed air flotation and SC-CO_2 extraction. The effectiveness of the surfactant treatments was evaluated by considering the FAME yield and composition.

CHAPTER 8 EFFECT OF DIFFERENT HARVESTING/ PRE-EXTRACTION CONDITIONS ON THE COMPOSITION OF LIPIDS EXTRACTED FROM *CHLORELLA SACCHAROPHILA* MICROALGAE BY SUPERCRITICAL CO_2

This chapter has been submitted to the journal of *Algal Research*.

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8.1 Abstract

Surfactant aided dispersed air flotation is a microalgae harvesting technique, where foam is produced from a surfactant solution for the recovery of microalgae, which can then be used for biodiesel production. However, during the harvesting process the surfactant may also cause cell disruption and enhance product recovery. In this study, *Chlorella saccharophila* cells were exposed to the surfactant CTAB both during and after harvesting via dispersed air flotation. Supercritical carbon dioxide (SC-CO_2) extraction was performed and the recovery of fatty acid methyl esters (FAME) from the biomass was determined and compared with the control which had not been exposed to surfactant. The control resulted in a total FAME (T-FAME) and biodiesel dominant (BD-FAME) content of 5.3 and 2.4% (dry cell weight (dcw)), respectively, whereas, the T-FAME and BD-FAME content from biomass harvested by dispersed air flotation with no hold time and 24 h hold time were 12.4±0.10 and 5.5±0.11% (dcw) and 16.6±0.51 and 7.6±0.33% (dcw), respectively. The composition of the T-FAME recovered from the various runs consisted predominantly (46% *w/w*) of the BD-FAME (C16:0, C18:0, C18:1n9*cis*, and C18:2n6*cis*). However, the proportion of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the lipids for the biomass control were 78, 10 and 12% (*w/w*), whereas, the proportions for the biomass harvested by dispersed air flotation with no hold time and 24 h hold time were 42, 19 and 39% (*w/w*), and 52, 16 and 32% (*w/w*), respectively. In all cases SFA and MUFA were most predominant, which is most desirable for biodiesel. Therefore, the FAME profile from centrifuged biomass without surfactant treatment, is most suited for biodiesel. However, surfactant treatment

was able to increase the levels of PUFAs, which could be recovered as high-value added byproducts for human health, and potentially improve the overall process economics.

8.2 Introduction

Microalgae-derived biodiesel is regarded as a renewable, environmentally sustainable form of energy, capable of substituting for diesel and meeting global demand (Huang, Zhou, & Lin, 2012; Milano et al., 2016). However, the high costs involved in the downstream processing of the biomass are a drawback of this technology (Davis, Aden, & Pienkos, 2011). One of the techniques for improving recovery yields is by using cell disruption. These methods include either chemical or mechanical techniques such as sonication, microwave radiation, enzymatic and surfactant (Günerken et al., 2015; Kim et al., 2016). However, the effectiveness of the technique is dependent on the microalgae species, cell wall composition, age of the culture (Byreddy, Gupta, Barrow, & Puri, 2015) as well as cell size and cell wall thickness (Wang, Yuan, Jiang, Jing, & Wang, 2014).

Cell disruption techniques that are used for the production of microalgae-derived biodiesel need to be scalable and low cost. With these considerations, some disruption techniques are less suitable than others due to their high energy requirements or poor scalability (Alhattab, Ghaly, & Hammoud, 2015; Huang & Kim, 2013). These include sonication treatments that have been noted to vary in energy from 1.7 to 119 Wh g^{-1} (Fu, Hung, Chen, Su, & Wu, 2010; Zheng et al., 2011; Cho, Oh, Park, Lee, & Park, 2013; Surendhiran & Vijay, 2014) as well as possess scale up difficulties (Guldhe, Singh, Rawat, Ramluckan, & Bux, 2014; Wang et al., 2014). Bead milling treatments have also been employed for cell disruption, however, this technique is not suitable for operation at an industrial scale (Lee & Shah, 2012). Microwave radiation is a scalable technique, however recovered products are susceptible to degradation as a result of thermal treatment (de Boer, Moheimani, Borowitzka, & Bahri, 2012; Munir, Sharif, Shagufta, Saleem, & Manzoor, 2013; Günerken et al., 2015). Alternatively, surfactant treatment for improving product recovery, has proven effective in disrupting microalgae cells (Huang & Kim, 2013; Lai, Francesco, Aguinaga, Parameswaran, & Rittmann, 2016), and is suitable for large scale operation, with food-grade surfactants, such as cetyl trimethylammonium bromide (CTAB), being available (Thimmaraju, Bhagyalakshmi, Narayan, & Ravishankar, 2003).

The proposed mechanism for surfactant cell disruption with CTAB is based on two interactions, electrostatic and hydrophobic (Rupprecht & Gu, 1991). Electrostatic interaction is the initial step of the adsorption process (Huang & Kim, 2013), whereby the positively charged cationic surfactant head groups present in CTAB, electrostatically bind to the negatively charged microalgae surface (Rupprecht & Gu, 1991; Gilbert & Moore, 2005). Hydrophobic interactions then occur, where the hydrophobic constituents of the cytoplasmic membrane interlock with the hydrophobic tail ends of the surfactant, forming micelles that result in extracellular disruption (Cabral, 1992; Gilbert & Moore, 2005; Brown & Audet, 2008; Ulloa et al., 2012; Huang & Kim, 2013; Nasirpour, Mousavi, & Shojaosadati, 2014).

A harvesting technique that requires surfactants to assist in the recovery of microalgae cells is foam-aided dispersed air flotation. It is a promising method due to its high cell recoveries and good concentration ratios, ease of operation, and its suitability for processing large volumes (Alhattab & Brooks, 2017). Recent studies have investigated the use of surfactants as agents in microalgae cell recovery from dilute liquid suspensions using foam-aided dispersed air flotation (Phoochinda & White, 2003; Phoochinda, White, & Briscoe, 2005; Coward, Lee, & Caldwell, 2013; Coward, Lee, & Caldwell, 2014; Garg, Wang, & Schenk, 2014; Garg, Wang, & Schenk, 2015). As the surfactant can act as a cell disruption agent in addition to a harvesting aid, this property can be exploited during the cell harvesting process. By combining these downstream processes of harvesting and cell disruption into a single operation, there is potential in improving the economics of microalgae-derived biodiesel production (Huang & Kim, 2013; Kim et al., 2016). Thus, the aim of this study was to investigate the effect of surfactant on the extraction of lipids from *Chlorella saccharophila* by comparing: (1) biomass without surfactant treatment; (2) biomass from dispersed air flotation with no hold time and (3) biomass from dispersed air flotation with 24 h hold time. SC-CO_2 was used to extract lipids from all the biomass samples and the lipids were analyzed for FAME content.

