

DEVELOPMENT OF AN ALGAL OIL SEPARATION PROCESS

A Thesis

by

NALIN UDAYANGA SAMARASINGHE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Biological and Agricultural Engineering

Development of an Algal Oil Separation Process

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August 2012

Major Subject: Biological and Agricultural Engineering

ABSTRACT

Development of an Algal Oil Separation Process. (August 2012)

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Chair of Advisory Committee: Dr. Sandun Fernando

Microalgae surpass the lipid productivity of terrestrial plants by several folds. However, due to the high moisture content and rigidity of algal cell walls, extraction of lipids from algae is still a significant technological challenge. In this research, an attempt was made to develop an algal lipid separation process which is energy efficient and effective.

Algal related research requires a unique set of knowledge in areas of algae culturing, measuring cell concentration, harvesting, cell rupturing and lipid quantification. The first section of this thesis focuses on the state of the art as well as knowledge gained during preliminary studies.

The second section of this thesis focuses on selecting a suitable measurement technique for quantification of algal cell disruption induced by homogenization. The selected method, hemocytometry was used to measure the degree of algal cell disruption induced by homogenization. In the third section, various homogenization treatments were evaluated for determining the fraction of cells disrupted during the homogenization.

Finally, lipid extraction efficiency of homogenized algae was evaluated using different extraction solvents under different homogenization conditions.

Preliminary research concluded that using cell counting is the most suitable technique to measure the effect of high pressure homogenization on concentrated microalgae.

It was observed that higher pressure and increased number of passes increase the degree of cell disruption. Concentrated, non stressed samples show best response to homogenization.

Out of the three solvents used for solvent extraction, chloroform gave a higher extraction yield at low intensity homogenizations. However at harsher homogenization levels the advantage of chloroform was not significant.

Lipid extraction efficiency increases with increased levels of homogenization. However, a significant increase in lipid yields was not detected beyond 20 000 psi and 2 passes of homogenization treatment.

DEDICATION

To my dear parents

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Fernando, and my committee members, Dr. Faulkner, Dr. Devarenne, and Dr. Thomasson, for their guidance and support throughout the course of this research.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

I also want to extend my gratitude to the staff of the Microscopy and Imaging Center (especially Dr. Stanislav Vitha for his helping us with the acquisition of optical microscopic images) and the Material Characterization Facility (especially Dr. Amanda Young for her assistance in obtaining spectrofluorometric data) at Texas A&M University

I appreciate Dr. Ronald Lacey's group from the Department of Agricultural Engineering-Texas A&M University, personnel of Texas Agrilife Research and its Algal Research facility, Pecos, Texas, for providing us with algal cultures for these experiments.

Finally I would like to thank the National Alliance of Advance Biofuels and Bioproducts (NAABB) via the Department of Energy, for providing funding for related research.

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1. INTRODUCTION: THE IMPORTANCE OF RESEARCH ON ALGAL LIPID EXTRACTION

Biodiesel can be considered to be one of the most attractive alternatives for fossil fuels in terms of emissions reduction, storability, renewability and safety¹⁻⁵. However, producing crop-based biodiesel is not sustainable^{6,7}. The major reasons for this are cost of feedstock and slow growth rate of terrestrial plants. Another limitation of using terrestrial oil seed crops for producing raw materials for fuel production is the direct competition for land available for production agriculture.

Fortunately, microalgae are available as an alternative to terrestrial crops for providing biomass and raw materials for fuels. Microalgae are a promising feed stock for biodiesel generation when compared to other conventional feed stocks due to their higher growth rate, high lipid composition, relatively lower cost and the potential to use marginal land that is not suitable for commercial agriculture². When subjected to nutrient deprivation; or when “Stressed”; microalgae accumulates lipids by compromising the gain of biomass⁸.

This thesis follows the style of *Energy and Fuels*.

However, there are no known algal oil producing plants operational at the moment – primarily due to the fact that most critical technical challenges related to algal lipid extraction are not yet fully overcome. Lipid extraction from algae is difficult in comparison to lipogenic terrestrial plants for two major reasons. Firstly, algal biomass contains about 99.9 % moisture. This amount can be reduced to about 90 percent using coagulation, but further moisture removal requires energy and capital-intensive operations like centrifugation and drying. High moisture decreases the effectiveness of conventional lipid extraction techniques like pressing and solvent extraction. Secondly, tough cell walls of microalgae make the lipid extraction process even more challenging.

Therefore, it is necessary to develop an algal lipid extraction method which can: (1) tolerate a considerable amount of moisture in its feedstock and (2) process cells that are small and have tough cell walls.

The hypothesis of this thesis is that high pressure homogenization of high-moisture algal slurry will liberate lipids from the cells. Once liberated, these lipids can be extracted via a suitable extraction solvent while the medium still contains significant quantities of moisture.

2. PROBLEM

Since lipid content and production efficiency of algae can surpass the lipid content of any lipogenic terrestrial plants⁹, using microalgal biomass constituents as substrates for fuel and chemicals production has attracted wide attention^{2, 10, 11}. However, there are several practical challenges that need to be overcome before algal based technologies become widely applicable. For example, the algal broth from a bioreactor contains close to 99% (w/w – wet basis) of moisture and prior to any type of processing, the moisture needs to be removed. Unfortunately, harvesting technologies like sedimentation and centrifugation can only reduce the amount of moisture to a level around 90% (w/w). Further removal of moisture can only be achieved via drying. Drying is energy intensive and removal of these magnitudes of moisture is cost prohibitive-especially if reliable and low cost solar energy is not available¹².

The small size of microalgae coupled with the presence of an intransigent cell wall¹³, require the cells to be ruptured prior to product extraction. The small cell size, thick cell walls and presence of large amounts of moisture makes such lysing extremely challenging and energy intensive¹⁴.

Accordingly, a cell extraction technique, to be successful for algal biomass, 1) needs to tolerate a high moisture environment and 2) needs adequate amount of cell rupture for the solvent to be accessible to lipid containing cellular matrix.

The hypothesis of this thesis is that high pressure homogenization of high-moisture algal slurry will liberate lipids from the cells. Once liberated, these lipids could be extracted via a suitable extraction solvent while the medium still contains significant quantities of moisture. Accordingly, the objectives of this study are:

- Objective 1: Measurement of the effect of homogenization conditions on the degree of cell breakage
- Objective 2: Evaluating the effect of the degree of cell breakage on oil extraction efficiency

Significant amount of preliminary research was needed for building experiences for doing this research. All the relevant preliminary research is listed under the Section 3 “Preliminary Research”

3. PRELIMINARY RESEARCH

Note: This work has been accepted to be published in Renewable Energy Journal (Elsevier).

Homogenization of Algae and Particle Size Detection

The rationale for homogenization is several fold. The most evident being the ability of a homogenizer to handle slurry phase materials in a continuous stream. It was hypothesized that the sudden pressure differential between the nozzle and the outside environment combined with shear forces generated when the high velocity fluid stream passes through the nozzle would induce rupturing of the cell walls. Similar cell lysing operations have been performed on yeast and *E. coli* cells¹⁵⁻¹⁸. Also, the increase of temperature during homogenization may weaken cell walls that in turn would help in the rupturing process. An attractive advantage of high pressure homogenization is the relative ease of scalability. For example, there are industrial scale systems that have throughput capacities over 50,000 liters/hr at moderate pressure (< 68.948 MPa) and up to 5,750 liters/hour at high pressure (up to 310.264 MPa).

The high-pressure homogenizer used for this study (NanoDeBEE, BEE International, (South Easton, Massachusetts)) is illustrated in Figure 1. The pressure applied on a sample could be varied between 10,000 (68.948 MPa) to 45,000 PSI (310,264 MPa). The average throughput of the equipment was 50 ml/min. For this study, three nozzles

with orifice diameters of 100, 130, and 190 μm were used. A reciprocating piston driven by pressurized hydraulic fluid was used to force the material through the nozzle creating a high velocity jet. This liquid jet was then forced through a homogenization cell where high intra-material shear forces were generated. These shear forces are dependent on the pressure applied, viscosity of the fluid, and the nozzle size used. In case of algae cell breakage, the resultant temperature (25 to 70⁰C) and the number of passes (1 to 6 passes) through the nozzle were also considered.

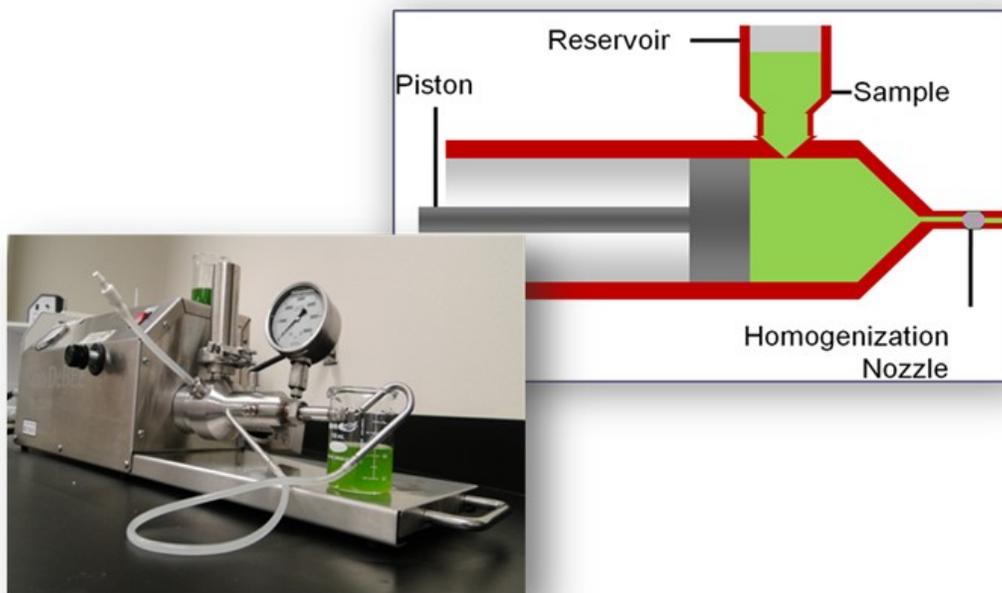


Figure 1. High-pressure homogenizer

An initial screening study was designed to detect whether homogenization had an effect on algal cell wall rupture. For this experiment, we used *Nannochloris oculata* (UTEX 1998) which have a particle size of approximately 1 – 2 μm per cell and were grown in Bold modified Basel media. During initial studies, a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Westborough, MA) with a Hydro SM attachment was used for quantitative particle sizing assessment while optical (Zeiss Axiphot with 20X and 100X objectives) and confocal laser (Olympus FV1000 with 60X objective) scanning microscopy was used for qualitative assessment.

Algal samples harvested using coagulation and centrifugation were used. The Malvern Mastersizer utilizes the Mie theory of laser diffraction¹⁹ and can detect particle sizes ranging from 0.1 μm to 200 μm . Particles were dispersed in methanol before injection into the Malvern. A particle refractive index of 1.46 (based on cellulose) and a dispersant refractive index of 1.36 were used. Particle size distributions of the algal samples were measured before and after homogenization at various pressures and number of passes Table 1 (A two level factorial design).

Figure 2 depicts the particle size distribution of algal broth after homogenization under varying conditions. The variables are named according to the labeling described in Table 2. The variable named “Algae-Raw” represents the control (non-homogenized) sample.

Table 1. Parameter variables used during the initial study

| Sample ID | Nozzle | Pressure | Number of Passes |
|------------|----------|--------------------------|------------------|
| Z5:25:2:1R | Z5-130um | 25 000 PSI (172.369 MPa) | 2 |
| Z5:40:1:1R | Z5-130um | 40 000 PSI (275.790 MPa) | 1 |
| Z5:40:2:1R | Z5-130um | 40 000 PSI (275.790 MPa) | 2 |
| Z5:25:1:1R | Z5-130um | 25 000 PSI (172.369 MPa) | 1 |

Initial algae concentration was approximately 35g/l solids.

A bimodal distribution was observed for homogenized algae. One peak was observed at 50 μm for the control algae, and 2 peaks were observed for homogenized algae around 38 and 58 μm . We conjecture that the peak at 50 μm represents algal flocs formed by the harvesting mechanism used (flocculation). However, some of those flocs may break into smaller ones during homogenization, yielding a bimodal distribution. In order to explain this observation, a series of microscopic images of samples prior to and after homogenization were analyzed. A representative selection of two such images is depicted in Figure 3. While some algal cells stayed segregated in the medium after homogenization, others tended to agglomerate, forming clusters. Also, larger cells were observed scattered throughout the sample, which we suspect to be contaminant (see Figure 3 (a) circled).

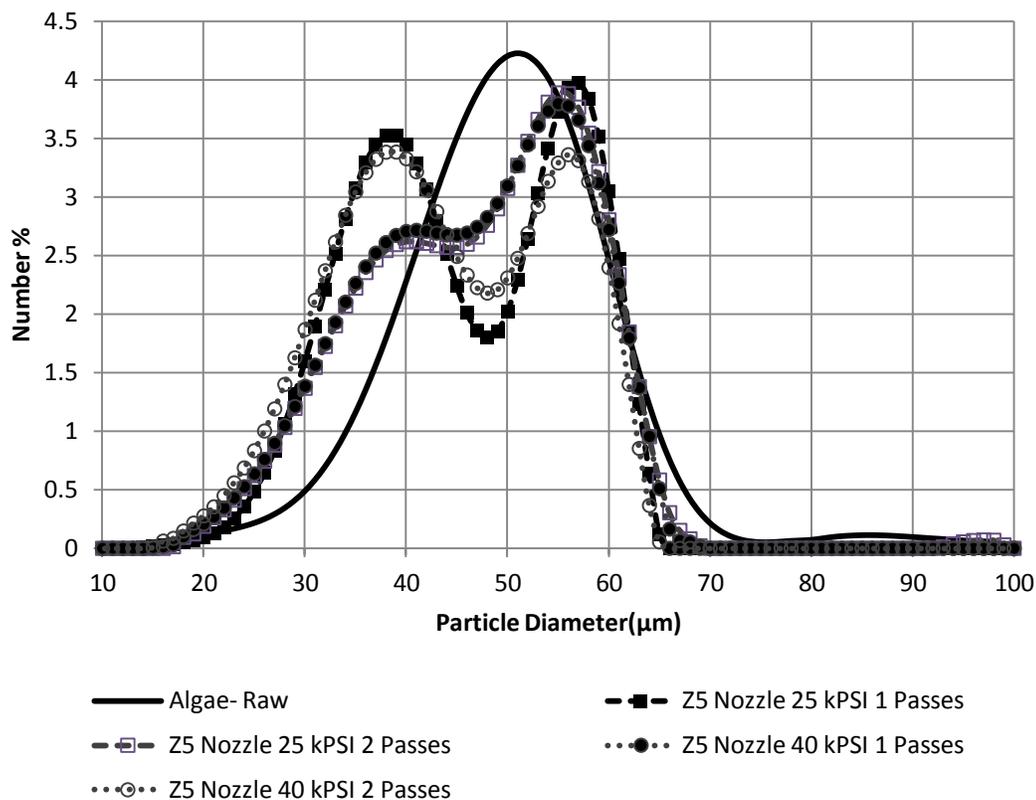


Figure 2. Particle size distributions of algal samples (from 10-100µm) after various homogenization treatments

Note: $25 \text{ PSI} \times 10^3 = 172.369 \text{ MPa}$ and $40 \text{ PSI} \times 10^3 = 310,264 \text{ MPa}$. Each line represents the average of three replicates.

Although a reduction of the average particle size was expected as a result of homogenization, we did not observe any significant, uniform shift of the particle size distributions. Regardless of the intensity variation of the variables, a significant correlation between any of the variables and particle size distribution was not observed. However, particle size distribution data did not match observations made with the

microscopic images, which showed that most of the algal cells were almost entirely ruptured after homogenization (see Figure 3(b)). Hypothesized explanations for this observation include:

1. The cells, though ruptured, retain their size and shape (analogous to a partially ruptured but intact rubber ball) while some agglomerate into flocs.
2. Since larger particles (in this case non disrupted cells and cell agglomerates) scatter a significantly larger amount of light as compared to smaller particles (in this case cell debris) at the same concentration, cell debris may get shielded thus not appearing in the particle size distribution data ²⁰.

In order to rule out the first possibility, a series of experiments were carried out with a particle sizer with a lower minimum detection limit (DelsaTM Nano-C, Beckman Coulter, Opa Locka, FL). If the first hypothesis is correct, particle size should increase or remain the same after homogenization. The detection range of this particle sizer was 0.6 nm to 10 μ m. A method was developed to measure the particle size of algae without using a special solvent. The following parameters for water were used in measurements: refractive index of 1.33; viscosity of 0.89 cP; and dielectric constant of 78.3.