8.3 MATERIALS AND METHODS

8.3.1 Chemicals

The chemicals used in this study were purchased from Sigma-Aldrich (Oakville, Ontario, Canada) and Thermo Fisher Scientific (Ontario, Canada).

8.3.2 Microalgae Cultivation

Chlorella saccharophila (ATCC® 30408^{TM}, Manassas, VA, USA) was grown in an open pond system (Al-Hattab & Ghaly, 2014) for a period of 10 d. A Fitzgerlad medium (Hughes, Gorham, & Zehnder, 1958) was used for cultivation, supplemented with 85.7 mg of NH_4NO_3, 194.3 mg of $(NH_4)_2HPO_4$, 94.3 mg of NH_4SO_4 and 1.3 g of sodium bicarbonate per litre of distilled water. A total of 10 L was grown in each batch for experiments. Biomass growth occurred at room temperatures and followed a photoperiod of 14 h light, 10 h dark cycle. The resultant liquid suspension had a total suspended solids content (TSS) of 0.02 % (*w/w*), which was harvested using surfactant aided dispersed air flotation at the end of the cultivation period.

8.3.3 Microalgae Harvesting

Microalgae cells were harvested from dilute liquid suspension (TSS of 0.02 % *w/w*) using different harvesting/pre-extraction conditions: (1) biomass without surfactant treatment; (2) biomass from dispersed air flotation with no hold time and (3) biomass from dispersed air flotation with 24 h hold time. Figure 8.1 summarizes the conditions used to prepare the different biomass samples.

8.3.3.1 Biomass without Surfactant Treatment (Control)

Biomass obtained at the end of the 10 d cultivation period was recovered by centrifugation (Sorvall T1, Thermo Fisher Scientific, Ontario, Canada) at 2000 rpm for 2 min to a TSS of 2.7% (*w/w*). The supernatant was removed and the biomass pellets were dried using a vacuum oven operating at 45°C and 26 psi to achieve a TSS of 3.6 % (*w/w*). The resulting biomass was collected for SC- CO_2 extraction.

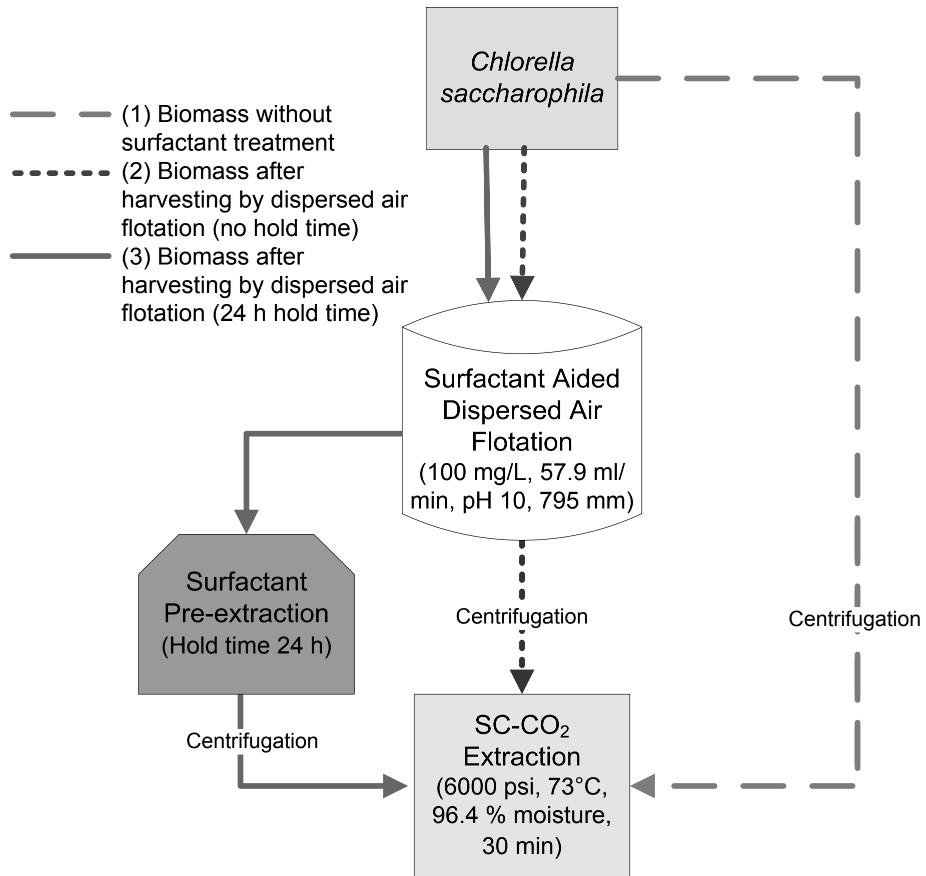


Figure 8.1. Experimental flow chart of the effects of harvesting/pre-extraction techniques on *Chlorella saccharophila* using SC-CO_2.

8.2.3.2 Biomass from Dispersed Air Flotation (No Hold Time)

The dispersed air flotation unit used to harvest the microalgae and simultaneously expose the biomass to surfactant is shown in Figure 8.2. The unit consisted of an air supply, diffuser to create bubbles, culture and foam collection chambers, and an adjustable column that was set to 795 mm in height. For each run, 3.5 L of dilute cell suspension obtained after the 10 d cultivation period was used. Initially, the suspension was adjusted to a pH of 10 using 0.5 M sodium hydroxide, followed by the addition of cetyl trimethylammonium bromide (CTAB) to make up a concentration of 100 mg/L. The mixture was stirred for 5 min using a Thermix Stirring Hot Plate (Model 310T, Fisher Scientific, Ottawa, Ontario), operating at 400 rpm. Once complete the suspension was transferred to the culture chamber of the unit, Figure 8.2. Air was supplied to the unit at a rate of 57.9 ml/min and left to run for 45 min to allow the foam and accompanying biomass to travel up the column to the foam collection chamber. At the end of the harvesting run, the biomass slurry in the foam collection chamber (resulting from the collapsed foam and associated microalgae) was collected then centrifuged at 2000 rpm for 2 min to a TSS of 3.6% (*w/w*). The supernatant was removed and biomass pellets were collected and combined to ensure homogeneity prior to SC-CO_2 extraction.

8.2.3.3 Biomass from Dispersed Air Flotation (24 h Hold Time)

Biomass was harvested using dispersed air flotation as previously described, however, the biomass slurry remained in the foam collection chamber, undisturbed for 24 h prior to centrifugation to ensure greater exposure to the CTAB surfactant. After the 24 h hold time, the biomass slurry was centrifuged at 2000 rpm for 2 min to a TSS of 3.6% (*w/w*). The supernatant was removed and biomass pellets were collected and combined to ensure homogeneity prior to SC-CO_2 extraction.

8.3.4 Procedure for SC-CO_2 Extraction

In order to prepare for SC-CO_2 extraction, 2.5 g of the centrifuged biomass samples with a TSS solids of 3.6 % (*w/w*) were placed on pre-weighed filter paper (415, VWR International, Ontario, Canada) such that 90 mg of total dry solids were deposited.

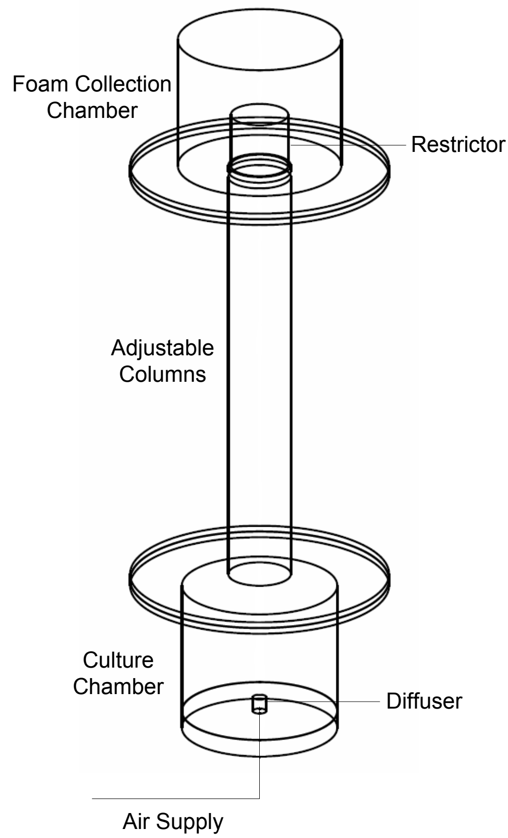


Figure 8.2. Schematic diagram of the unit used for harvesting microalgae via surfactant aided dispersed air flotation.

Two replicates were prepared in this manner, for each biomass type and stored at -20°C until SC-CO_2 extractions were conducted. SC-CO_2 extraction was carried out using a bench top supercritical fluid extractor (SFT-110, Supercritical Fluid Technologies, Inc., Delaware, USA) at the operational conditions of 6000 psi, 73°C, 96.4 % moisture content (*w/w*) and 30 min run time.

8.3.5 Transesterification of the Extract

The extract obtained from SC-CO_2 extraction was transesterified to form FAME for quantification. The procedure was adapted from Laurens et al. (2012) for use on biomass quantities of 90 mg (dry solids). Briefly, the recovered extract, was initially transferred to a 10 ml test tube, to which 2.6 mL of chloroform/methanol (2:1 *v/v*) was added, followed by 3.9 ml of sulfuric acid/methanol (5 % *v/v*) mixture. Following this addition, the tube was flushed with N_2 and vortexed. Finally, the tubes were placed in a heating block (No. 88871002, Thermo Scientific, Ontario, Canada) set at 85°C for 1 h. Once the reaction was complete, the tubes were removed from the heating unit and left to cool at ambient temperature for 15 mins. Next, the samples were transferred to a 50 mL conical tube and 13 ml of both hexane and distilled deionized water were added. The tubes were flushed with N_2 and vortexed and centrifuged at 1,200 *g* for 10 min. The organic layer was transferred to a 15 ml conical tube, a small scoop of anhydrous sodium sulfate was added and allowed to settle for 10 min. Finally, the top layer was again transferred, leaving behind the sodium sulfate crystals. The hexane was evaporated using a rotary evaporator (HiTEC RE-51, Yamato Scientific America, California, USA) set at 32°C, and then 1 mL of hexane was added to the sample for GC-FID quantification.

8.3.6 FAME Quantification

Quantification of FAME was performed by gas chromatography with a flame ionization detector (GC-FID) (Agilent 7890B chromatograph, Mississauga, Ontario, Canada). Peak identification was performed using Supelco 37 Component FAME Mix (CRM47885, Sigma Aldrich, Oakville, Ontario, Canada). Standard curves were prepared for quantification of each FAME peak by diluting the Supelco 37 by factors of 0, 2, 2.5, 3.33, 5 and 10, using GC grade hexane (97 %, Sigma Aldrich, Oakville, Ontario, Canada). A DB-23 column (60 m length x 0.25 mm ID, 0.15 μm film) was loaded with 1 μL of

sample using a split ratio of 1/50 heated to 250°C. The column temperature was held at 50°C for 1 min, and increased by 25°C/min to 175°C, where a hold time of 4 min was placed. After which, an increase in temperature of 4°C/min was maintained until 230°C, and held for 5 min. The detector temperature was set to 280°C with hydrogen as the carrier gas flowing at 40 mL/min. Samples were integrated to determine yields using SD ChemStation® F.01.03.2357 Software (Agilent Technologies Inc.) set to auto-integrate with an initial threshold of 13, initial peak width of 0.013, and with the shoulder detection turned off. All GC-FID sample analysis was performed in duplicates, in this manner.

8.3.7 Data Analysis

Variation in FAME content in the SC-CO_2 extracts for the different biomass samples were compared for statistical differences using the Tukey grouping comparison (α=0.05) in Minitab® (version 17.3.1, Minitab Inc., State College, PA, USA).

8.4 Results and Discussion

SC-CO_2 extraction of T-FAME and BD-FAME from *Chlorella saccharophila* using various harvesting/pre-extraction techniques was explored in this work. The results of the T-FAME and BD-FAME extraction using SC-CO_2 at operational parameters of 6000 psi, 30 min, 96.4 % moisture content (*w/w*) and 73°C, are tabulated in Table 8.1. The averages are based on two sample replicates each with GC-FID analysis performed in duplicate. The results obtained indicate that the mean values for each sample type were significantly different from one another. The T-FAME and BD-FAME content of the biomass without surfactant treatment was 5.3±0.50 and 2.4±0.24 % (dcw), respectively. In comparison, biomass from dispersed air flotation with no hold time, resulted in a T-FAME and BD-FAME content of 12.4±0.10 and 5.5±0.11 % (dcw), respectively, while the additional 24 h hold time resulted in contents of 16.6±0.51 and 7.6±0.33 % (dcw), respectively. This indicates that the T-FAME yields from the biomass harvested using dispersed air flotation with a 24 h hold time (and the longest exposure to surfactant) improved both T-FAME and BD-FAME contents by a factor of 3 times that of the control biomass. Similar findings were attained for surfactant exposed biomass (without a pre-extraction hold time), in that T-FAME and BD- FAME yields improved by 2.3 times that of biomass recovered by