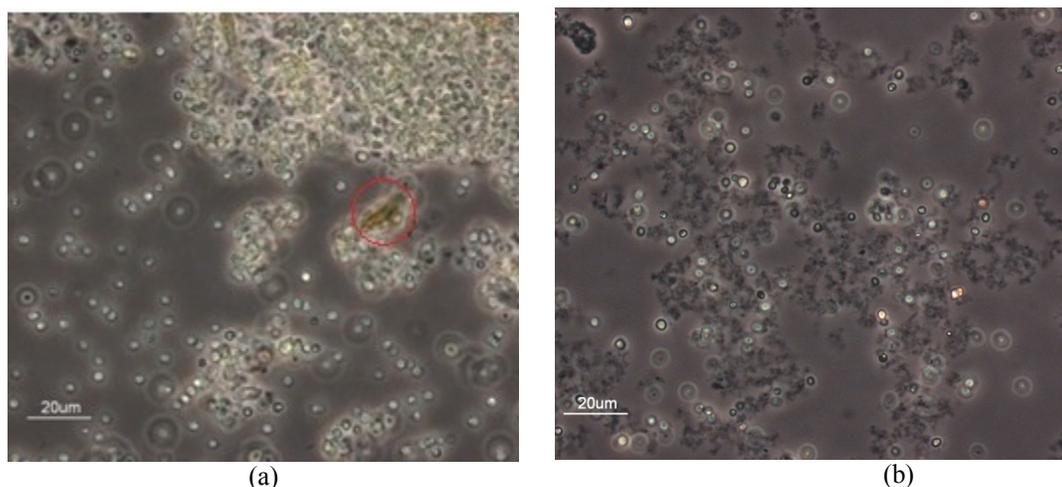


Figure 3. Live algae (left) and a homogenized algal sample (right)

(a; Stressed concentrated algae 35 g/l – Non treated, b; Stressed concentrated algae 35 g/l – Homogenized using Z8 (195 μm) Nozzle, 2 passes, 15 000 PSI (103.421 MPa))

A sample particle size distribution of *N. oculata* containing medium in its native state (non-homogenized) and after homogenizing four times through a Z8 Nozzle (195 μm diameter) at 10,000 PSI (68.948 MPa) is shown in Figure 4. Note that the samples were taken directly from the bio reactor, without using any harvesting mechanism like coagulation and/or centrifugation. Here, it was observed that the peak particle size was around 2 μm -unlike 50 μm in the previous study, which likely resulted from different harvesting procedures. Also, average particle size decreased after homogenization. Although we were able to obtain precise particle size readings for native algae, experiments with the addition of standardized particles of 0.1 μm (much smaller than that of algal cells) indicated that the particle sizer was not able to detect the smaller

particles when these co-existed with larger particles. It is hypothesized that the larger particles shield the smaller particles from detection (confirming our initial suspicion). This suggests that even if some fraction of algal cells is broken, the fraction of broken cells could escape undetected as the particle sizing equipment generates size distribution(s) as fractions (or percentages) of only detectable material. For example, if 50% of cells with initial diameter D were ruptured and all the broken cell material was shielded since those were small, the particle sizer would still predict that 100% cells are of diameter D after cell rupture. Thus the size distribution results before and after cell breakage may not give adequate insights to make effective, quantitative comparisons on how many cells were broken. As a result, we decided to abandon using particle sizers for detecting algal cell rupturing. Another consideration is that the whole algae has a negative surface charge and may actually attract and bond with the smaller particles.

Quantification of Cell Breakage via Hemocytometry

Subsequent to the first round of experiments, the suitability of using cell counting via hemocytometry to quantify the degree of algal cell disruption after homogenization was evaluated. A hemocytometer is a slide with a gridded chamber that attaches onto an optical microscope. The gridded chamber accepts a known volume of liquid sample. The number of microscopic bodies (such as algae) can then be counted via observation under the microscope. Since the volume of the sample is known, it is possible to calculate the cell density with reasonable accuracy.

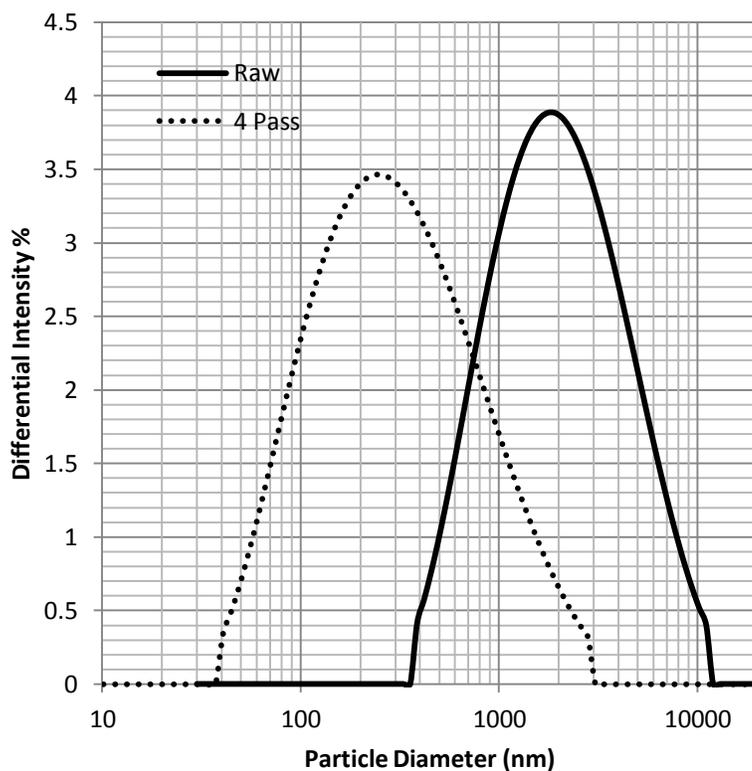


Figure 4. Particle size distribution of a raw *N. oculata* algal sample

Initial observations were conducted using a reusable hemocytometer with 100 μm chamber depth, for algal cell counting. However, this option was not considered to be feasible due to difficulties in cell counting resulting from overlapping cells in the 100 μm deep chamber. Alternatively, a disposable version of the cell counter (C-Chip DHC-S01 semen counting chambers from Incyto, Chungnam-do, Korea) was used with shallower chambers. These are Neubauer Improved counting chambers with 10 μl volumes designed for accommodating smaller cells.

For imaging the cells in the counting chamber, a Zeiss Axiphot optical microscope was used with 20x objective resolution. For an aperture size (NA) of the used objective lens (NA = 0.4) and for the minimum wavelength (λ) of visible light, the minimum lateral resolution can be calculated using Rayleigh Criterion as show in Equation 6. The value of NA was estimated to be 0.57 μm . This means that a particle less than 0.57 μm is not visible under given conditions. Since algal cells are between 1 to 3 μm in diameter, they are visible under visible light in 20x objective lens. However, when cells are broken down to particles smaller than 0.57 μm , they are not visible under given imaging conditions and were omitted from the cell count.

$$R_L = \frac{0.61 \lambda}{NA} \quad (1)$$

A digital, black and white camera was used for capturing sections of the counting chamber separately as the entire counting grid was not able to be captured in one image using the required magnification. A freeware with image processing capabilities (ImageJ 1.42q; National Institute of Health, USA) was used for counting cells in the images. The degree of cell breakage was characterized as a percentage of the total number of intact cells observed per unit volume. Double counting of cells in multiple images was avoided by cropping images along the gridline.

For growing algae used in this experiment, Bold-Modified Basel ²¹ fresh water concentrated solution (Sigma Aldrich, St. Louis, Missouri) was used as the nutrient medium. This was selected because other collaborating research groups were successfully using it for growing *N. oculata*. Ammonium chloride and potassium phosphate was used as the nitrogen and phosphorus sources. Although using CO₂ enriched air stream is desired, there are reports where successful algae culturing was done using air without CO₂ enrichment. In this study, 12-hour day and night illumination cycle was simulated using 2, 15 Watt florescent bulbs (Figure 5).

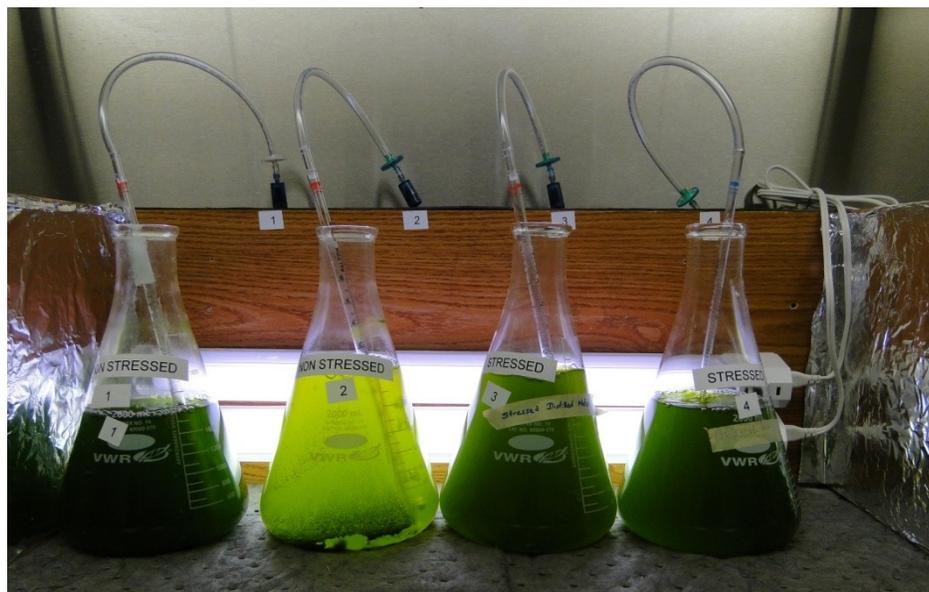


Figure 5. Algal photobioreactor

Screening Study for Algal Breakage Using High Pressure Homogenization

Parameters listed in Table 2 were identified as possible variables that can affect the degree of cell rupture during homogenization. A two-level fractional factorial design (2^{5-2}) with three replicate at each design point was designed to investigate the preliminary effect of these factors. In the experimental design, the generator terms $D = AB$ and $E = AC$ were used to construct the alias structure in the statistical analytical software. The software generates aliases as shown in the Table 3²². Any pattern observed in a term should be expected from its alias terms as well²².

Table 2. List of variables used for the screening study

| Factor | Variable | High | Low |
|------------------|-----------------|--------------------------|-------------------------|
| Extent of stress | A | Stressed | Non Stressed |
| Concentration | B | 100 g/l | 1 g/l |
| No of Passes | C | 1 | 4 |
| Pressure | D | 25 000 PSI (172.369 MPa) | 10 000 PSI (68.948 MPa) |
| Nozzle size | E | 195 μm (Z8) | 100 μm (Z4) |

Table 3. Aliases for the screening study

| Term | Aliases |
|-----------------|----------------|
| A Stress | BD, CE |
| B Concentration | AD |
| C No of Passes | AE |
| D Pressure | AB |
| E Nozzle | AC |
| BC | DE |
| BE | CD |

After processing the samples, the number of remaining intact cells was counted using the aforementioned hemocytometry technique.

The difference of the intact cell count before and after homogenization directly correlates to the extent of cell disruption. A collection of microscopic images with the cells in the gridded counting chambers are depicted in Figure 6. Ruptured cells resulting after subjecting algal cells to pressure treatment, disappear in the solution matrix. This is because ruptured cell particles are smaller than the resolution selected to image the intact cells.

In order to capture the degree of cell breakage, the intact “Cell Fraction” after homogenization was calculated according to the following formula:

$$\text{Cell Fraction} = \frac{\text{Cell density in sample after treatment}}{\text{Cell density in sample before treatment}} \quad (2)$$

Results were analyzed using Stat-ease Design Expert software (Stat-Ease, Inc., Minneapolis, Minnesota). Results of an analysis of variance (ANOVA) are depicted in Table 4. Nozzle size did not significantly affect the degree of cell breakage (however, note that the interaction term BC, Concentration – Nozzle size, is significant since the concentration term is significant. This observation is further illustrated in the Figure 7. Stress, number of passes and pressure are inversely related to “intact cell fraction” and concentration (negative effects), while the two interaction terms are directly related to the “intact cell fraction” (positive effects).

Plots shown in Figure 8 demonstrate the variation of cell fraction with the variation of different factors. A square root transformation was performed on intact cell fraction to obtain an approximately normal distribution. Individual plots depict the variation of cell fraction in relation to selected two factors while the other factors were kept fixed. Lower algal cell concentrations and higher number of passes (Figure 8 (a) and (b)) resulted in higher degree of cell breakage. At lower concentrations, nozzle size had an inversely proportional relationship with cell fraction while at higher algal concentrations the relationship was proportional. This may be due to cohesive as well as adhesive shear effects being more dominant at higher algal concentrations in addition to the pressure effects.

To further elucidate the effect of homogenization conditions on the degree of cell breakage, a full factorial experiment was performed using selected variables. Algal concentration and the extent of stress were fixed by using non stressed algal broth having 0.675 an optical density (0.15 mg/ml) for all experimental units. These variables were fixed at given values since at the onset of the study, access to stressed or concentrated algal samples were limited. The effect of variation of these values will be investigated in a later study. However, the homogenizer nozzle size was varied, primarily to confirm the findings of the previous screening study (2^{5-2} two-level fractional factorial design). The list of variables for this full-factorial-design is shown in Table 5.

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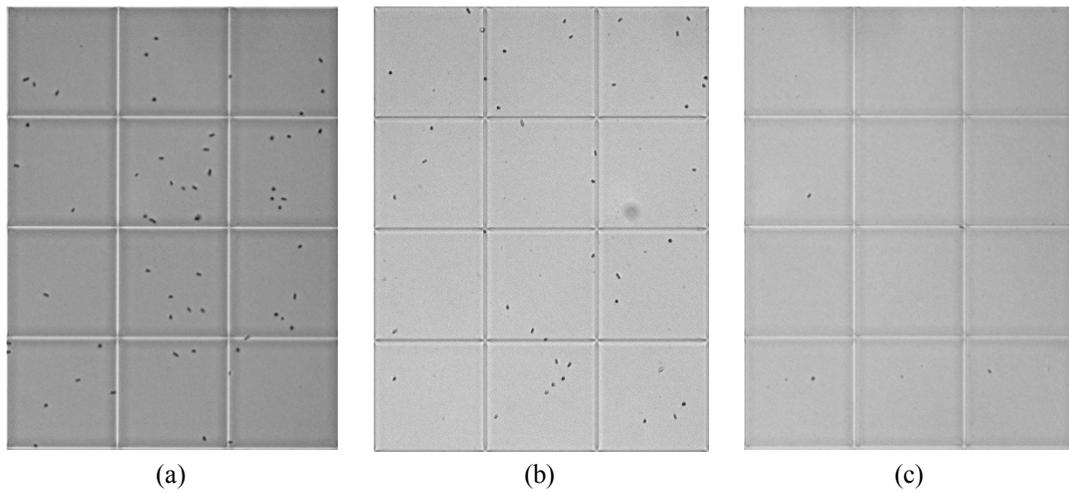


Figure 6. Counting chambers loaded with algal broth

(a; no processing, b; 1 pass through the 195 μm homogenising nozzle at $10 \text{ PSI} \times 10^3$ (68.948 MPa) ,c; 2 pass through the 195 μm homogenising nozzle at $10 \text{ PSI} \times 10^3$ (68.948 MPa)

Table 4. ANOVA table for the homogenization experiment [Partial sum of squares – type III]

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob> F |
|-----------------|-----------------------|-----------|--------------------|----------------|---------------------------|
| Model | 2.996 | 7 | 0.428 | 561.116 | < 0.0001 |
| A-Stress | 0.058 | 1 | 0.058 | 76.478 | < 0.0001 |
| B-Concentration | 0.510 | 1 | 0.510 | 668.904 | < 0.0001 |
| C-No of Pass | 0.362 | 1 | 0.362 | 474.067 | < 0.0001 |
| D-Pressure | 0.974 | 1 | 0.974 | 1276.643 | < 0.0001 |
| E-Nozzle | 0.003 | 1 | 0.003 | 3.302 | 0.0880 |
| BC | 0.725 | 1 | 0.725 | 950.783 | < 0.0001 |
| BE | 0.364 | 1 | 0.364 | 477.638 | < 0.0001 |
| Pure Error | 0.012 | 16 | 0.001 | | |
| Cor Total | 3.008 | 23 | | | |

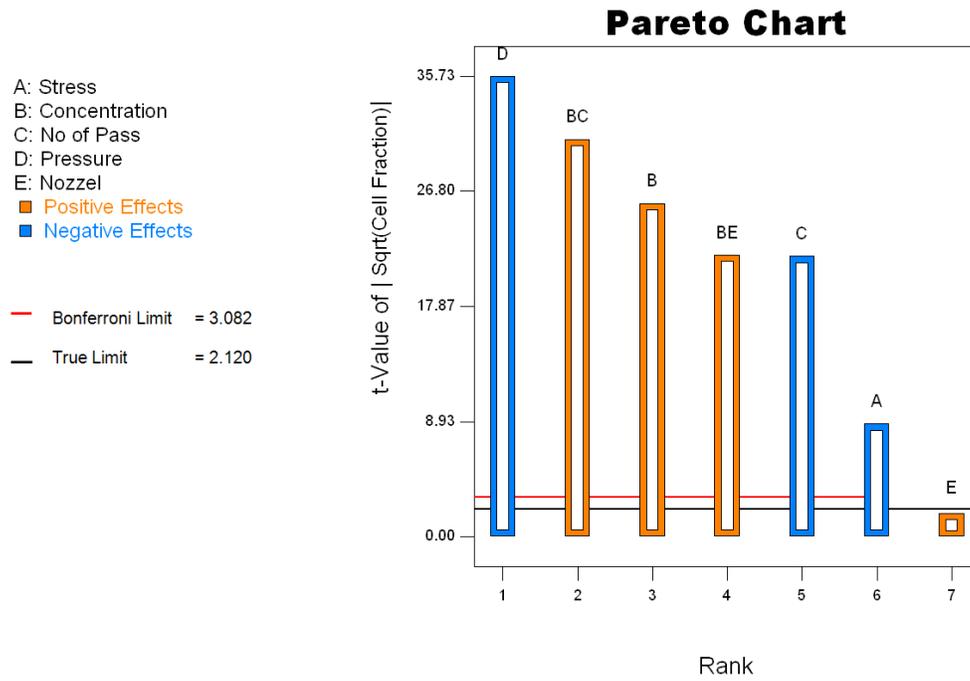


Figure 7. Pareto analysis of the effects of various variables on degree of cell breakage

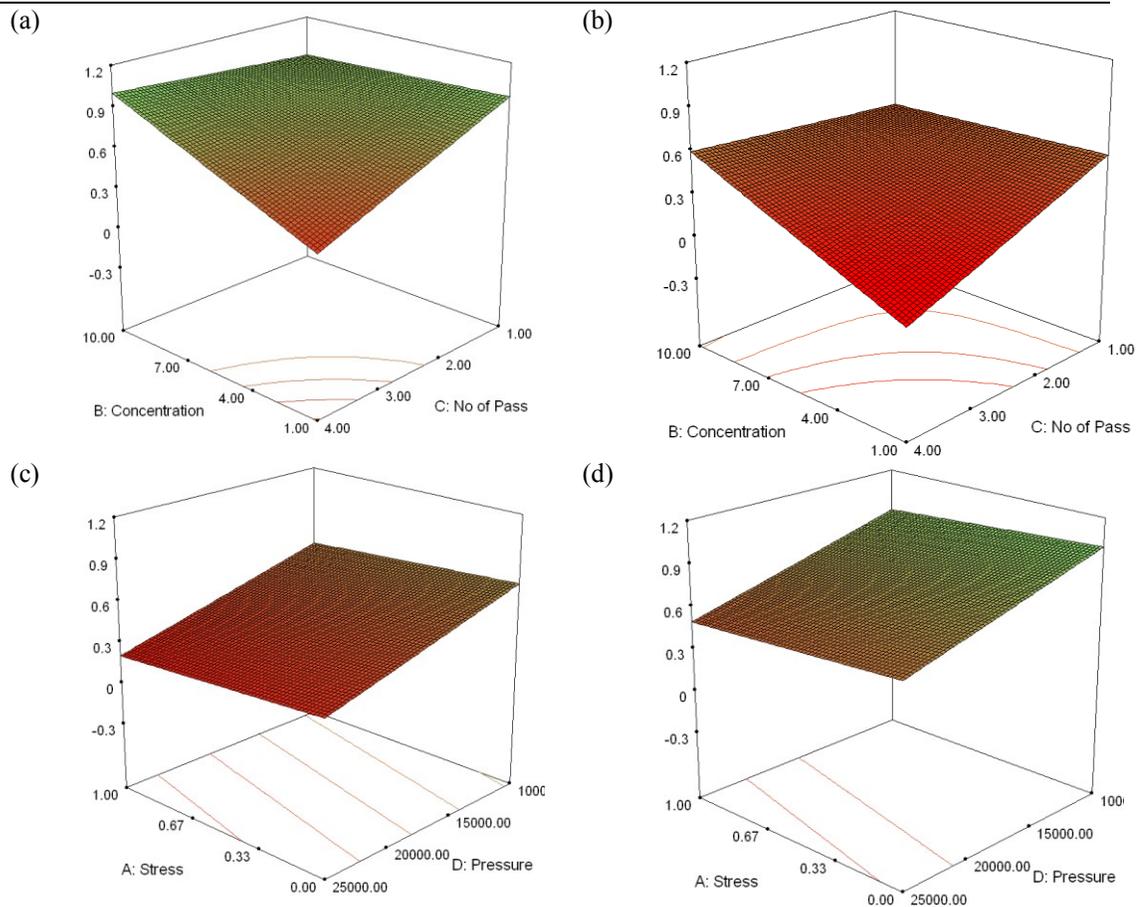


Figure 8. Variation of cell fraction with concentration, nozzle size and pressure (Y axis is square root of cell fraction)

Variation of Cell fraction with 2 selected factors, when other factors are fixed at given value. (Pressure in PSI and Nozzle size in μm) (a; pressure = 10000.00 PSI (68.948 MPa), Stress = 0.50, Nozzle size = 147.50 μm , b; pressure = 25000.00 PSI (172.364 MPa), Stress = 0.50, Nozzle size = 147.50 μm , c; Concentration = 1.00, No of Passes = 2.50, Nozzle = 147.50 μm , d; Concentration = 10.00, No of Passes = 2.50, Nozzle = 147.50 μm , e; No of Passes = 1.00, Stress = 0.50, Pressure = 17500.00 PSI (120.658 MPa) (f) No of Passes = 4.00, Stress = 0.50, Pressure = 17500.00 PSI (120.658 MPa)

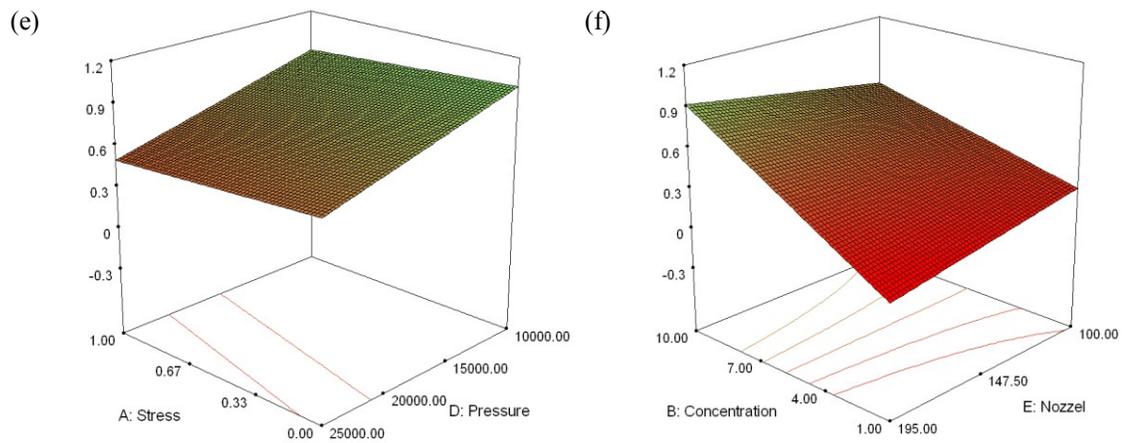


Figure 8. Continued

Table 5. List of variables for full factorial design

| Nozzle Size | Pressure | Number of Passes |
|------------------------|------------------------------------|------------------|
| 100 μm (Z4) | 10 PSI $\times 10^3$ (68.948 MPa) | 1-6 (six levels) |
| 130 μm (Z5) | 20 PSI $\times 10^3$ (137.895 MPa) | |
| 195 μm (Z8) | 30 PSI $\times 10^3$ (206.843 MPa) | |
| | 40 PSI $\times 10^3$ (275.790 MPa) | |

Each sample was then placed in a Neubauer improved counting chamber and the number of cells remaining intact were counted as described earlier.

Figure 9 depicts that the increase of pressure and the number of passes reduces the visible cell count, which correlates to increased cell disruption. This is confirmed from

the optical microscopic images as depicted in Figure 6 as well. Even in this experiment, nozzle size was determined to be insignificant-confirming the observations from previous experiments.

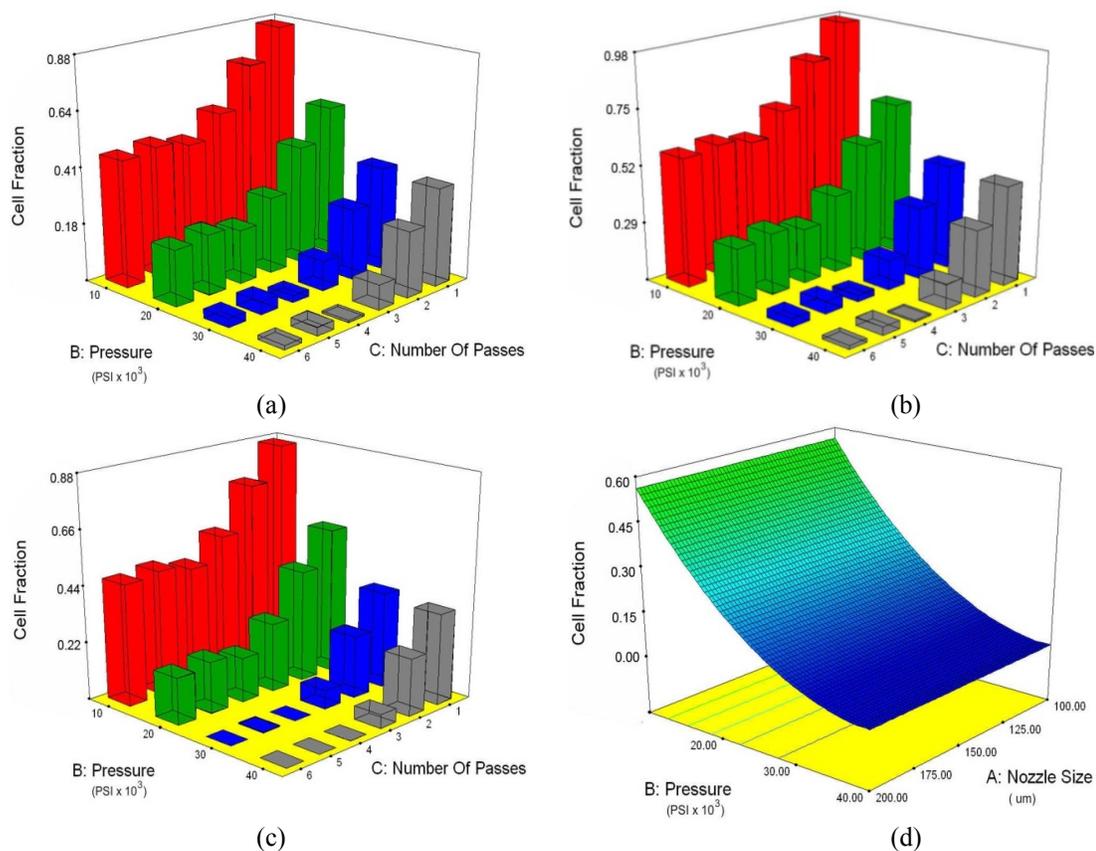


Figure 9. Fraction of cells remaining intact at different pressures and number of passes using different nozzle diameters

(a; 195 μm Nozzle, b; 130 μm Nozzle, c; 100 μm Nozzle, d; change in Cell Fraction With Nozzle size and pressure after 2 passes.)

It was observed that the cell count increased after first and second runs through the homogenizer at 10,000 PSI (68.948 MPa). This can be attributed to breakage of cell coagulations into individual cells.

Nozzle size did not affect the degree of cell rupture, which implies that the impact of shear exerted upon the cell walls from the nozzle walls was minimal. Intuitively, smaller nozzles should impart more resistance to forward movement of the cell mass rendering more shear force onto the algal cell walls. However, this is not the case, and the pressure differential alone has a significant effect on rupturing the cell walls. This is a significant finding, since it shows the possibility of using a larger nozzle size without compromising the cell lyses, given that the pressure differential across the nozzle is maintained. Larger nozzle sizes are desirable for the processes, because a larger nozzle is less likely to clog during operation.

Energy and Other Considerations

One of the basic questions consistently raised about homogenization is what the energy requirement of high pressure homogenization is. Work done by the piston can be calculated by multiplying operating pressure (P) by the volume (V) of the algal broth processed. Accordingly, the theoretical energy requirement for processing 1 m³ of algal slurry in 10,000 PSI (68.95 MPa) pressure in a single pass is 69 MJ in the unit used for the experiment. This should be compared with the energy available in algal broth to evaluate the energy efficiency of the process. If it is assumed that the specific energy of dried algae is 20 MJ/kg, solids concentration (wt) of algal slurry should be at least

0.345% to obtain a net energy output from the homogenization process. Since efficiency losses must be considered (multiple passes may be required to obtain adequate cell rupture), the homogenizer must be able to handle slurries of much higher solid concentrations than 0.345% (w/w) in order to realize a net energy gain. In fact, during preliminary studies, we processed slurries up to 10% solids (directly from centrifugation) in the homogenizer without any difficulty. These preliminary calculations project the likelihood of using homogenization for commercial scale algae processing. However a more accurate energy consumption analysis is necessary after determining the optimal conditions (e.g. number of passes and pressure) that maximize oil yields.

Another point to ponder is the fate of non-lipid algal debris after homogenization. Although increased cell rupture maximizes solvent accessibility to lipids, this also increases the number of smaller-sized non-lipid particles suspended in the matrix. Preliminary studies with solvent extraction have indicated that heavily homogenized algal slurry tends to make a hard-to-separate single-phase solution when mixed, while unhomogenized slurry rapidly separates. This may be a result of compounds from cellular matrix acting as amphiphiles making emulsions. Accordingly, it is imperative that there is an optimum level of cell disruption to cause adequate exposure of lipid bodies, while not causing downstream separation issues.

4. MATERIALS AND METHODS

The general methods pertinent to objectives 1 and 2 are presented initially. Information pertinent to each individual objective is presented subsequently.

Algal Samples

For this research *N. oculata* algal cells (obtained from Texas Agrilife Research Algal Research facility, Pecos, Texas) were used. The samples contained algae that were subjected to physiological stress via nutrient depletion. Stressing of algae is known to increase the lipid content^{8,9}.

The solids content of an algal broth obtained from a photo bioreactor or an open pond is around 0.1% (Total Suspended Solids, wet weight basis). Due to this highly diluted state, such samples need to be concentrated prior to subjecting to any form of processing. Accordingly, the cells were centrifuged in a large scale homogenizer at the growth facility prior to shipping. Apart from centrifugation, it is reported that numerous methods used to harvest algae, such as flocculation, can reduce moisture content to 70% (wet basis). Typical centrifugation can reduce moisture content to approximately 85~90% wet basis²³. Unfortunately, further removal of moisture can be extremely costly⁸. Consequently, it was decided to carryout lipid extraction studies with incoming algae at a moisture content of approximately 90%. In instances where the solids content was higher, it was adjusted to 90% by dilution.

Homogenization

Microalgae possess an intransigent cell wall that needs to be ruptured to facilitate solvent access to lipids. For this, high pressure homogenization was used.

A NanoDeBEE bench top high pressure homogenizer manufactured by Bee international, MA, was used for homogenizing algal samples. The schematic representation of the cross section of the homogenizer is given in the Figure 1.

When homogenizing algae, the aqueous sample is initially placed in the sample reservoir and when the hydraulically operated piston moves back in the piston, the sample is sucked in to the cylinder through a check valve. When the piston moves forward in the cylinder, the sample is forced through the orifice of the homogenization nozzle. As a result of the high shear forces and the sudden pressure drop across the nozzle, algal cells disintegrate during the passage through the orifice.

Objective 1: Elucidating the Effect of Homogenization Conditions on the Degree of Cell Breakage

Variable parameters used in the study are pressure, number of passes, stress of the sample and the algal concentration. Levels of these variables are shown in Table 6. A Full factorial design was utilized with three replicates at each design point.

Table 6. List of variables for determining effect of high pressure homogenization on *N. oculata*

| | Variable | | | |
|--------|------------------------------------|--------------|----------------|---------------------|
| | Solid content (w/w – wet basis) | Stress | Pressure (Psi) | Number of passes |
| Levels | 0.1% (Non Concentrated) | Non Stressed | 10000 | 1 |
| | 1% (Concentrated) | Stressed | 20000 | 2 |
| | | | 30000 | 3 |
| | | | 40000 | 4 |

Disposable cell counters (C-Chip DHC-S01 semen counting chambers from Incyto, Chungnam-do, Korea) with shallower chambers were used for cell counting. These are Neubauer Improved counting chambers with 10 μ l volumes and designed for accommodating small cells.