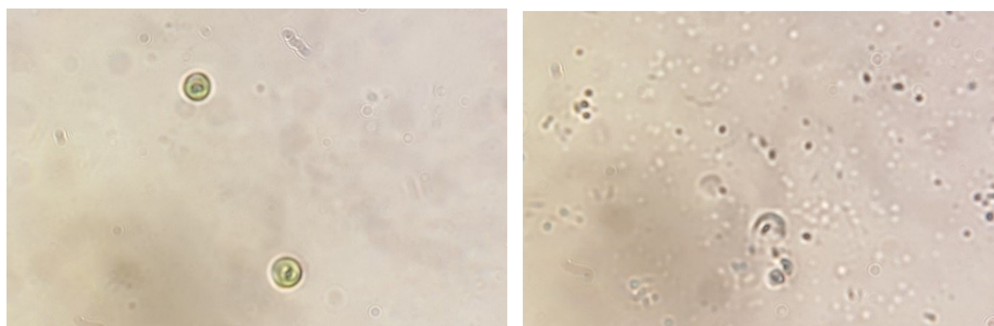
Table 8.1. Effect of various harvesting/pre-extraction techniques on the SC-CO_2 extraction of T-FAME and BD-FAME from *Chlorella saccharophila*.

Sample Type	SC-CO_2 Operational Parameters				FAME (% dcw) $^{\alpha}$		Tukey Pairwise (α=0.05)*
	Ti (min)	*P* (psi)	*T* (°C)	*M* (%)	Total	Biodiesel Dominant	
Without surfactant	30	6000	68	96.4	5.3±0.50	2.4±0.24	A
Dispersed air flotation - no hold time	30	6000	68	96.4	12.4±0.10	5.5±0.11	B
Dispersed air flotation – 24 h hold time	30	6000	68	96.4	16.6±0.51	7.6±0.33	C

Ti: time; *P*: pressure; *T*: temperature; *M*: moisture; $^{\alpha}$Mean of replicates ± standard deviation; *Means are significantly different if they do not share the same letter.

centrifugation without surfactant treatment. The variation in FAME content achieved across the various sample types can be attributed to the presence of the surfactant CTAB used in the harvesting step to recover the biomass, where the additional surfactant exposure time of 24 h resulted in greater T-FAME and BD-FAME yields. The mechanism for which involves the electrostatic and hydrophobic interactions between the microalgae cell and surfactant (as discussed in Section 2.3.14). Here, it is thought that cell wall disruption occurs as a result of the surfactant's hydrophobic interaction with the phospholipid microalgae cell walls, in the formation of micelle structures that cause irreversible damage. The light microscopy images in Figure 8.3 show a comparison of *Chlorella saccharophila* cells before and after exposure to CTAB. It can be seen that the cells prior to surfactant treatment are circular with a defined shape, however after surfactant exposure, only fragments are visible, no well-defined cells were noted. The T-FAME contents obtained in this study are similar to those reported for *Chlorella saccharophila* by Chinnasamy et al. (2010) of 12.9 to 18.1 % (dcw).

There have been various studies in the literature showing that cell disruption of microalgae via chemical methods have improved product yields. For example, in the study by Lai et al. (2016), surfactant treatment of *Scenedesmus* resulted in as much as a 16 fold increase in FAME compared to undisturbed biomass, using isopropanol extraction. Similarly, Ulloa et al. (2012) found that the antioxidant extraction of α-Tocopherol, β-Carotene and gallic acid from *Tetraselmis suecica* was 3.1, 32.5 and 1.6 times greater, respectively, using the surfactant, Triton 114, compared to disruption with ultrasound. The recovery of lipids from wet *Scenedesmus* biomass using SC-CO_2 was studied by Taher et al. (2014). They found that wet biomass (TSS of 6.8 % *w/w*) extraction resulted in 4.3 % (dcw) lipid recovery yield, which was lower than that of freeze dried biomass (11 % (dcw) yield). However, they also reported that lysozyme cell disruption prior to SC-CO_2 extraction, improved the yields to 12.5 % (dcw). Similar results were observed in this study in that the recovery of FAME using SC-CO_2 extraction of wet *Chlorella saccharophila* biomass (TSS of 3.6 % *w/w*), harvested by dispersed air flotation with no hold time, was capable of improving yields by 2.3 times that of the non-surfactant exposed



(a) (b)

Figure 8.3. Light microscope (1000x magnification) images of *Chlorella saccharophila* (a) no surfactant and (b) after 24 h of cationic CTAB surfactant exposure.

biomass (control). To our knowledge, this is the first study reporting on the use of surfactant as a means for improving FAME yields in microalgae using SC-CO_2 extraction.

The composition of the T-FAME achieved using the various types of biomass are graphically depicted in Figures 8.4-8.6, and tabulated in Table 8.2. It can be seen that the composition of the extract was primarily of BD-FAME (46 % of T-FAME), which consists of palmitic (C16:0), stearic (C18:0), oleic (C18:1n9*cis*), and linoleic acid (C18:2n6*cis*) (Knothe, 2008; Rakesh et al., 2015). Interestingly, saturated fatty acids (SFA) were the predominant fatty acids in all sample types and made up 78±0.94 % of the T-FAME recovered from the biomass without surfactant treatment, and 42±0.57 and 52±0.55 %, of the T-FAME from biomass harvested by dispersed air flotation with no hold time and 24 h hold time, respectively. Polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA), however, were greatest in the biomass obtained from dispersed air flotation with no hold time, followed by biomass from dispersed air flotation with 24 h hold time, and were lowest in biomass without surfactant treatment.

In a similar study performed by Coward et al. (2014) using Folch extraction (Folch, Lees, & Sloane-Stanley, 1957), they observed that the FAME composition of biomass without surfactant treatment (recovered by centrifugation), was highest in PUFA (33 %), followed by MUFA (6.7 %) and finally SFA (6.0 %). They also compared this to surfactant exposed *Chlorella* biomass during harvesting by dispersed air flotation and reported that the SFA, PUFA and MUFA composition in the biomass was 6.4, 25.3 and 9.7 %, respectively. The BD-FAME (C18:0, C18:1n9*cis* and C18:2n6*cis*) made up 24.7 and 23.3 % of the FAME in biomass without surfactant treatment and surfactant exposed biomass, respectively. Furthermore, the authors noted that the greater SFA and MUFA of 0.4 % and 3 %, respectively, obtained from surfactant exposed biomass were significantly greater than that of untreated biomass. On the other hand, Taher et al. (2014) used SC-CO_2 extraction on wet *Scenedesmus* sp. biomass (TSS of 6.8 % *w/w*) and reported SFA, MUFA and PUFA contents of 29.6, 11.4 and 60.1 %, respectively. In addition, they also noted that disruption of the same biomass using lysozyme under SC-CO_2 extraction resulted in SFA, MUFA, and PUFA contents of 41.9, 4.7 and 53.6 %, respectively.

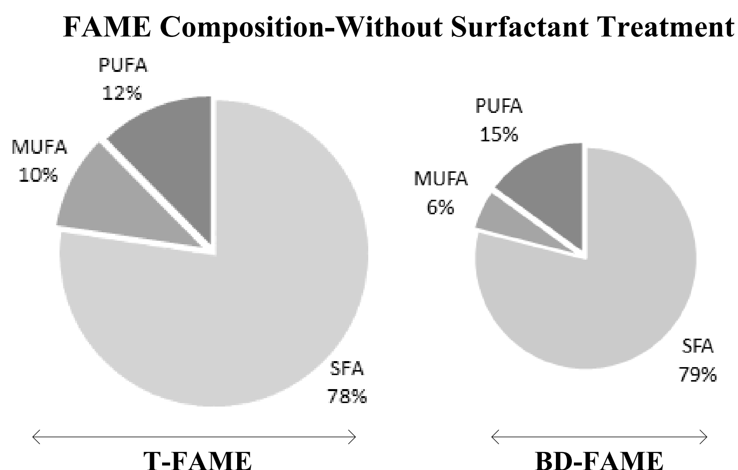


Figure 8.4. FAME composition of *Chlorella saccharophila* biomass without surfactant treatment, as percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), extracted using SC-CO_2 (30 min, 6000 psi, 68°C, 96.4 % (*w/w*) moisture content).

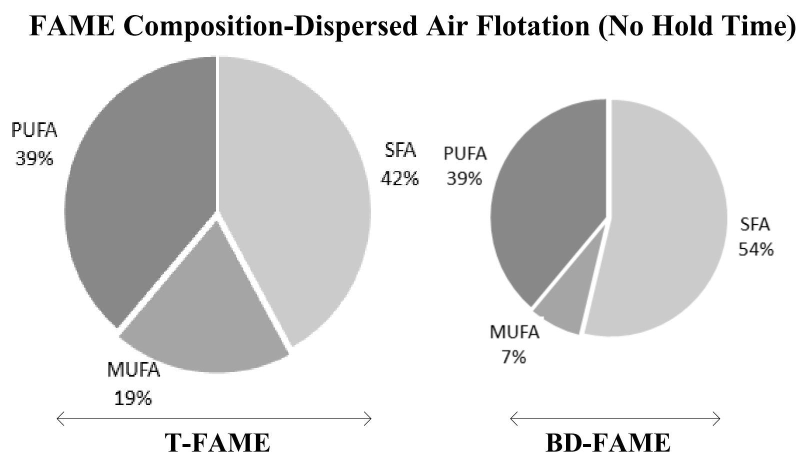


Figure 8.5. FAME composition of *Chlorella saccharophila* biomass from dispersed air flotation with no hold time, as percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), extracted using SC-CO_2 (30 min, 6000 psi, 68°C, 96.4 % (*w/w*) moisture content).

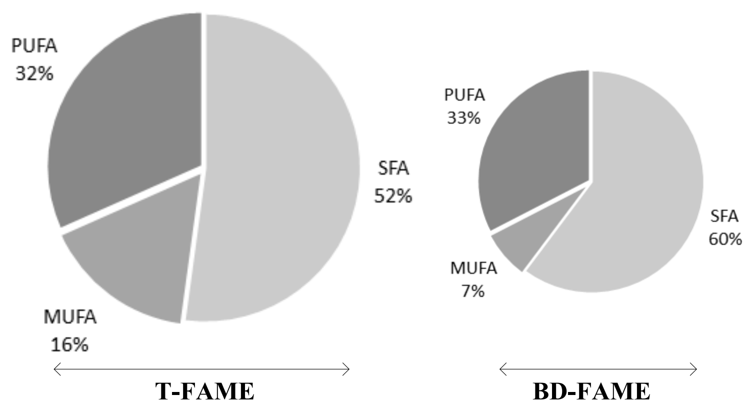


Figure 8.6. FAME composition of *Chlorella saccharophila* biomass from dispersed air flotation with a 24 h hold time, as percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), extracted using SC-CO_2 (30 min, 6000 psi, 68°C, 96.4 % (*w/w*) moisture content).

Table 8.2. Fatty acid composition (% *w/w*) of lipids extracted from *Chlorella saccharophila* using SC-CO_2 (30 min, 6000 psi, 68°C, 96.4 % moisture (*w/w*)) with different harvesting/pre-extraction methods.

Fatty Acid Type	Fatty Acid	Fatty Acid Composition (% (*w/w*) of T-FAME)		
		Without Surfactant Treatment	Biomass from Dispersed Air Flotation	
			No Hold Time	24 h Hold Time
SFA	C6:0	1.63	0.77	0.55
	C8:0	-	-	0.27
	C10:0	9.94	4.05	6.09
	C11:0	1.90	1.35	0.68
	C12:0	-	-	0.56
	*C16:0	16.31	16.78	15.13
	*C18:0	19.50	6.90	12.55
	C20:0	9.93	5.14	6.63
	C21:0	9.85	2.95	3.82
	C22:0	8.37	4.08	5.86
MUFA	C15:1	2.92	1.21	1.00
	C16:1	-	4.73	2.72
	C17:1	3.27	9.57	6.98
	*C18:1n9*cis*	2.86	3.26	3.31
	C22:1	1.16	0.46	2.16
PUFA	*C18:2n6*cis*	6.85	17.10	15.01
	C18:3n3	5.51	21.64	16.00
	C20:2	-	-	0.69

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; *BD-FAME

The significantly greater proportion of SFA in the biomass without surfactant treatment achieved in this study, may be attributed to the fact that complete extraction of FAME was not achieved (Table 7.3), as only 5.3 % of FAME were recovered (Table 8.1), therefore, unsaturated fatty acids may not have been extracted in detectable amounts to be integrated using the GC-FID integration parameters specified (threshold of 13). As for the MUFA and PUFA, the composition dramatically increased in biomass from dispersed air flotation with no hold time compared to biomass without surfactant treatment, which again maybe as a result of incomplete extraction of lipids in biomass without surfactant exposure. However, in comparing the two samples that were exposed to surfactant, we see that the pre-extraction treatment of 24 h liberated greater quantities of SFA, and lowered the PUFA contents. These results are in agreement with those of Lai et al. (2016), they also noted that increasing the pre-extraction surfactant treatment exposure time to 24 h, using hexane/isopropanol solvent system, lowered the PUFA composition from 38 to 18 %, and increased both the MUFA and SFA contents from 30 to 35 % and 32 to 47%, respectively, in intermediate-lipid biomass. This may also be attributed to the incomplete recovery of lipids, since the use of surfactants increased the recovery by up to 30 %. Unfortunately, no comparison in composition to untreated biomass was made. Furthermore, it was also observed by Taher et al. (2014), that compared to untreated biomass, disrupted biomass resulted in greater SFA and lowered the PUFA content. The results from this study are different from those observed by Coward et al. (2014) in that they reported no significant difference in the FAME content as a result of harvesting technique used. This variation maybe attributed to the lower quantity of CTAB used (10 mg/L) compared to that used in this study of 100 mg/L, as well as the lower treatment time of 30 min compared to the 45 min used in this study. In addition, the differences maybe a result of the different cultivation media used, and the difference in growth period of 12 d used by Coward et al. (2014) compared to that of 10 d used in this study.