For imaging the cells in the counting chamber, a Zeiss Axiphot optical microscope with 20x object resolution was used. A digital, black and white camera was used for capturing sections of the counting chamber separately as the entire counting grid was not able to be captured in one image using the required magnification. A freeware with image processing capabilities (“ImageJ1.42q” from National Institute of Health, USA) was

used for counting cells in the images. The degree of cell breakage was characterized as a percentage of the total number of intact cells observed per unit volume. Double counting of cells in multiple images was avoided by cropping images along the gridline.

The intact cell percentage after homogenization (which inversely correlates to the degree of cell breakage) was calculated according to Equation 1:

$$\% \text{ Cells Remaining} = \frac{\text{Cell density in sample after treatment}}{\text{Cell density in sample before treatment}} \times 100\% \quad (3)$$

Results and conclusions of this experiment are shown in “Results and Discussion” section under the topic “effect of homogenization on *N. oculata*”

Objective 2: Elucidating the Effect of Degree of Cell Breakage on the Oil

Extraction Efficiency

Our previous experiments have shown that the extent of cell lyses depends on the pressure used, number of passes through the homogenizer and the concentration of the sample. However, in this experiment, the concentration of the samples was kept fixed at 10% because the collaborating research group working on electro coagulation concluded that 10% is the optimum concentration for electro coagulation. The combinations of pressure and number of passes through the nozzle used for this experiment are depicted

in Table 7 and the amount of cell disruption was calculated using the cell counting procedure explained in the section “Preliminary Research” and given in the Table 11.

Table 7. Homogenizations conditions

| Pressure | Number of passes | Cell Disruption |
|-----------------|-------------------------|------------------------|
| 10 000 | 2 | 10% |
| 20 000 | 2 | 20% |
| 30 000 | 3 | 54% |
| 40 000 | 4 | 67% |

Solvents Used

Three types of organic solvents were used for the extraction of algal lipids from the homogenized algae, namely hexane, dichloromethane and chloroform. Hexane was selected because it is the industry standard for extraction of lipids from bio renewable substrates. Chloroform was used due to its slightly higher polarity as compared to hexane. It was hypothesized that moisture-laden algae would be more compatible with chloroform due to its slightly polar nature and ability to form hydrogen bonding. As chloroform is highly toxic and the ability for it to be used in commercial scale operations

Table 8. Physical properties of solvents

| Property | Hexane | Dichloromethane | Chloroform |
|--|---|-----------------|--------------------------------|
| Assay | 98.5% | 99.5% | 99.8% |
| CAS Number | 110-54-3 | 75-09-2 | 67-66-3 |
| Dialectic Constant | 1.88 | 9.81 | 4.81 |
| R-phrases [†] | R11, R38, R48/20, R62 ,R65 , R67R51/53 | R40 | R22, R38, R40, R48/20/22 |
| Boiling Point (c) | 69 °C | 39.6 °C | 61.2 °C |
| Specific heat capacity (kJ/kg.K) | 2.26 | 1.02 | 1.05 |
| Density (kg/l) | 0.6548 | 1.33 | 1.483 |
| Enthalpy of evaporation(kJ/mol) | 28.85 | 28.6 | 31.4 |
| Molecular weight(g/mol) | 86.18 | 84.93 | 119.38 |
| Energy needed for evaporating one liter of solvent(kJ) | 284.32 | 467.68 | 446.59 |
| Hansen solubility parameter (HSPiP) ^{24, 25} | | | |
| δP | 0.0 | 7.3 | 3.1 |
| δH | 0.0 | 7.1 | 5.7 |

[†]**Risk phrases** defined by European Union Directive 67/548/EEC²⁶; **R11** ; Highly flammable, **R20** ; Harmful by inhalation, **R22**; Harmful if swallowed, **R38**; Irritating to skin, **R40**; Limited evidence of a carcinogenic effect, **R48/20**; Harmful: danger of serious damage to health by prolonged exposure if swallowed, **R48/20/22**; Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed, **R62**; Possible risk of impaired fertility, **R65**; Harmful: may cause lung damage if swallowed, **R67R51/53**; Vapors may cause drowsiness and dizziness, Toxic to bees, May cause long-term adverse effects in the aquatic environment

would be remote, dichloromethane was tested as an environmentally friendlier alternative. Hexane and chloroform are considered non-polar solvents while dichloromethane is a polar aprotic solvent. Some of the relevant physical properties of these solvents are shown in Table 8.

Extraction Procedure

Initially, 10 ml of 10 % (w/w – wet basis) algae (in aqueous environment) were homogenized using a NanoDeBee High pressure homogenizer according to predetermined pressures and number of passes. Subsequently, the homogenized algae were vortexed with solvent for one minute at 10,000 rpm. Then, the mixture was centrifuged to separate solvent from water before 7 ml of solvent was pipetted into an 8 ml high pressure reaction vial. Lipid exhausted algae and remaining solvent with additional 10 ml of solvent was mixed together to obtain another extraction. This procedure was repeated to obtain three sequential extractions from a single sample. The solvent in the reaction vial was evaporated to obtain the (lipid-containing) residue.

Transesterification

The residue was dried in an oven at 105°C to remove moisture until the weight of the sample became constant (Around 1 hour). Then 4ml of 4% methanolic H₂SO₄, the transesterification catalyst, was added to the dried residue. One ml of 0.75 mg/ml methanolic C15 Fatty Acid was also added as the internal standard (This was used as the internal standard for calculating the transesterification efficiency). This mixture was

heated at 110°C for 2 hours while vortexing for 20 sec every 15 minutes at 10,000 rpm. After 2 hours, the sample was mixed with 3 ml of hexane and 4 ml of water and vortexed for one minute at 10,000 rpm. After vortexing, the sample was centrifuged at 2,500 rpm for five minutes to separate the hexane layer. Hexane was used to separate esters from water soluble components. The hexane layer was pipetted out and filtered using a 0.2 µm PTFE syringe filter. 1 ml of this sample was transferred to a 2 ml vial and 50 µl of 1mg/ml C13 C19 Fatty Acid Methyl Esters (FAME) in methanol mixture was added as the internal standard (These was used to calculate the area to concentration ratio of other FA). This sample was injected to a GC equipped with a Flame Ionization Detector (FID) and the signal pattern was recorded. GC was calibrated using a FAME slandered 68A (NU-Check Prep, MN, USA).

In the GC analysis, the area under the signal for each peak for the FID signal was calculated and the resulting area was compared with the signal obtained for a standard fatty acid mixture. Since the amount of C15 FA used in the original sample is known, it is possible to determine the efficiency of the transesterification. Using this information it is possible to calculate the amount of FA available in the original sample.

Methodology described in the above section is illustrated in Figure 10.

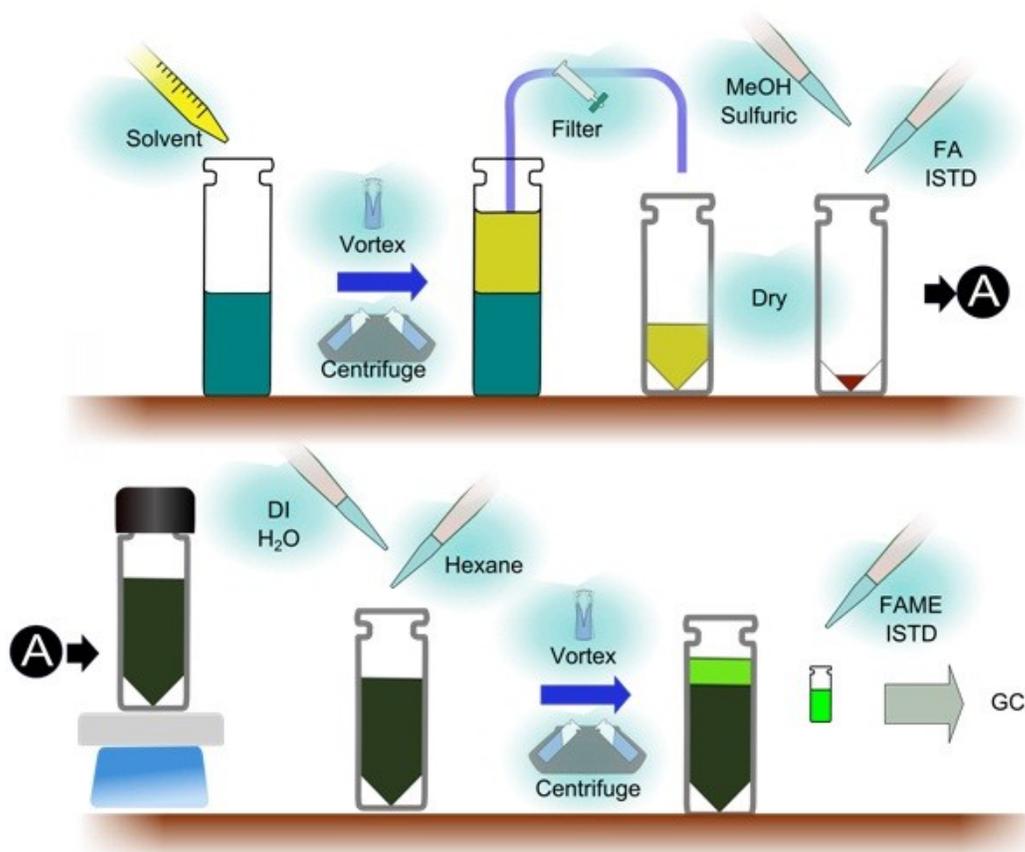


Figure 10. Extraction methodology

Experimental Design

A full factorial design was utilized with extent of stress and solvent type as variables. Table 9 shows the entire distribution of the variables used. An asterisk (*) represents a single design point. Three replicates of each design point were performed.

Table 9. Experimental design for determining the behavior of the selected extraction solvents in extracting partially ruptured algal biomass

| Pressure | Extraction Number of passes | Solvent | | | | | | | | | | |
|----------|-----------------------------------|---------|---|---|-----------------|---|---|------------|---|---|---|---|
| | | Hexane | | | Dichloromethane | | | Chloroform | | | | |
| | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | | |
| N/A | N/A | * | * | * | * | * | * | * | * | * | * | * |
| 10 000 | 2 | * | * | * | * | * | * | * | * | * | * | * |
| 20 000 | 2 | * | * | * | * | * | * | * | * | * | * | * |
| 30 000 | 3 | * | * | * | * | * | * | * | * | * | * | * |
| 40 000 | 4 | * | * | * | * | * | * | * | * | * | * | * |

Design Expert 8 (Stat-Ease, Inc, MN) was used to conduct statistical analysis of the results.

In this particular segment of studies a square-root transformation was used to obtain a proper normal distribution.

5. RESULTS AND DISCUSSION

Objective 1: Elucidating the Effect of Homogenization Conditions on the Degree of Cell Breakage

The difference of the intact cell count before and after homogenization directly correlates to the extent of cell disruption. A collection of microscopic images with the cells in the gridded counting chambers are depicted in Figure 6. It is apparent that the number of intact cells reduced in the solution matrix with increasing pressure treatment. This is because ruptured cell particles are smaller than the resolution selected to image the intact cells.

Results of an analysis of variance (ANOVA) are depicted in Table 10. All of the variables are statistically significant.

Figure 11 shows the variation of percentage of remaining cells with various combinations homogenization conditions. Greater cell disruption is induced by a higher number of passes and higher pressures. Some remaining cell fraction values were higher than 100%. We anticipate that this happens because of the breakage of the cell conglomerates without significant disruption of cells during low intensity homogenization.

Table 10. Analysis of variance table [Classical sum of squares - Type II]

| Source | Sum of Squares | df | Mean Square | F Value | p-value (Prob> F) |
|--------------------|----------------|-----|-------------|---------|-------------------|
| Block | 228.95 | 2 | 114.48 | | |
| Model | 73539.41 | 8 | 9192.43 | 75.55 | < 0.0001 |
| A-Concentration | 10118.49 | 1 | 10118.49 | 83.16 | < 0.0001 |
| B-Extent of Stress | 551.66 | 1 | 551.66 | 4.53 | 0.0346 |
| C-Pressure | 45837.27 | 3 | 15279.09 | 125.58 | < 0.0001 |
| D-Number of Passes | 17032 | 3 | 5677.33 | 46.66 | < 0.0001 |
| Residual | 22022.02 | 181 | 121.67 | | |
| Cor Total | 95790.38 | 191 | | | |

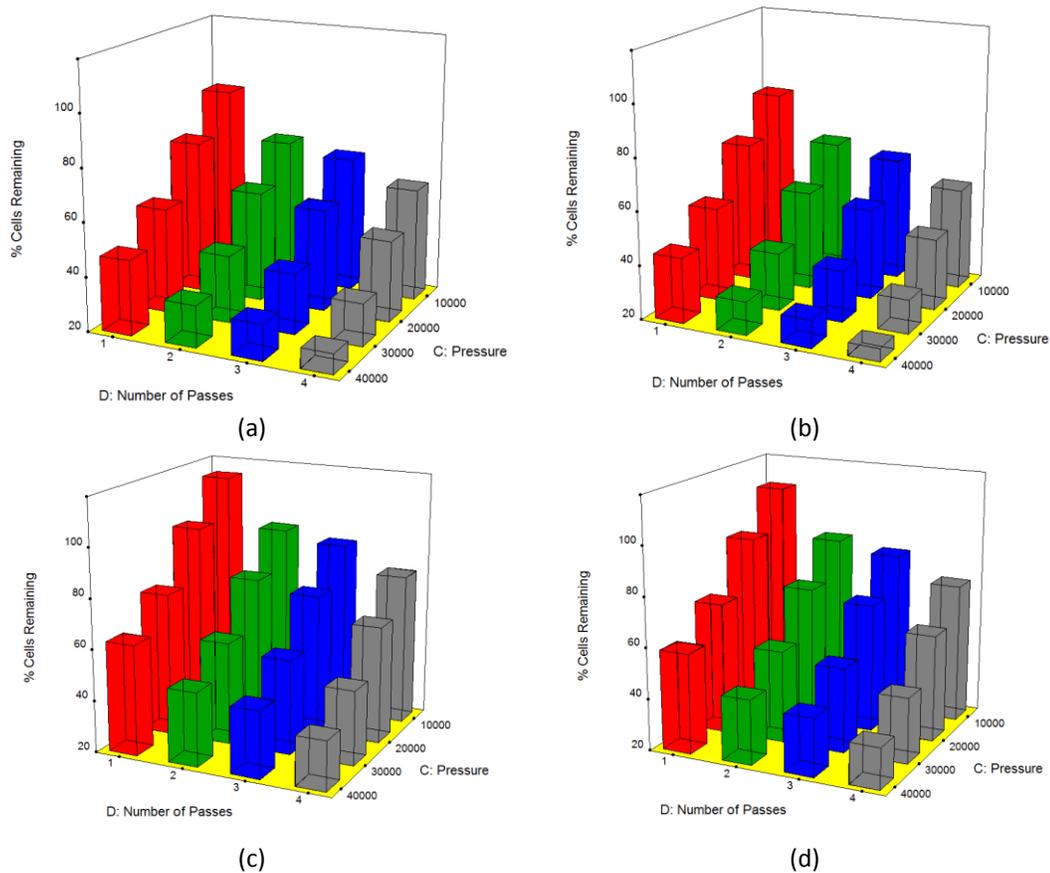


Figure 11. Percentage of cells remaining after homogenization

(a; 0.1% concentration, unstressed, b; 0.1% concentration, stressed, c; 1% concentration, unstressed, d; 1% concentration, stressed)

Objective 2: Elucidating the Effect of Degree of Cell Breakage on the Oil

Extraction Efficiency

Statistical analysis was utilized to ascertain whether the variables used, i.e., level of cell disruption and the type of solvent, had any significant impact on the amount of

extractable lipids. According to the ANOVA, (Table 11) it is clear that all of the variables affect the response variable.

Table 11. ANOVA table for extraction experiment

| Source | Sum of Squares | df | Mean Square | F Value | p-value (Prob> F) |
|--------------|----------------|-----|-------------|---------|-------------------|
| Block | 0.025 | 2 | 0.014 | | |
| Model | 18.99 | 8 | 2.37 | 103.53 | <0.0001 |
| A-Solvent | 0.73 | 2 | 0.37 | 16.01 | <0.0001 |
| B-Disruption | 4.56 | 4 | 1.15 | 50.09 | <0.0001 |
| C-Extraction | 12.56 | 2 | 6.28 | 273.86 | <0.0001 |
| Residual | 2.48 | 108 | 0.023 | | |
| Cor. Total | 21.49 | 118 | | | |

It is worthwhile to look in to diagnostic plots to confirm the statistical validity of the experiment. Relevant statistical diagnostic plots are depicted in Figure 12. Close to linear distribution of normal plot of residuals and the Box-Cox shows that the selected square root transformation is acceptable. Residual vs. predicted plot and residual vs. run number plots demonstrate that the randomization process of the design is acceptable and that there is no obvious bias in the experiment.

The lipid yield reduces with each extraction iteration (Figure 13). The highest amount of lipids extracted was 48.15mg of FA from 1 g of algae (Dry basis) with chloroform in the first extraction. In this instance, the third extraction resulted in only 5.7 mg of FAs from 1g of algae lipids. Although the solvents behaved differently for the initial extraction, they behaved very similar during subsequent extractions.