With regards to biodiesel quality, it is desirable to have a greater proportion of SFA and MUFA in the FAME, as they increase the energy yield of the fuel, cetane number and also improve the oxidative stability (Coward et al., 2014; Renaud, Parry, & Thinh, 1994). In the biomass obtained from dispersed air flotation with no hold time and that with a 24 h hold time, the SFA and MUFA made up a total of 61 and 68 % (*w/w*) of the T-FAME,

respectively. These proportions are comparable to those obtained by Makareviciene et al. (2014), whereby they reported SFA, MUFA, and PUFA (% of T-FAME) in *Chlorella protothecoides* oil, cultivated under heterotrophic conditions, of 7.6, 65 and 27 %, respectively. Although the SFA and MUFA made up 72 % of the FAME composition, in this study a greater proportion of SFA were recovered compared to MUFA, with the opposite being true for the study by Makereviciene *et al*. This variation may be attributed to the different cultivation conditions used and the characteristics of the species, as it has been documented in literature that the microalgae fatty acid profile is a function of the culturing conditions used (Andrich, Nesti, Venturi, Zinnai, & Fiorentini, 2005; Guzman, de la Jara Valido, Adelina, Duarte, & Presmanes, 2010; Halim, Gladman, Danquah, & Webley, 2011). Further, although the harvesting technique used for the recovery of the biomass was not specified by Makereviciene *et al.*, this could also have affected the resultant FAME composition. It was demonstrated by Borges et al. (2011) that the two different types of flocculants (anionic and cationic polyacrylamide) used for harvesting *Nannochloropsis oculata* and *Thalassiosira weissflogii* resulted in varying FAME compositions based on type of flocculant used. They concluded that the harvesting conditions should be based on the level of saturation desired in the FAME. Coward et al. (2014) also reached a similar conclusion in their work on dispersed air flotation, where they determined that the harvest technique can impact the quality and quantity of the lipids.

The results of this study illustrate how the presence of the surfactant (CTAB), from microalgae harvesting or dewatering by dispersed air flotation, can be coupled as a pre-extraction treatment for cell disruption to improve FAME content from SC-CO_2 lipid extraction technology. The FAME yields improved significantly using biomass from dispersed air flotation with no hold time, compared to biomass obtained without surfactant treatment. The addition of a hold time of 24 h after dispersed air flotation, further improved FAME yields beyond those achieved with no hold time. Although the increase in FAME yields obtained from biomass harvested using dispersed air flotation, is partly attributed to the release of higher amounts of PUFA which are less desirable for biodiesel, they are considered essential fatty acids for human health and are used in pharmaceutical products (Pereira et al., 2012). Therefore, these fatty acids could be recovered as highly valued byproducts, which would improve the quality of biodiesel and provide an additional

revenue stream. This suggests that potential improvements in the overall economics of the process could be achieved, through the combination of cell disruption and harvesting processes for microalgae-derived biodiesel.

8.5 CONCLUSIONS

The results of this study demonstrate how the use of CTAB surfactant, present in biomass harvested using surfactant aided dispersed air flotation, can couple as a cell disruption technique for improving FAME recoveries using SC-CO_2 extraction from *Chlorella saccharophila*. Centrifuged biomass without surfactant treatment, resulted in a T-FAME and BD-FAME content of 5.3 and 2.4 % (dcw), respectively. These values were significantly improved by factors of 2.3 and 3 after harvesting by dispersed air flotation with no hold time and 24 h hold time, respectively. In this manner, the economics of microalgae processing can potentially be improved by using surfactant to simultaneously aid in the recovery of dilute microalgae cells from liquid suspension using the low energy consuming technique of dispersed air flotation, and aiding in microalgae cell disruption for improving FAME recoveries. The FAME composition recovered from the various types of biomass consisted predominantly (46 %) of the BD-FAME. However, the SFA, MUFA and PUFA compositions for the biomass control were 78, 10 and 12 % (*w/w*), whereas, the biomass obtained after harvesting by dispersed air flotation with no hold time and 24 h hold time were 42, 19 and 39 %, and 52, 16 and 32 % (*w/w*), respectively. These suggest that the biomass recovered without surfactant exposure resulted in a FAME composition most suitable for biodiesel production, however, surfactant treatment liberated greater quantities of PUFA which can be recovered as value added products for pharmaceuticals that are beneficial to human health. This would further improve the economics of the process as it offers an alternate source of revenue.

CHAPTER 9 CONCLUSIONS

9.1 Summary and Conclusions

This study investigated and developed a process for the large scale production of microalgae-derived biodiesel, focusing on harvesting, cell disruption and extraction stages. In this work, care was taken to select the most promising technologies for large scale production and these were then experimentally tested to predict the optimum FAME yields and resultant FAME composition.

To begin, **Chapter 2** reviewed the current literature on techniques used for microalgae harvesting, pre-extraction treatment and oil extraction methods. The information was used in **Chapter 3** to conduct a comparative analysis for the harvesting methods, where the assessment of large scale suitability was based on several criteria: dewatering efficiency, cost, toxicity, suitability for large scale use, time, species specificity, reusability of media and maintenance. The highest scoring techniques consisted of centrifugation, organic flocculation, surfactant aided dispersed air flotation, and filtration.

In **Chapter 4**, the cell wall structure of selected microalgae species and the effectiveness of various cell wall disruption techniques on product recovery were reviewed. Although the effectiveness of the technique is dependent on the composition and structure of the cell wall, it is difficult to compare the results from different studies due to the wide range in operating conditions and inconsistencies in the cultivation and harvesting conditions. Despite this, surfactant exposure was identified as a promising cell disruption method for *Chlorella saccharophila*, with the added benefit of the surfactant already being present in the harvesting method of surfactant aided dispersed air flotation. The use of surfactant as an aid in both harvesting and cell wall disruption couples two downstream processes into one, potentially limiting the costs typically necessary for other techniques.