The effect of the degree of cell disruption on the lipid extraction efficiency is depicted in Figure 14. The results indicate the amount of lipids extractable for a given combination of percentage cell disruption via homogenization and the solvent type used. The degree of cell disruption significantly affects the efficacy of lipid extraction. Once disrupted, the higher amounts of cell breakage (compare 67% vs. 54%) do not necessarily increase the amount of lipids extracted. However, although statistically not significant, an increase of lipid yield is still observable with increasing cell disruption. This may be happening because at 20% disruption most of the cells are significantly damaged in their cell wall, sufficient enough to allow extraction solvents into the cell but not sufficient enough for cells to break into parts.

The response of lipid yields to different types of solvents for different number of sequential extractions is illustrated in Figure 15.

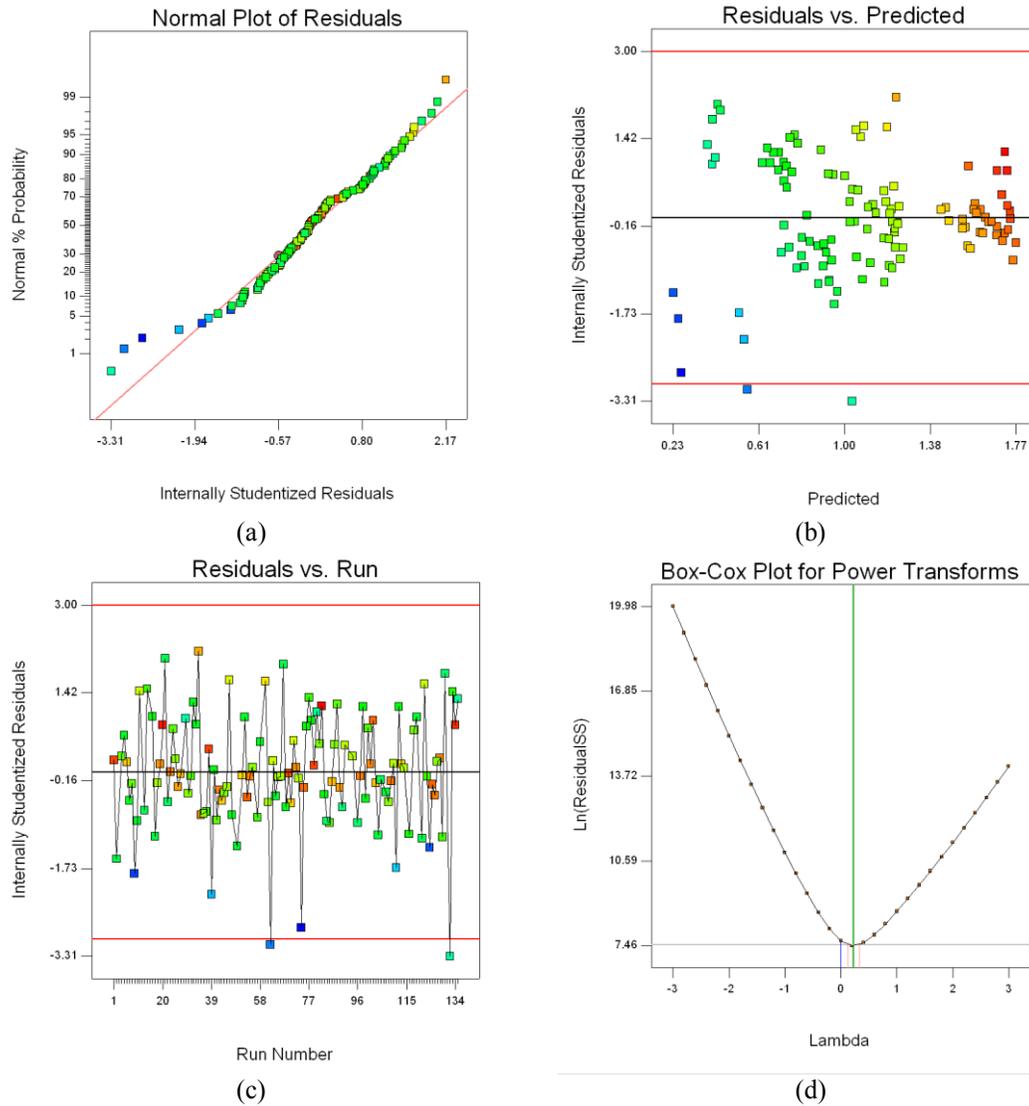


Figure 12. Statistical diagnostic graphs

(a; Normal Plot of residuals, b; Residual vs. Predicted, c; Residual vs. Run and d; Box-Cox Plot)

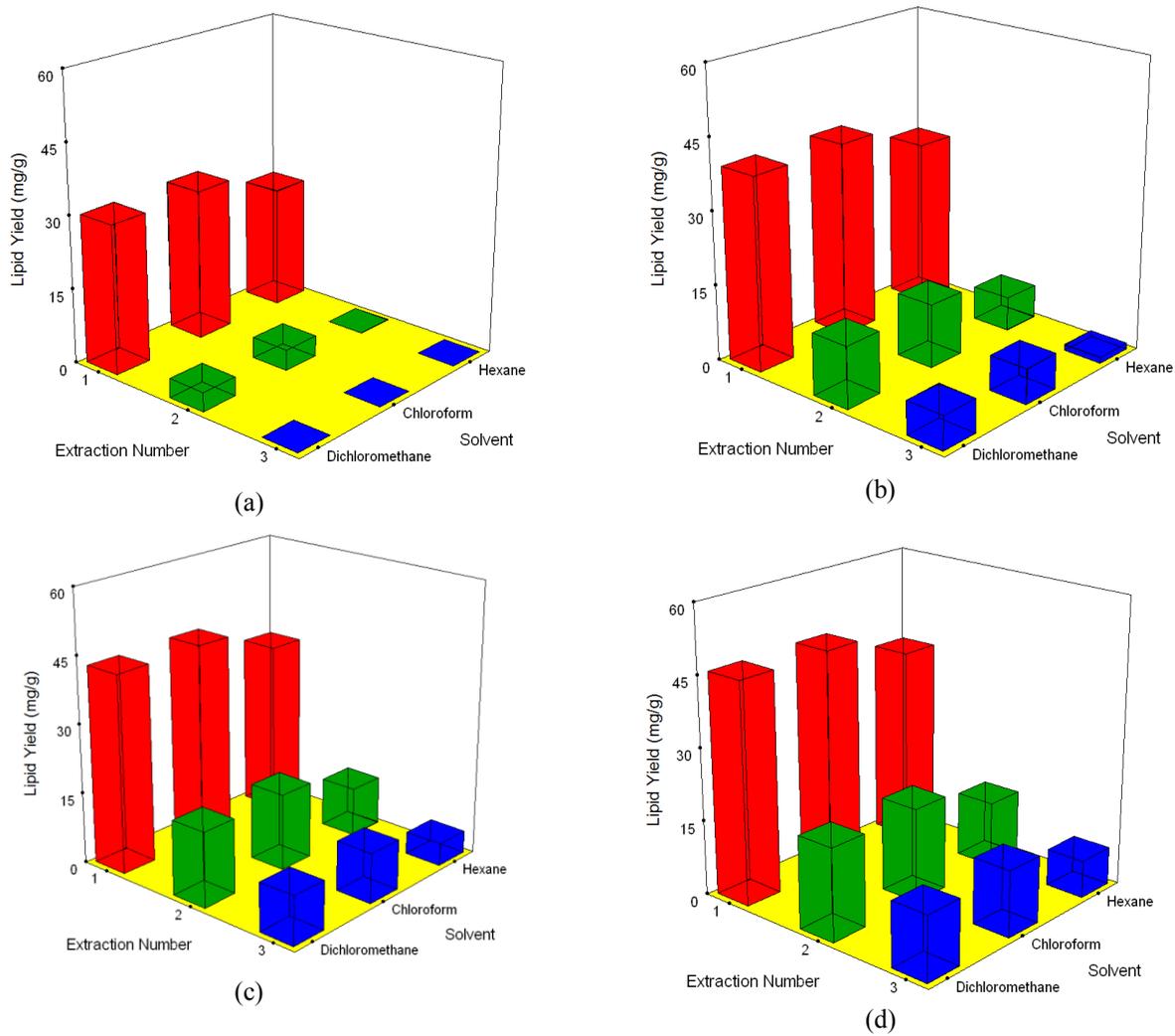
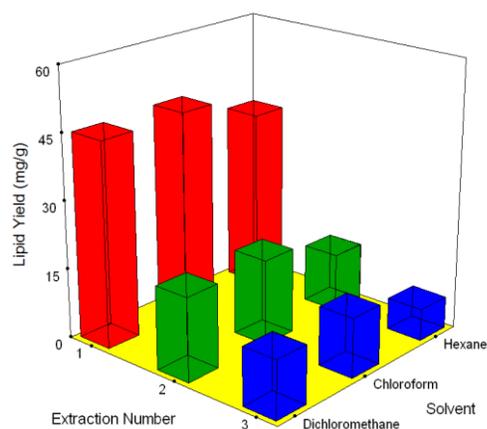


Figure 13. Variation of amount of lipids extracted when different amounts of cells are disrupted

a; Raw Algae, b; 10 000 Psi, 2 Pass, c; 20 000 Psi, 2 Pass, d; 30 000 Psi, 3 Pass and e; 40 000 Psi, 4 Pass



(e)

Figure 13. Continued

Hexane has a lower extraction efficiency compared to dichloromethane and chloroform. Although the amounts of lipids extracted from chloroform in the first extraction are slightly more than that extracted from dichloromethane (both series are close together and cannot be separately identified in the graph), the responses are within the range of the standard error. Therefore, it is possible to conclude that chloroform and dichloromethane are equally effective in extracting algal lipids for the first extraction.

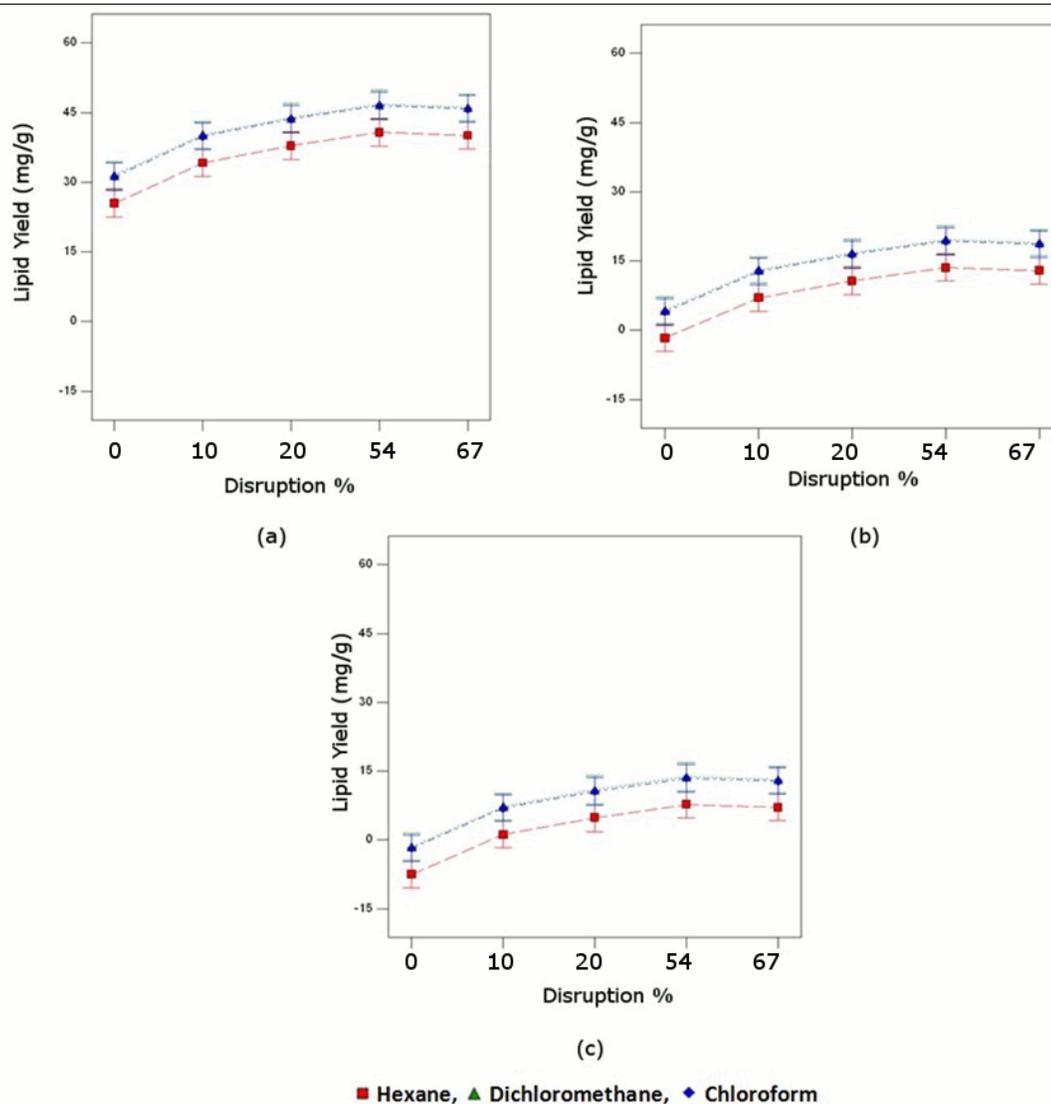


Figure 14. Effect of percentage disruption of algal cells and effect of solvent

(a; First Extraction, b; Second Extraction and c; Third Extraction)

The degree of cell disruption has a strong impact on the amount of lipids extracted in the first extraction (Figure 14). However, the increase of the amount of lipids extracted when the percentage of cell breakage is increased from 0% to 10 % is much higher than

the increase in the amount of lipids extracted when the percentage of cell breakage is increased from 10% to 67%.

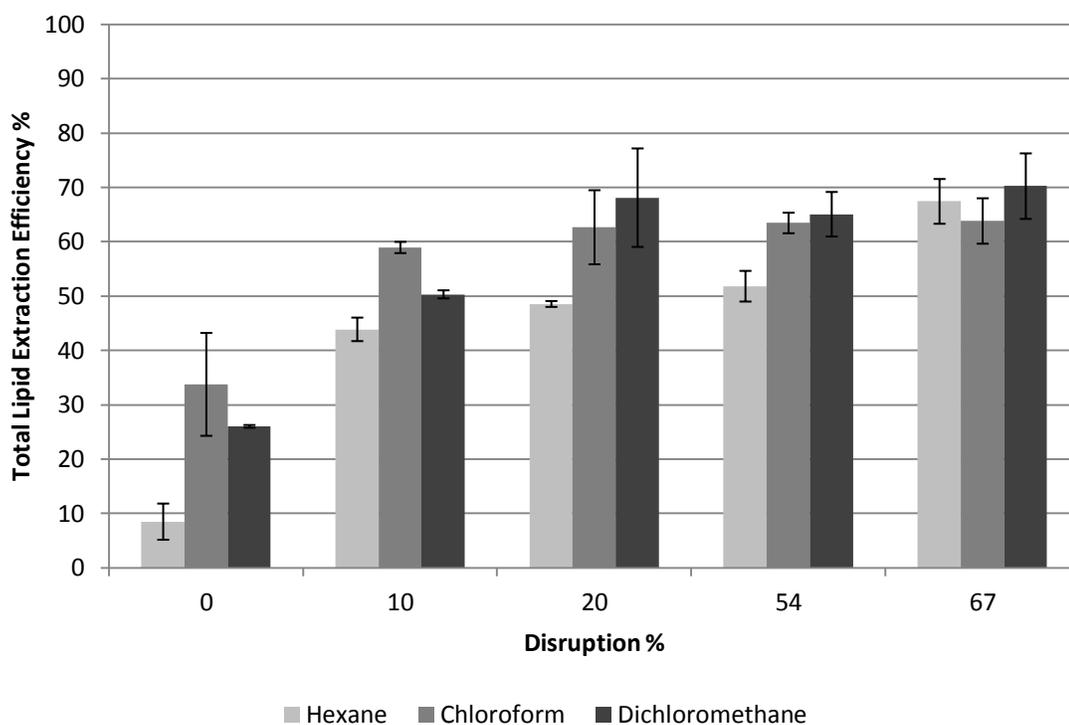


Figure 15. Lipids extracted from 1 gram of biomass during three consecutive extractions

Figure 15 depicts the amount of lipids extracted with different cell breakage–solvent combinations as a percentage of extractable lipids from non-homogenized algae in hexane. In this case, hexane was selected as the benchmark for the comparison because it is the industry standard. The analysis was done by simple addition of the amounts of

lipids extracted in each successive extraction. The analysis confirms that it is possible to extract around 75 milligrams of lipids from 1 gram of algae (approximately 7.5% of the dry weight of algae). In Figure 16, total lipid yield from hexane for non-disrupted algae was considered to be the standard and was benchmarked to be 100%.

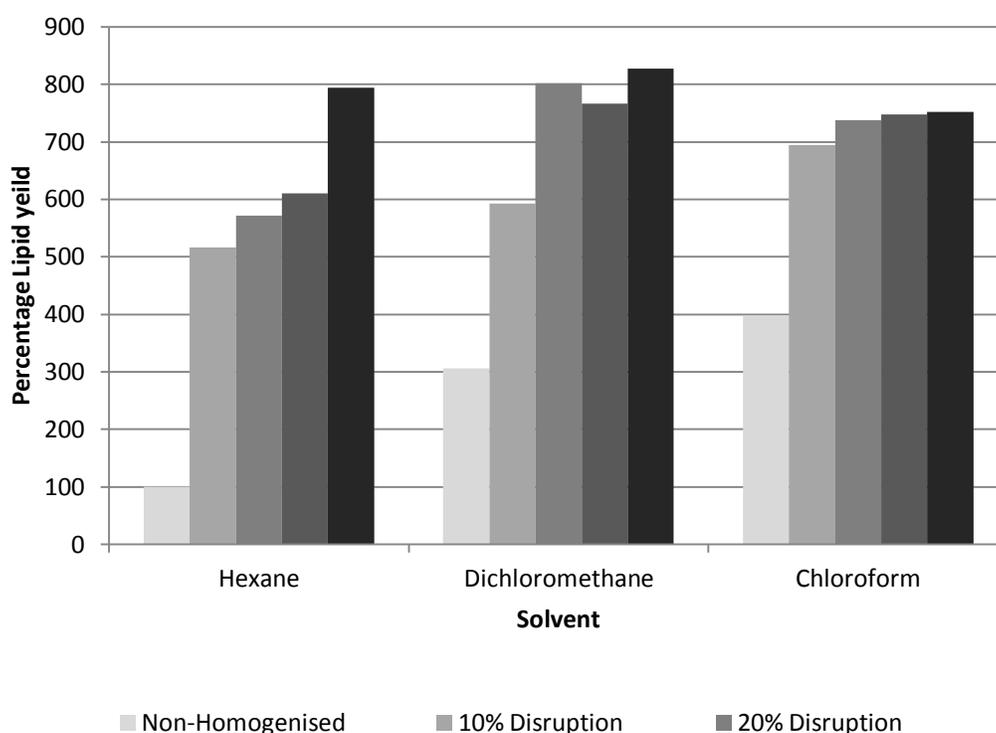


Figure 16. The amount of lipids extracted from 1 gram of algal biomass during three consecutive extractions compared (as a percentage) to the amount of lipids extracted from non-homogenized algae using hexane

Highly disrupted algae (when at least 67% of the cells are broken), when extracted with chloroform, results in almost 8.5 times more oil than using undisrupted algae in hexane. However, when the cells were disrupted, the differences between the total amounts of lipids extracted (in three consecutive extractions) were minimal. The reason for hexane behaving better during the second and the third extractions may be due to the removal of most of the moisture from the system during the first extraction. Hexane, being a highly hydrophobic solvent does not perform well in high moisture environments ^{27, 28}. Consequently, the advantage that chloroform and dichloromethane had during the first iteration decreases in subsequent extractions.

Chloroform, although immiscible with water, is known to perform better in somewhat aqueous extractions. This may be due to the slight polarity exerted by chlorine coupled with debris from algae acting as amphiphiles to emulsify and stabilize the mixture. Dichloromethane is more polar than chloroform and is expected to perform analogously (or better) in an aqueous environment.

Energy Considerations

Higher pressures and number of passes allow a higher lipid yield for both stressed and unstressed algae. However, energy consumption increases with increasing pressures and number of passes. Therefore it is important to compare the amount of energy available in recovered lipids and the amount of energy spent for homogenizing an algal suspension.

For this, information illustrated in Figure 15 was used. The amount of energy spent for homogenizing a 10 ml algal sample was calculated using Equation 2.

$$W = PVn \quad (4)$$

Where W is Work done in J, P is Pressure in Pa, V is volume of algal broth homogenized and N is number of passes through the homogenizer.

The amount of energy available in extracted lipids from 10 ml of algal broth (equivalent of 1 g of dried algae) was calculated assuming energy content of 41.70 MJ/kg for algal lipids²⁹.

The amount of energy available from lipids vs. the amount of energy used during homogenization is depicted in Figure 17. The diagonal dashed line indicates zero energy recovery from the homogenization operation. Locations above and left to the dashed line indicate a positive energy (gain) from the operation and locations below and to the right to the line shows a net loss of energy from the operation.

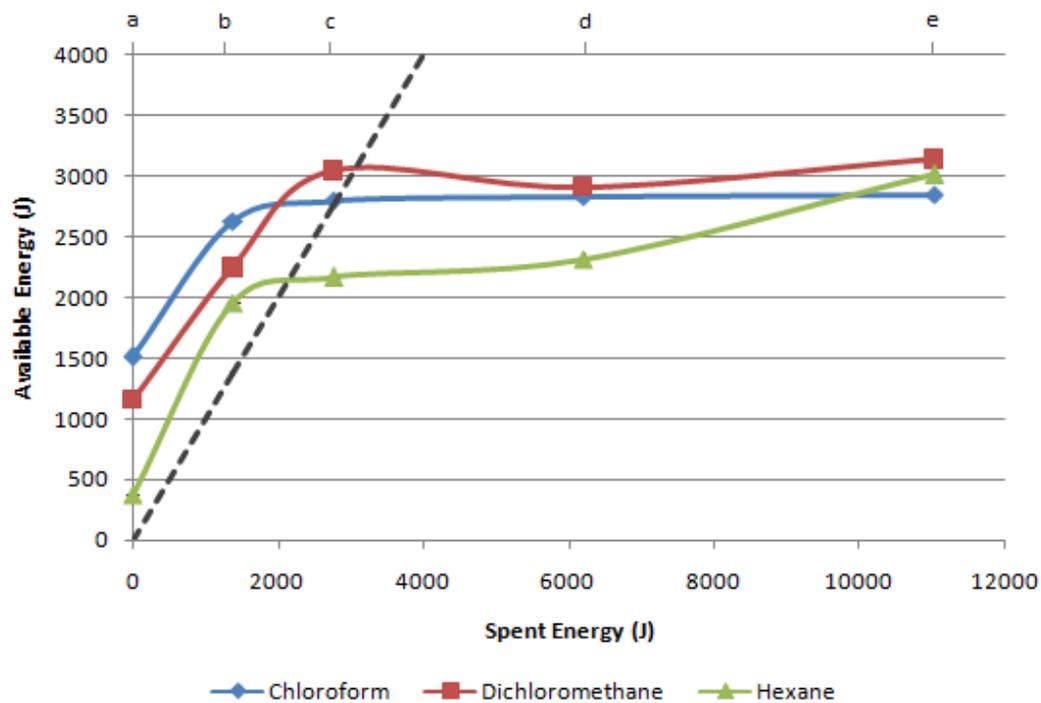


Figure 17. Comparison of available energy in algal lipids vs. energy spent during homogenization

Key for secondary X axis: a, algae with no treatment ; b, 10 000 Psi, 2 pass ; c, 20 000 Psi, 2 pass ; d, 30 000 Psi, 3 pass ; e, 40 000 Psi, 4 pass.

6. SUMMARY AND CONCLUSIONS

Preliminary Research

Initially, two particle sizers with 0.1 μm to 200 μm and 0.6 μm to 10 μm size detection ranges were used to analyze the particle size distributions of algae after and before homogenization. Cell breakage during homogenization was evident because it was observed in microscopic imaging. It was expected to see a leftward shift of the particle size distribution after homogenization. Although a shift was detected, the values did not correlate to significant cell breakage. The observed shift of particle size distribution is likely due to the breakage of cell agglomerates rather than individual cells. The reason for the inability to observe the expected results from homogenization via particle sizing apparatus may be due to the fact that larger particles (in this case non-disrupted cells and cell agglomerates) scatter a significantly larger amount of light as compared to smaller particles (in this case cell debris) at the same concentration. Therefore, cell debris may get shielded from appearing in the particle size distribution data. Therefore, particle sizing cannot be used as a means of measuring the effectiveness of *N. oculata* algal cell homogenization.

Studies indicated that cell counting using a cell-counting-chamber is an alternative way of measuring homogenization effectiveness. By screening studies, it was observed that the homogenization pressure, number of passes through the homogenizer, and

concentration of culture are significantly affect the degree of cell breakage while the nozzle diameter (between 100 to 195 μm diameter) does not.

Therefore it was concluded that nozzle size can be changed in the homogenizer to increase the nozzle flow without affecting the homogenization effectiveness.

Objective 1: Elucidating the Effect of Homogenization Conditions on the Degree of Cell Breakage

The analysis confirms that a higher number of passes and higher pressures through the high pressure homogenizer results in higher levels of cell disruption. Maximum cell disruption was observed for non-stressed 1% concentrated algal samples while minimum cell disruption was observed for stressed 0.1% samples. Non-stressed samples, in general, showed a higher degree of disruption compared to stressed samples. The reason for this can be changes of the structure of cell wall of algae. Change of the structural strength of the cell wall, specifically the shear resistance, from such changes is still to be investigated. Also, non-concentrated samples disrupted less compared to concentrated samples, probably due to increased shear forces resulting from increased viscosity.

Objective 2: Elucidating the Effect of Degree of Cell Breakage on the Oil Extraction Efficiency

As expected, the amount of total lipids extracted reduced with each extraction iteration. Although solvent chemistry affected the total amount lipids extracted varied during the initial extraction, the variability was low during subsequent extractions. It was observed

that greater cell breakage does not necessarily increase the amount of lipids extracted. Hexane has a lower extraction efficiency compared to dichloromethane and chloroform. Chloroform and dichloromethane were equally effective in extracting algal lipids, in the first extraction. However, when the second and third extractions are considered, it is observed that the effect of the solvent become less significant. The analysis confirms that it is possible to extract around 75 milligrams of lipids from 1 gram of algae (7.5% of the dry weight of algae).

Highly disrupted algae (when at least 67% of the cells are broken), when extracted with chloroform, results in almost 8.5 times more oil than using undisrupted algae in hexane. However, when the cells were disrupted, the differences between the total amounts of lipids extracted (in three consecutive extractions) were minimal.

Therefore it is possible to use mildly homogenized algal samples for lipid extraction without a significant reduction of lipid extraction efficiency.

Even though this study illustrates the possibility of using high pressure homogenization for lipid extraction from aqueous microalgae, energy demand for homogenization can exceed the energy content of extracted algal lipids in harsher homogenization conditions. Since this study concludes that the nozzle size can be increased without a significant loss of homogenization efficiency, it is necessary to investigate the possibility of using larger nozzle sizes for homogenization. Increased nozzle sizes can allow higher flow rates which in turn may lead to reduced overall energy demand.^{11, 30}

REFERENCES

- (1) Hill, J.; Nelson, E.; Tilman, D.; Polasky, S.; Tiffany, D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences* **2006**, 103 (30), 11206-11210.
- (2) Ross, A. B.; Anastasakis, K.; Kubacki, M.; Jones, J. M. Investigation of the pyrolysis behaviour of brown algae before and after pre-treatment using PY-GC/MS and TGA. *Journal of Analytical and Applied Pyrolysis* **2009**, 85 (1-2), 3-10.
- (3) Dote, Y.; Sawayama, S.; Inoue, S.; Minowa, T.; Yokoyama, S.-y. Recovery of liquid fuel from hydrocarbon-rich microalgae by thermochemical liquefaction. *Fuel* **1994**, 73 (12), 1855-1857.
- (4) Aresta, M.; Dibenedetto, A.; Barberio, G. Utilization of macro-algae for enhanced CO₂ fixation and biofuels production: Development of a computing software for an LCA study. *Fuel Processing Technology* **2005**, 86 (14-15), 1679-1693.
- (5) Bhatti, H. N.; Hanif, M. A.; Qasim, M.; Ata ur, R. Biodiesel production from waste tallow. *Fuel* **2008**, 87 (13-14), 2961-2966.
- (6) Miao, X.; Wu, Q. Biodiesel production from heterotrophic microalgal oil. *Bioresource Technology* **2006**, 97 (6), 841-846.
- (7) Demirbas, M. F.; Balat, M. Recent advances on the production and utilization trends of bio-fuels: a global perspective. *Energy Conversion and Management* **2006**, 47 (15-16), 2371-2381.
- (8) Sheehan, J.; Dunahay, T.; Benemann, J.; Roessler, P. *Look back at the U.S. Department of Energy's aquatic species program: biodiesel from algae; close-out report*; 1998.
- (9) Chisti, Y. Biodiesel from microalgae. *Biotechnology Advances* **2007**, 25 (3), 294-306.
- (10) Agrawal, R.; Singh, N. R.; Ribeiro, F. H.; Delgass, W. N. Sustainable fuel for the transportation sector. *Proceedings of the National Academy of Sciences* **2007**, 104 (12), 4828-4833.
- (11) Beer, L. L.; Boyd, E. S.; Peters, J. W.; Posewitz, M. C. Engineering algae for biohydrogen and biofuel production. *Current Opinion in Biotechnology* **2009**, 20 (3), 264-271.

- (12) Golueke, C. G.; Oswald, W. J. Harvesting and Processing Sewage-Grown Planktonic Algae. *Water Pollution Control Federation* **1965**, 37 (4), 471-498.
- (13) Sukenik, A.; Zmora, O.; Carmeli, Y. Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II. *Nannochloropsis* sp. *Aquaculture* **1993**, 117 (3-4), 313-326.
- (14) Mendes-Pinto, M. M.; Raposo, M. F. J.; Bowen, J.; Young, A. J.; Morais, R. Evaluation of different cell disruption processes on encysted cells of *Haematococcus pluvialis*: effects on astaxanthin recovery and implications for bio-availability. *Journal of Applied Phycology* **2001**, 13 (1), 19-24.
- (15) Currie, J. A.; Dunnill, P.; Lilly, M. D. Release of protein from bakers' yeast - *Saccharomyces cerevisiae*- by disruption in an industrial agitator mill. *Biotechnology and Bioengineering* **1972**, 14 (5), 725-736.
- (16) Engler, C. R., Cell breakage. In *Protein Purification Process Engineering*, Marcel Dekker: New York, 1994; pp 37-57.
- (17) Roger, G. H.; Todd, P.; Rudge, S. R.; Petrides, D. P., *Bioseparations Science and Engineering*. Oxford University Press: New York, 2003.
- (18) Sauer, T.; Robinson, C. W.; Glick, B. R. Disruption of native and recombinant *Escherichia coli* in a high-pressure homogenizer. *Biotechnology and Bioengineering* **1989**, 33 (10), 1330-1342.
- (19) de Boer, G. B. J.; de Weerd, C.; Thoenes, D.; Goossens, H. W. J. Laser diffraction spectrometry: fraunhofer diffraction versus mie scattering. *Particle & Particle Systems Characterization* **1987**, 4 (1-4), 14-19.
- (20) Li, S.-D.; Huang, L. Nanoparticles evading the reticuloendothelial system: role of the supported bilayer. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2009**, 1788 (10), 2259-2266.
- (21) Peña-Castro, J. M.; Martínez-Jerónimo, F.; Esparza-García, F.; Cañizares-Villanueva, R. O. Heavy metals removal by the microalga *Scenedesmus incrassatulus* in continuous cultures. *Bioresource Technology* **2004**, 94 (2), 219-222.
- (22) Montgomery, D. C., *Design and Analysis of Experiments*. 7 ed.; John Wiley & Sons Pte Ltd: Hoboken, NJ, 2009.
- (23) Minowa, T.; Yokoyama, S.; Kishimoto, M.; Okakura, T. Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction. *Fuel* **1995**, 74 (12), 1735-1738.