Similarly, in **Chapter 5**, a comparative analysis was performed to determine a suitable large scale process for microalgae lipid extraction, which would be used in the experimental section of this thesis. Assessment was based on oil extraction efficiency, cost, toxicity, suitability for large scale use, time, pre-treatment requirement, reusability and maintenance. Supercritical CO_2 extraction scored the highest and had added benefits

over the other methods due to easy removal of the solvent and separation of the biomass. Although supercritical CO_2 suffers from high operational costs, these can be lowered by improving the process through cell disruption and optimization, furthermore, supercritical CO_2 technology is regarded as a green solvent as it can be recycled and reused.

In **Chapter 6**, an experimental investigation of surfactant aided dispersed air flotation for harvesting *Chlorella saccharophila* was conducted. This was performed by the optimization of the process parameters (surfactant concentration, pH, flow rate and column height), based on maximizing both the recovery and enrichment ratio, simultaneously. The results indicated that recovery was significantly influenced by the surfactant concentration and flow rate, however all tested variables were significant in enrichment. A maximum recovery and enrichment ratio of 95 % and 13, respectively, was predicted and experimentally validated at a CTAB concentration of 100 mg/L, air flow rate of 57.9 mL/min, pH of 10 and a column height of 795 mm. It was determined that a further reduction in water content would be necessary to concentrate the slurry to a TSS of 3.6 % (*w/w*) prior to supercritical CO_2 extraction in **Chapters 7** and **8**. However, the use of this technique as a preliminary step is beneficial as it is uses less energy compared to other methods, and reduces the processing volumes by up to 14 times, resulting in a lower usage of more energy intensive techniques, such as centrifugation.

Then in **Chapter 7**, the harvested *Chlorella saccharophila* biomass was used to investigate the SC-CO_2 lipid extraction process. The process parameters (reaction time, pressure, moisture content and temperature) were optimized using RSM to achieve maximum FAME recoveries. It was determined that greater FAME yields were attained using lower pressures, greater temperatures, higher processing time and greater biomass moisture content. The optimized operational conditions of 86.4 min, 3500 psi, 73°C and 86.2 % moisture suggested a T-FAME yield of 20 % (dcw), while further analysis to reduce the reaction time to 30 min and eliminate the need to dry the biomass (moisture content of 96.4 %), resulted in a comparable T-FAME yield of 16.5 % (dcw). From an industrial standpoint, the latter conditions are more favorable as the process economics can be improved by reducing the processing time to one third of that required for the optimized conditions and by eliminating the need to dry the biomass. This is significant, as a major

impediment for using supercritical CO_2 is the requirement of the biomass to be fully dried for effective extraction.

Finally, in **Chapter 8**, the effect of surfactant CTAB on the extraction of lipids from *Chlorella saccharophila* was investigated by comparing: (1) biomass without surfactant treatment; (2) biomass from dispersed air flotation with no hold time and (3) biomass from dispersed air flotation with 24 h hold time. The results from SC-CO_2 extraction showed that the biomass exposed to CTAB during harvesting with dispersed air flotation, resulted in FAME yields of 2.3 times higher compared to biomass without surfactant treatment. Furthermore, the biomass from dispersed air flotation with a hold time of 24 h, resulted in FAME yields that were 3 times higher than biomass without surfactant treatment. The surfactant exposure resulted in different FAME profiles, as biomass recovered without surfactant exposure had a FAME profile most suitable for biodiesel production, whereas, surfactant treatment resulted in more PUFA. This could further improve the economics of the process by offering an alternate source of revenue, as PUFA can be recovered as value added products for pharmaceuticals that are beneficial to human health.

To summarize, the specific conclusions to the original research objectives are as follows:

i) Surfactant aided dispersed air flotation was the most suitable harvesting and pretreatment technique, while SC-CO_2 extraction was the best oil extraction method for large scale processing of microalgae-derived biodiesel.

ii) The optimal conditions determined for both recovery and enrichment ratio using surfactant aided dispersed air flotation as a harvesting technique for *Chlorella saccharophila* were 100 mg/L CTAB, air flow rate of 57.9 mL/min, pH of 10 and a column height of 795 mm. At these conditions, a recovery and enrichment ratio of 95 % and 13, respectively, were attained.

iii) The optimized conditions for T-FAME (20 % dcw) extraction from *Chlorella saccharophila* using SC-CO_2 were 86.4 min, 3500 psi, 73°C and 86.2 % moisture. However, it was determined that lowering the run time to 30 min and eliminating the need to dry the biomass, resulted in good yields (16.5% dcw of

T-FAME). The latter conditions are more suitable for industrial scale as they improve the processing economics.

iv) The surfactant CTAB is effective for disrupting *Chlorella saccharophila* cells, as it does increase the recovery of FAME extracted with SC-CO_2. This finding is significant as the processing costs can be reduced when CTAB is used both during harvesting with surfactant aided dispersed air flotation and as a cell disruption agent. In addition, more PUFA are recovered with surfactant exposure, creating an added potential revenue stream.

9.2 Novel Contributions to Science

This study encompasses various avenues of novelty, since suitable means for microalgae downstream processing that are economically viable, have yet to be determined. The novel contributions are summarized below:

- A novel approach was used to assess and optimize the performance of surfactant aided dispersed air flotation on *Chlorella saccharophila.* This was based on maximizing both enrichment ratio and recovery of the biomass, simultaneously. Many of the recent studies employing this dewatering technique only investigate one of these response variables, even though both performance indicators are necessary to fully assess the effectiveness of the technique's dewatering ability. This is of particular significance since the method offers a promising low-cost technique that can be implemented for large scale use.
- The experimental results from this work indicate that good lipid recoveries can be achieved from SC-CO_2 extraction using "wet" biomass harvested by surfactant aided dispersed air flotation. This discovery is an important contribution, as it is generally accepted by most studies that the biomass should be completely dried prior to SC-CO_2 extraction, as moisture hinders the process. This is especially significant in identifying areas for process cost reductions, since most drying methods are energy intensive and can be expensive.
- The use of surfactant as a pretreatment for microalgae prior to lipid extraction using supercritical CO_2 is also unique. Studies in the literature have only

reported on the use of other cell wall disruption techniques with supercritical CO_2 extraction for lipid recovery.

- This study illustrates the feasibility of using surfactant to combine two microalgae downstream processes (harvesting and cell disruption) for improved lipid recovery with supercritical CO_2 extraction. Although some studies have shown that disruption treatments can improve FAME recovery from biomass, most of these require different equipment in comparison to the harvesting process. This is a significant contribution as the combined process has advantages of reduced costs and energy consumption in comparison to alternative processes involving two separate harvesting and cell disruption technologies.