- (24) Hansen, C. M., *Hansen Solubility Parameters: a User's Handbook*. CRC Press: Boca Raton, FL, 2007.
- (25) Abbott, S.; Hansen, C. M., *Hansen Solubility Parameters in Practice*. Hansen-Solubility.com: Hørsholm, Denmark, 2008.
- (26) Nature of special risks attributed to dangerous substances and preparations In *Council Directive 67/548/EEC* Agency, E. C., Ed. European Chemicals Agency: Helsinki, Finland, 1967.
- (27) Nagle, N.; Lemke, P. Production of methyl ester fuel from microalgae. *Applied Biochemistry and Biotechnology* **1990**, 24-25 (1), 355-361.
- (28) Grima, E.; Medina, A.; Giménez, A.; Sánchez Pérez, J.; Camacho, F.; García Sánchez, J. Comparison between extraction of lipids and fatty acids from microalgal biomass. *Journal of the American Oil Chemists' Society* **1994**, 71 (9), 955-959.
- (29) Weyer, K.; Bush, D.; Darzins, A.; Willson, B. Theoretical maximum algal oil production. *BioEnergy Research* **2009**, 3 (2), 204-213.
- (30) de Vries, R. P.; Visser, J. Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews* **2001**, 65 (4), 497-522.
- (31) Wijffels, R. H.; Barbosa, M. J. An outlook on microalgal biofuels. *Science* **2010**, 329 (5993), 796-799.
- (32) Dismukes, G. C.; Carrieri, D.; Bennette, N.; Ananyev, G. M.; Posewitz, M. C. Aquatic phototrophs: Efficient alternatives to land-based crops for biofuels. *Current Opinion in Biotechnology* **2008**, 19 (3), 235-240.
- (33) Rodolfi, L.; Chini Zittelli, G.; Bassi, N.; Padovani, G.; Biondi, N.; Bonini, G.; Tredici, M. R. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering* **2009**, 102 (1), 100-112.
- (34) Wolf, F. R.; Nonomura, A. M.; Bassham, J. A. Growth and branched hydrocarbon production in a strain of *Botryococcus braunii* (chlorophyta)1. *Journal of Phycology* **1985**, 21 (3), 388-396.
- (35) Vazhappilly, R.; Chen, F. Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth. *Journal of the American Oil Chemists' Society* **1998**, 75 (3), 393-397.

- (36) Renaud, S.; Parry, D.; Thinh, L.-V.; Kuo, C.; Padovan, A.; Sammy, N. Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. *Journal of Applied Phycology* **1991**, 3 (1), 43-53.
- (37) Flynn, K.; Davidson, K.; Leftley, J. Carbon-nitrogen relations during batch growth of *Nannochloropsis oculata* (Eustigmatophyceae) under alternating light and dark. *Journal of Applied Phycology* **1993**, 5 (4), 465-475.
- (38) Merrett, M. J.; Nimer, N. A.; Dong, L. F. The utilization of bicarbonate ions by the marine microalga *Nannochloropsis oculata* (Droop) Hibberd. *Plant, Cell and Environment* **1996**, 19 (4), 478-484.
- (39) Myers, J., Growth characteristics of algae in relation to the problems of mass culture In *Algal Culture from Laboratory to Pilot Plant*, Burlew, J. S., Ed. Carnegie Institution of Washington Publication: Washington, DC, 1953; pp 37-55.
- (40) Rosenberg, J. N.; Oyler, G. A.; Wilkinson, L.; Betenbaugh, M. J. A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. *Current Opinion in Biotechnology* **2008**, 19 (5), 430-436.
- (41) Ragonese, F. P.; Williams, J. A. A mathematical model for the batch reactor kinetics of algae growth. *Biotechnology and Bioengineering* **1968**, 10 (1), 83-88.
- (42) Zittelli, G. C.; Pastorelli, R.; Tredici, M. R. A modular flat panel photobioreactor (MFPP) for indoor mass cultivation of *Nannochloropsis* sp. Under artificial illumination. *Journal of Applied Phycology* **2000**, 12, 521-527.
- (43) Converti, A.; Casazza, A. A.; Ortiz, E. Y.; Perego, P.; Del Borghi, M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification* **2009**, 48 (6), 1146-1151.
- (44) Hagiwara, A.; Hamada, K.; Hori, S.; Hirayama, K. Increased sexual reproduction in *brachionus plicatilis* (rotifera) with the addition of bacteria and rotifer extracts. *Journal of Experimental Marine Biology and Ecology* **1994**, 181 (1), 1-8.
- (45) Sukenik, A. Ecophysiological considerations in the optimization of eicosapentaenoic acid production by *Nannochloropsis* sp. (Eustigmatophyceae). *Bioresource Technology* **1991**, 35 (3), 263-269.
- (46) Durmaz, Y. Vitamin E ([alpha]-tocopherol) production by the marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae) in nitrogen limitation. *Aquaculture* **2007**, 272 (1-4), 717-722.

- (47) Dunstan, G.; Volkman, J.; Barrett, S.; Garland, C. Changes in the lipid composition and maximisation of the polyunsaturated fatty acid content of three microalgae grown in mass culture. *Journal of Applied Phycology* **1993**, 5 (1), 71-83.
- (48) Araujo, A.; McNair, J. Individual and population level effects of antibiotics on the rotifers, *Brachionus calyciflorus* and *B. plicatilis*. *Hydrobiologia* **2007**, 593 (1), 185-199.
- (49) Okauchi, M.; Yamada, T.; Ozaki, A. Optimum media for outdoor large-scale and indoor small-scale batch style culture of *Nannochloropsis oculata*. *Aquaculture Science* **2008**, 56 (2), 147-155.
- (50) Suen, Y.; Hubbard, J. S.; Holzer, G.; Tornabene, T. G. Total lipid production of the green alga *Nannochloropsis* sp. Qii under different nitrogen regimes. *Journal of Phycology* **1987**, 23 (s2), 289-296.
- (51) Moazami, N.; Ranjbar, R.; Ashori, A.; Tangestani, M.; Nejad, A. S. Biomass and lipid productivities of marine microalgae isolated from the Persian Gulf and the Qeshm Island. *Biomass and Bioenergy* **2011**, 35 (5), 1935-1939.
- (52) Hu, H.; Gao, K. Response of growth and fatty acid compositions of *Nannochloropsis* sp. To environmental factors under elevated CO₂ concentration. *Biotechnology Letters* **2006**, 28 (13), 987-992.
- (53) Hodgson, P.; Henderson, R.; Sargent, J.; Leftley, J. Patterns of variation in the lipid class and fatty acid composition of *Nannochloropsis oculata* (Eustigmatophyceae) during batch culture. *Journal of Applied Phycology* **1991**, 3 (2), 169-181.
- (54) Fábregas, J.; Domínguez, A.; Regueiro, M.; Maseda, A.; Otero, A. Optimization of culture medium for the continuous cultivation of the microalga *Haematococcus pluvialis*. *Applied Microbiology and Biotechnology* **2000**, 53 (5), 530-535.
- (55) Rodolfi, L.; Zittelli, G. C.; Barsanti, L.; Rosati, G.; Tredici, M. R. Growth medium recycling in *Nannochloropsis* sp. mass cultivation. *Biomolecular Engineering* **2003**, 20 (4-6), 243-248.
- (56) Forján, E.; Garbayo, I.; Casal, C.; Vilchez, C., Enhancement of carotenoid production in *Nannochloropsis* by phosphate and sulphur limitation. In *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, Méndez-Vilas, A., Ed. Formates: Badajoz, Spain, 2007; pp 356-365.

- (57) Guillard, R. R. L.; Ryther, J. H. Studies of marine planktonic diatoms: *I. Cyclotella nana* hustedt, and *Detonula confervacea* (cleve) gran. *Canadian Journal of Microbiology* **1962**, 8 (2), 229-239.
- (58) Harrison, P. J.; Waters, R. E.; Taylor, F. J. R. A broad spectrum artificial sea water medium for coastal and open ocean phytoplankton. *Journal of Phycology* **1980**, 16 (1), 28-35.
- (59) Provasoli, L. Media and prospects for the cultivation of marine algae. *The Japanese Society of Plant Physiologists* **1966**, 12 (3), 63-75.
- (60) Karlson, B.; Potter, D.; Kuylenstierna, M.; Andersen, R. A. Ultrastructure, pigment composition, and 18S rRNA gene sequence for *Nannochloropsis granulata* sp. nov. (Monodopsidaceae, Eustigmatophyceae), a marine ultraplankton isolated from the Skagerrak, northeast Atlantic Ocean. *Phycologia* **1996**, 35 (3), 253-260.
- (61) Lund, J. W. G.; Kipling, C.; Cren, E. D. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologia* **1958**, 11 (2), 143-170.
- (62) Andersen, R. A., *Algal Culturing Techniques*. Elsevier/Academic Press: Burlington, MA, 2005.
- (63) Nalewajko, C. Photosynthesis and excretion in various planktonic algae. *Limnology and Oceanography* **1966**, 11 (1), 1-10.
- (64) Gouveia, L.; Oliveira, A. Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology & Biotechnology* **2009**, 36 (2), 269-274.
- (65) Amin, S. Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management* **2009**, 50 (7), 1834-1840.
- (66) Levine, R. B.; Pinnarat, T.; Savage, P. E. Biodiesel production from wet algal biomass through in situ lipid hydrolysis and supercritical transesterification. *Energy & Fuels* **2010**, 24 (9), 5235-5243.
- (67) Tredici, M. R.; Materassi, R. From open ponds to vertical alveolar panels - the italian experience in the development of reactors for the mass cultivation of phototrophic microorganisms. *Journal of Applied Phycology* **1992**, 4 (3), 221-231.
- (68) Ginzburg, B.-Z. Liquid fuel (oil) from halophilic algae: A renewable source of non-polluting energy. *Renewable Energy* **1993**, 3 (2-3), 249-252.

- (69) Grima, E. M.; Belarbi, E. H.; Fernandez, F. G. A.; Medina, A. R.; Chisti, Y. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* **2003**, 20 (7-8), 491-515.
- (70) Crist, R. H.; Oberholser, K.; Shank, N.; Ming, N. Nature of bonding between metallic ions and algal cell walls. *Environmental Science & Technology* **1981**, 15 (10), 1212-1217.
- (71) Blumreisinger, M.; Meindl, D.; Loos, E. Cell wall composition of chlorococcal algae. *Phytochemistry* **1983**, 22 (7), 1603-1604.
- (72) Zhang, G.; Zhang, P.; Wang, B.; Liu, H. Ultrasonic frequency effects on the removal of *Microcystis aeruginosa*. *Ultrasonics Sonochemistry* **2006**, 13 (5), 446-450.
- (73) Sazdanoff, N. Modeling and simulation of the algae to biodiesel fuel cycle. The Ohio State University, Columbus, OH, 2006.
- (74) Gualtieri, P.; Barsanti, L.; Passarelli, V. Chitosan as flocculant for concentrating *Euglena gracilis* cultures. *Annales de l'Institut Pasteur / Microbiologie* **1988**, 139 (6), 717-726.
- (75) Buelna, G.; Bhattarai, K. K.; de la Noue, J.; Taiganides, E. P. Evaluation of various flocculants for the recovery of algal biomass grown on pig-waste. *Biological Wastes* **1990**, 31 (3), 211-222.
- (76) Israelachvili, J. N., *Intermolecular Surface Forces*. Elsevier: Burlington, MA, 2011.
- (77) Shammas, N. K.; Wang, L. K.; Hung, Y.-T., Coagulation and flocculation physicochemical treatment processes. In *Handbook of Environmental Engineering*, Humana Press: New York, 2005; Vol. 3, pp 103-139.
- (78) Tenney, M. W.; Echelberger, W. F., Jr.; Schuessler, R. G.; Pavoni, J. L. Algal flocculation with synthetic organic polyelectrolytes. *Applied and Environmental Microbiology* **1969**, 18 (6), 965-971.
- (79) Ayoub, G. M.; Lee, S.-I.; Koopman, B. Seawater induced algal flocculation. *Water Research* **1986**, 20 (10), 1265-1271.
- (80) McGarry, M. G. Algal flocculation with aluminum sulfate and polyelectrolytes supercritical transesterification. *Journal (Water Pollution Control Federation)* **1970**, 42 (5), R191-R201.
- (81) Sukenik, A.; Teltch, B.; Wachs, A. W.; Shelef, G.; Nir, I.; Levanon, D. Effect of oxidants on microalgal flocculation. *Water Research* **1987**, 21 (5), 533-539.

- (82) Tilton, R. C.; Murphy, J.; Dixon, J. K. The flocculation of algae with synthetic polymeric flocculants. *Water Research* **1972**, 6 (2), 155-164.
- (83) Han, M. Y.; Kim, W. A theoretical consideration of algae removal with clays. *Microchemical Journal* **2001**, 68 (2-3), 157-161.
- (84) Seuront, L.; Vincent, D.; Mitchell, J. G. Biologically induced modification of seawater viscosity in the Eastern English Channel during a *Phaeocystis globosa* spring bloom. *Journal of Marine Systems* **2006**, 61 (3-4), 118-133.
- (85) Chisti, Y. Response to Reijnders: Do biofuels from microalgae beat biofuels from terrestrial plants? *Trends in Biotechnology* **2008**, 26 (7), 351-352.
- (86) Berry, A. E. Removal of algae by microstrainers. *Jour, of the. American Water Works Association* **1961**, 53 (12), 1503-1508.
- (87) Levin, G. V.; Clendenning, J. R.; Gibor, A.; Bogar, F. D. Harvesting of algae by froth flotation. *Applied and Environmental Microbiology* **1962**, 10 (2), 169-175.
- (88) Olaizola, M. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. *Journal of Applied Phycology* **2000**, 12 (3), 499-506.
- (89) Went, H. A. A "micro" homogenizer for small-volume samples. *Biochimica et Biophysica Acta* **1958**, 27 (1), 165-167.
- (90) Simon, D.; Helliwell, S. Extraction and quantification of chlorophyll a from freshwater green algae. *Water Research* **1998**, 32 (7), 2220-2223.
- (91) Matsuda, Y.; Musgrave, A.; van den Ende, H.; Roberts, K. Cell walls of algae in the volvocales: Their sensitivity to a cell wall lytic enzyme and labeling with an anti-cell wall glycopeptide of *Chlamydomonas reinhardtii*. *Journal of Plant Research* **1987**, 100 (4), 373-384.
- (92) Doan, T.-T.; Obbard, J. Improved Nile red staining of *Nannochloropsis sp.* *Journal of Applied Phycology* **2010**, 23 (5), 1-7.
- (93) Ahlgren, G.; Gustafsson, I.-B.; Boberg, M. Fatty acid content and chemical composition of freshwater microalgae. *Journal of Phycology* **1991**, 28 (1), 37-50.
- (94) Folch, J.; Lees, M.; Stanley, G. H. S. A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* **1957**, 226 (1), 497-509.
- (95) Boberg, M.; Croon, L. B.; Gustafsson, I. B.; Vessby, B. Platelet fatty acid composition in relation to fatty acid composition in plasma and to serum lipoprotein

lipids in healthy subjects with special reference to the linoleic acid pathway. *Clinical Science* **1985**, 68 (5), 581-587.

(96) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Canadian Journal of Physiology and Pharmacology* **1959**, 37 (8), 911-917.

(97) Dunstan, G. A.; Volkman, J. K.; Jeffrey, S. W.; Barrett, a. S. M. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 2. Lipid classes and fatty acids *Journal of Experimental Marine Biology and Ecology* **1992**, 161 (1), 115-135.

(98) Vyas, A. P.; Verma, J. L.; Subrahmanyam, N. A review on FAME production processes. *Fuel* **2010**, 89 (1), 1-9.

(99) Dahmer, M. L.; Fleming, P. D.; Collins, G. B.; Hildebrand, D. F. A rapid screening technique for determining the lipid composition of soybean seeds. *Journal of the American Oil Chemists' Society* **1989**, 66 (4), 543-549.

(100) Mourente, G.; Lubián, L. M.; Odriozola, J. M. Total fatty acid composition as a taxonomic index of some marine microalgae used as food in marine aquaculture. *Hydrobiologia* **1990**, 203 (3), 147-154.

(101) Cooksey, K. E.; Guckert, J. B.; Williams, S. A.; Callis, P. R. Fluorometric determination of the neutral lipid content of microalgal cells using Nile red. *Journal of Microbiological Methods* **1987**, 6 (6), 333-345.

(102) Lee, S.; Yoon, B.-D.; Oh, H.-M. Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology Techniques* **1998**, 12 (7), 553-556.

(103) Huang, G.-H.; Chen, G.; Chen, F. Rapid screening method for lipid production in alga based on Nile red fluorescence. *Biomass and Bioenergy* **2009**, 33 (10), 1386-1392.

(104) Cooper, M. S.; Hardin, W. R.; Petersen, T. W.; Cattolico, R. A. Visualizing "Green Oil" in live algal cells. *Journal of Bioscience and Bioengineering* **2010**, 109 (2), 198-201.

(105) Chen, W.; Sommerfeld, M.; Hu, Q. Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. *Bioresource Technology* **2011**, 102 (1), 135-141.

(106) Poncet, J.-M.; Véron, B.; et Cryopreservation of the unicellular marine alga, *Nannochloropsis Oculata*. *Biotechnology Letters* **2003**, 25 (23), 2017-2022.

(107) Tzovenis, I.; Triantaphyllidis, G.; Naihong, X.; Chatzinikolaou, E.; Papadopoulou, K.; Xouri, G.; Tafas, T. Cryopreservation of marine microalgae and potential toxicity of cryoprotectants to the primary steps of the aquacultural food chain. *Aquaculture* **2004**, 230 (1-4), 457-473.

APPENDIX

LITERATURE SURVEY: CURRENT STATUS AND FUTURE DIRECTIONS OF BIOFUELS FROM MICROALGAE

Introduction

Microalgae-based energy production has attracted considerable attention in recent times primarily due to the ability of microalgae to assimilate carbon at substantially higher rates than terrestrial plants, their general ability to convert this carbon to lipids with unmatched efficiency, and the ability to cultivate algae in marginal land that is unsuitable for any other agricultural activity^{2, 9, 11, 31, 32}. The lipids recovered from algae could be directly converted to hydrocarbon fuels or biodiesel via deoxygenation and transesterification respectively, while the lipid-exhausted biomass, subsequent to high-value product recovery, could further be utilized as a raw material for energy production.