9.3 Recommendations for Future Work

Surfactant aided dispersed air flotation and SC-CO_2 extraction show promise as a suitable large scale process for microalgae-derived biodiesel, as shown in this study. However, further work is recommended to further improve the process. These are as follows:

- During surfactant aided dispersed air flotation, the foam volume recovered was determined to be a function of flow rate, which implies that changing the runtime will also affect the foam volume as less foam would reach the collection chamber. Therefore it is suggested that the recovery and enrichment ratio be investigated over elapsed time to determine that necessary for maximizing these two indicators. It could be determined that the majority of the cells are harvested in less than the 45 mins tested here, which would result in greater enrichment since less additional foam (less total volume) will be carried to the surface.
- The cell biomass achieved in the cultivation process was dilute with a total suspended solids content of 0.02%. Further tests should be conducted on different samples obtained throughout the cultivation process and on more concentrated suspensions to determine whether the optimal conditions change.

- The feasibility of a continuous foam fractionation and dispersed air flotation system should also be tested, as large scale processes are more efficient in this form as opposed to batch runs.
- The remaining surfactant in the culture chamber of the batch run should be further foamed after treatment to recover the surfactant from water. This would limit the discharge of surfactant into the environment. Studies on recycling the collected foam in the process should also be investigated, as this could reduce the operating costs.
- The extract recovered from supercritical CO_2 extraction should also be analysed for other viable products that could be extracted from the biomass. This would also improve the economics of the process by providing other value added products.
- The impact of surfactant in improving the yield of other potential byproducts (such as various pigments) from microalgae should also be investigated, as microalgae offer a wide range of value added products, and the concept of a bio-refinery would significantly improve economics by offering multiple revenues.

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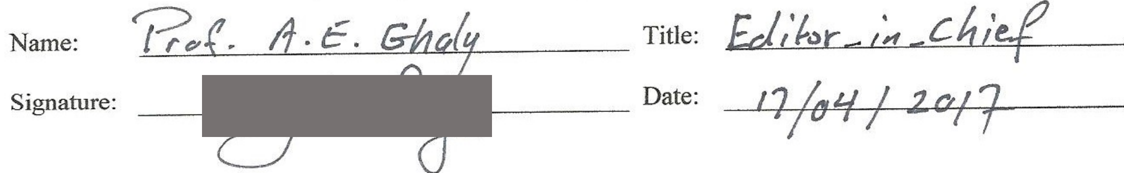

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APPENDIX B LIPID QUANTIFICATION USING COLORIMETRY PROCEDURE

A rapid lipid quantification colorimetric technique (Mishra et al., 2014) was used to determine lipid content in the microalgae culture after the 10 day cultivation period, to ensure that there was consistency between the biomass obtained from different production runs prior to SC-CO_2 extraction. This method is based on a sulpho-phospho-vanillin (SPV) reaction for direct quantification of lipids. Briefly, a standard curve (Figure B1) from commercial 100 % canola oil was prepared in 10 ml of chloroform, giving a final lipid concentration of 2.77 mg/mL. Aliquots of 0, 5, 10, 20, 30 and 35 µL were taken from the stock solution and transferred to 10 mL glass test tubes, and the solvent was subsequently evaporated for 10 min at 60°C. To each test tube, 100 µL of deionized water was added, and processed further using the SPV method described below.

The phospho-vanillin reagent was prepared by dissolving 0.6 g of vanillin in 10 ml of ethanol and 90 ml of deionized water, with continuous stirring (Thermix Stirring Hot Plate, Model 310T, Fisher Scientific, Ottawa, Ontario). Once dissolved, 400 mL of concentrated phosphoric acid was added and the reagent was stored in the dark until use. Aliquots of 100 µL of standard and cultured biomass (known cell amount) were placed in 10 mL test tubes, to which 2 mL of concentrated sulfuric acid were added. The mixture was heated at 100°C for 10 mins using a heating block (No. 88871002, Thermo Scientific, Ontario, Canada), and cooled for 10 mins. Following this, 5 mL of the phospho-vanillin reagent was added to the samples, which were then incubated at 37°C and 200 rpm in an incubator shaker (Series 25 New Brunswick Scientific Co., Inc. New Jersey, United States). The absorbance was read for all samples at 530 nm using a UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific, Ontario, Canada). All samples were prepared in duplicates.

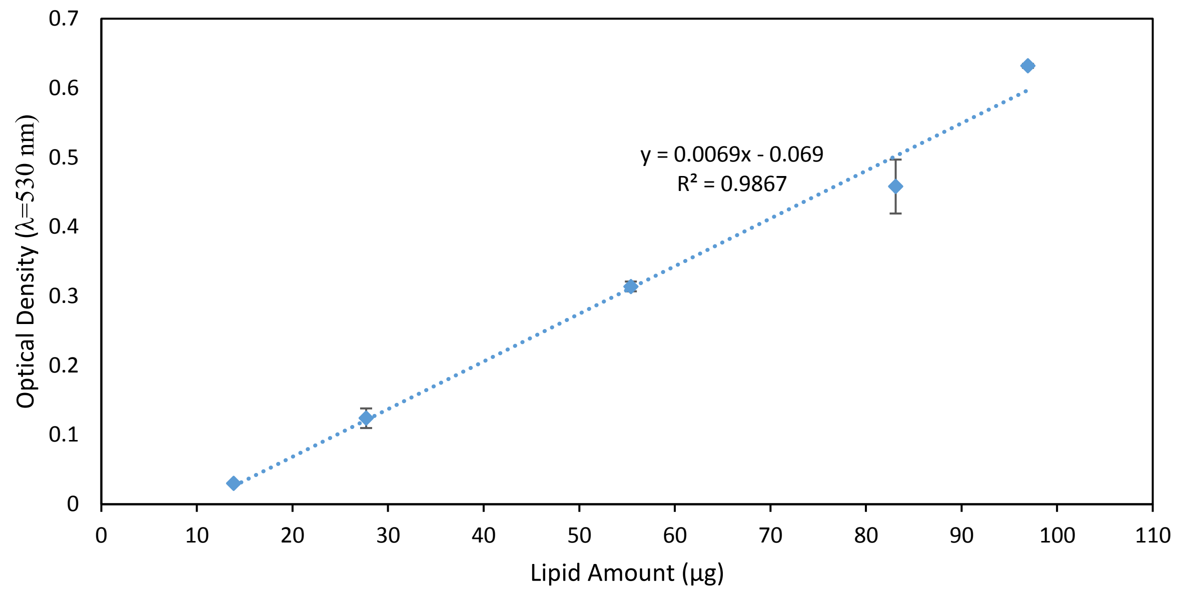

Figure B1. Correlation between lipid amount (µg) and optical density (λ=530 nm) using sulpho-phospho-vanillin technique.