Despite these advantages, microalgae based energy production is yet far from being a commercial reality due to a multitude of challenges. In a broader sense, challenges have been identified in stages including growth, harvesting and processing. The overall objective of this analysis is to critically review the state-of-the-art of this technology, identify key challenges in these stages, and suggest potential strategies to overcome some of the more critical ones.

Growth of High Lipid Producing Microalgae

Although there are numerous microalgal species, only those that have following qualities are of interest in the context of bio-energy production:

- High growth rates (with high sunlight and carbon-dioxide assimilation efficiencies).
- Ability to assimilate lipids with relative ease.
- High tolerance to environmental variations.

A list of microalgal species that have been identified as having a high potency for bio-energy production is shown in ³³ for comparison.

Table 12. Characteristics of selected microalgal species that have a high potential in bio-energy industry

| Species | Origin | Biomass Productivity (g/l/day) | Lipid Productivity (mg/l/day) | Cell size (um) | Remarks |
|----------------------|---------------------|--------------------------------|-------------------------------|----------------|---------------------------------------|
| Nannochloropsis spp. | Marine / Soft water | 0.17~0.21 | 37.6~61.0 | 2~5 | Spherical single-celled green algae |
| Chlorella spp. | Soft water | 0.23 | 42.1~44.7 | 2~10 | Spherical single-celled green algae |
| Tetraselmis spp. | Marine | 0.28~0.23 | 36.4~43.4 | 5~10 | phytoplankton |
| Botryococcus Spp. | Marine | 0.17 ³⁴ | 51 | 10 | pyramid shaped, Planktonic microalgae |

Nannochloropsis sp. are purported to show highest lipid productivities-which can range from 40 to 60 mg lipids/l of broth/day³³.

Bioreactors for Microalgae

The two techniques that are believed to be most practical for commercial scale algae cultivation are open raceways and photo bioreactors⁹.

Raceway Ponds

Raceway ponds are the simplest method of culturing algae. A simple looped channel of approximately 0.3 m depth, constructed with concrete or compacted earth lined with

clear plastic, as shown in, is called a raceway pond. Ponds are made shallow in order to facilitate maximum light penetration to the depths⁹.

In these ponds, flow and agitation are required for preventing algal settling. Mixing is typically performed with a paddle wheel. During growth, nutrient media is added before the paddle wheel to facilitate good mixing while harvesting is performed down-stream from the paddle to maximize effective yields⁹.

Raceway ponds are relatively inexpensive to construct and operate. However, because of their open design, wide temperature fluctuations, loss of moisture and CO₂ losses and contamination from other algal species reduces the efficiency of raceway ponds. Additionally, power mixing and inability to maintain a dark zone reduces the maximum culture concentration⁹.

Photo Bioreactors

Photo bioreactors (Figure 19) consist of an array of transparent tubes with a diameter around 0.1 m. To increase the algae production efficiency it is necessary to orient the array of tubes to collect maximum solar energy⁹.

Algal broth is continuously pumped through the array of transparent tubes and a reservoir which acts as the dark zone. For pumping algae a mechanical pump or airlift pump can be used⁹.

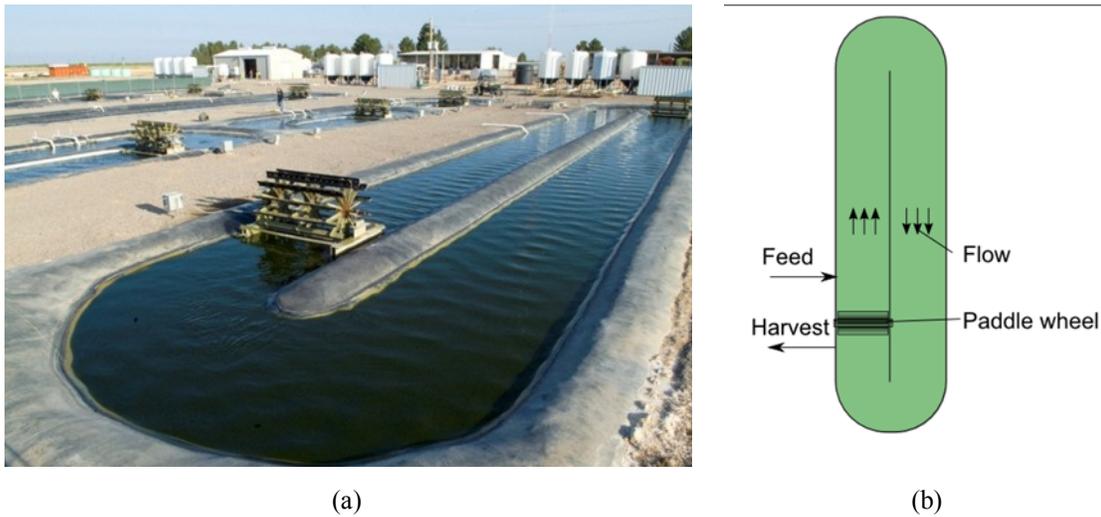


Figure 18. Raceway ponds

(a, Open raceway*; b, Schematic diagram of a raceway)

Courtesy : Texas Agrilife Research

Photo Bioreactors

Photo bioreactors (Figure 19) consist of an array of transparent tubes with a diameter around 0.1 m. To increase the algae production efficiency it is necessary to orient the array of tubes to collect maximum solar energy⁹.

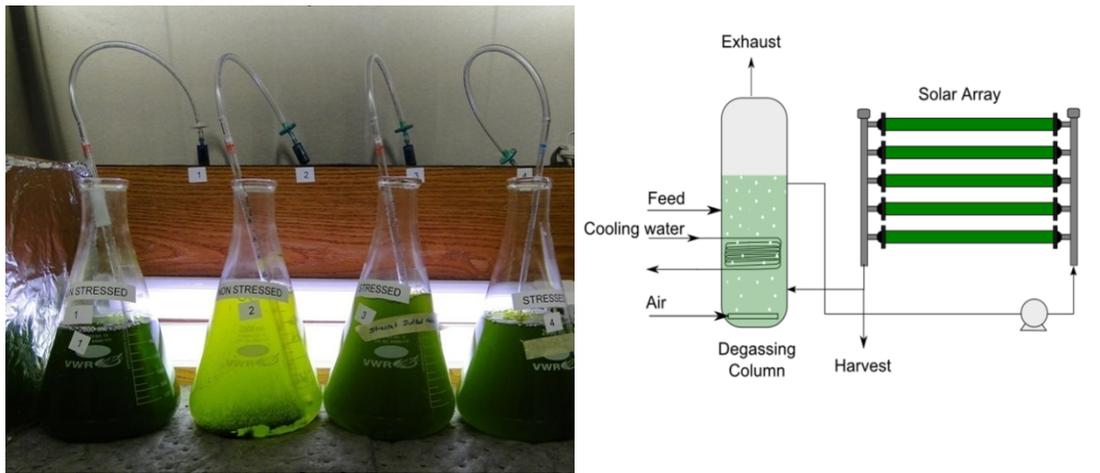
Algal broth is continuously pumped through the array of transparent tubes and a reservoir which acts as the dark zone. For pumping algae a mechanical pump or airlift pump can be used⁹.

Since bioreactors are sealed. They are resilient for contamination and have a higher CO₂ efficiency than raceway ponds. However the cost of operation and construction can be higher than raceway ponds⁹.

Sedimentation of cells in the tubes should be prevented by maintaining higher turbulence in the bioreactor. Also periodic cleaning may be necessary to prevent algal deposition on reactor wall. Due to photosynthesis, photo bioreactors generate a high amount of oxygen. If oxygen in the broth exceeds air saturation concentration by 300%, inhibition of photosynthesis may occur. Therefore a degassing apparatus should be operated in line with the photo bioreactor to remove oxygen and air bubbles. Temperature controlling can be done using a heating/ cooling coil in the algae reservoir. Because of the ease of controlling various parameters which affect photosynthesis, photo bioreactors surpass raceway ponds with efficiency.

Growth Conditions

The ability of algae to assimilate carbon is directly proportional to the availability of visible light. Therefore it is of paramount importance that photoautotrophic conditions are maintained³⁵. In the laboratory, illumination of the growth culture is normally done by cool white fluorescent bulbs to prevent heating of the culture.



(a)

(b)

Figure 19. Schematic diagram of a photo bioreactor

(a; Photo bioreactor used to culture algae in lab, b; schematic diagram of a tubular photo bioreactor)

Logically illumination should simulate natural solar light in pattern (day night cycle) and average photon flux density. Normally, 12-hour day time and 12-hour night time is assumed³⁶. Switching on and off the illumination system can be done by using electrical timing devices. Generally, a photon flux density of $50 \sim 100 \mu\text{molm}^{-2}\text{s}^{-1}$ is supplied for *N. Oculata* cultures^{37, 38}. 300 w/m^2 is a good estimate for the maximum solar energy availability on most of the places on earth and an equivalent amount should reach cells at the far side of the reactor/pond for maximum effectiveness. Measuring the light intensity can be done using underwater quantum sensors³⁶.

Studies have confirmed that when the amount of light available for an algal culture is increased, the growth rate increases. However, when a certain illumination level is exceeded, there is no further increase of the growth rate. This illumination level is referred as saturation level ³⁹. Since algal cells are physiologically and biochemically adapted for low light conditions, under higher illumination, they tend to form reactive oxygen species, reduce photosynthetic efficiency, and increase deleterious cell stress ⁴⁰[55]. Einsteinian's law of photochemical equivalence can be used to model the effect of illumination on algal cultures ⁴¹. It should also be noted that the higher irradiance levels contributes to higher amounts of fatty acid content ⁴².

For optimal growth of algal cells, a carbon source is equally important as light. In the case of algae, this is carbon dioxide (CO₂). Although CO₂ naturally dissolves in water (0.1% w/w), these levels deplete quite fast at high algal concentrations. To circumvent this issue, a common practice is to use aerators (Figure 20) ⁴³. Mechanical agitation can be used in this instance since microalgal cells are not shear sensitive ³⁶.

It should be noted that despite the amount of aeration, there is a maximum amount of CO₂ that can be dissolved in water and as a result, continuous aeration may not be necessary.



Figure 20. CO₂ supplementation via aeration (Source: Agrilife Research)

An effective strategy is to supplement concentrated CO₂ whenever available. For an example effluent gases from coal power plants and petrochemical refineries are rich with CO₂. Strategically locating algal production plants in close proximity to these plants may open up cheap sources of CO₂.

It should be noted that CO₂ can become toxic to the algal stream when the levels are above 5% (w/w) in the gas stream in equilibrium with the suspension ³⁹.

Culture Media

Although CO₂ and sunlight are the main ingredients necessary for algal survival, for optimal growth, algae needs nutrients just like any terrestrial crop. For this many nutrient formulations have been developed. The primary ingredients in these formulations include supplements that provide nitrogen (N), phosphorus (P), potassium (K), minerals and vitamins.

Example nutrient mediums that are sea water based are “f/2 media” and “Erd-Schreiber medium”⁴⁴⁻⁴⁶. These are prepared using artificial or natural sea water^{47,48}. These media can be purchased from chemical suppliers like Sigma Aldrich, St. Louis, USA. in concentrated liquid or powder forms. Preparation of media is done in sterile conditions to eliminate bacterial contamination. Sterilization can be done using filtration by 0.2 μm micro filters or autoclaving using 103.4 kPa for 15 minutes^{36,37}. It is also suggested that it is possible to use direct artificial seawater or sterilized seawater for culturing marine alga cost effectively^{37,49}.

“Bold modified Basel Mixture” is another soft water based nutrient medium²¹. GPM Medium,⁵⁰ RM medium⁵¹ are other mediums which can be used to cultivate algae.

Preparation of Algal Culturing Medium

The compositions of a few culture mediums are presented below. For example, F and F/2 mediums are the most commonly used for the culturing *Nannochloropsis sp.*^{36,37,45,47,52-57}. These are prepared by enriching seawater with a vitamin, trace metal and nutrient

solution. Natural seawater or artificial seawater can be used in the place of sea salt ⁵⁸.

Artificial seawater is prepared according to the following procedure:

Add following amounts of anhydrous salts to 500 ml of distilled water. Autoclave (Label as A-Salts STK”)

| Solute | Weight to 500 ml Distilled water |
|---------------------------------|----------------------------------|
| NaCl | 20.758 g |
| Na ₂ SO ₄ | 3.477 g |
| KCl | 0.587 g |
| NaHCO ₄ | 0.170 g |
| KBr | 0.0845 g |
| H ₂ BO ₃ | 0.0225 g |
| NaF | 0.0027 g |

Add following amounts of hydrated salts to 500 ml of distilled water and autoclave it.
(Label as “H-Salts STK”)

| Solute | Weight to 500 ml Distilled water |
|--------------------------------------|----------------------------------|
| MgCl ₂ .6H ₂ O | 9.395 g |
| CaCl ₂ .2H ₂ O | 1.326 g |
| SrCl ₂ .6H ₂ O | 0.0214 g |

Prepare artificial seawater mixture by adding A-Salts STK and H-Salts STK together.
(Label as “ASW STK”)

Enrichment Solution

Several enrichment solutions are available for different growth conditions. For example, f enrichment solution, published by Guillard ⁵⁷, is prepared with high N, Si and Fe concentrations while Provasoli's ES ⁵⁹ solution is made with intermediate amounts. An example recipe for preparation of an enrichment solution is given below:

It should be noted that it is difficult to mix all of these components in to seawater without precipitation. Therefore components should be dissolved with distilled water to obtain several stock mixtures and then should be sterilized and mixed together to obtain the final product. For example, following procedure is followed to obtain medium f or medium f/2.

| Name Component | Concentration per 1 l of sea water. | | |
|--|-------------------------------------|-------------------------|--------------------------------|
| | Medium f ⁵⁷ | ES Medium ⁵⁸ | Stressing Medium ⁵² |
| NaNO ₃ | 150 mg | 46.67 mg | 12.74mg |
| NaH ₂ PO ₄ .2H ₂ O | 10 mg | 6.67 mg | 0.71 mg |
| Fe Sequestrene | 10 mg | | |
| Na ₂ SiO ₃ .5H ₂ O | 30-60 mg | 30 mg | 30 mg |
| Na ₂ EDTA.2H=O | | 5.53 mg | 5.53 mg |
| Trace Metal | | | |
| CuSO ₄ .5H ₂ O | 0.0196 mg | 0.016 mg | 0.016 mg |
| ZnSO ₄ .7H ₂ O | 0.044mg | 0.073 mg | 0.073 mg |
| CoCl ₂ .6H ₂ O | 0.022 mg | | |
| MnCl ₂ .4H ₂ O | 0.360 mg | | |
| Na ₂ MoO ₄ .2H ₂ O | 0.0126 mg | | |
| H ₃ BO ₃ | | 3.8 mg | 3.8 mg |
| Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O | | 2.4 mg | 2.4 mg |
| Vitamins | | | |
| Thiamine. HCL | 0.2 mg | 0.1 mg | 0.1 mg |
| Biotin | 1 µg | 1 µg | 1 µg |
| B ₁₂ | 1 µg | 2µg | 2µg |

Note : Instead of Fe Sequestrene, Ferric Chloride and Na₂EDTA.2H₂O can be mixed to give the same amount of Ferric and chelating agent.

1. Prepare a 150.0 g/l solution of NaNO_3 (Name it as “ NaNO_3 STK”).
2. Prepare 10 g/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ solution (Name it as “ $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ STK”).
3. Prepare a 30 g/l solution of $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ (Name it as “ $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ STK”).
4. Prepare 30 g/l $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ solution (name it as “ Na_2EDTA STK”).
5. Add following amounts of trace metal solutions in to 750 ml of distilled water while mixing thoroughly. Finally, add distilled water to make up to 1 L (Name it as “Trace metal STK”).

| Solute | Weight (mg) per 1 L Distilled Water |
|---|-------------------------------------|
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 19.6 |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | 44.0 |
| $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ | 22.0 |
| $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ | 360.0 |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ | 12.6 |

6. Add 9 g of Ferric citrate and citric acid to 1 l of distilled water and autoclave at 15 psi, 121 °C for 15 min to dissolve(Name it as “Ferric STK”)
7. Prepare 10.0 mg /100 ml dH₂O solution of Vitamin B12 (Name it as “Vitamin B12 stock solution”)
8. Prepare 10.0 mg /100 ml dH₂O solution of Biotin. (Name it as “Biotin stock solution”)
9. Add 1 ml “Vitamin B12 stock solution” and 1 ml “Biotin Stock Solution” and 20 mg of thiamine HCL solution to 100 ml of distilled water (Name it as “ Vitamin STK”)
10. Add following amounts of stock mixtures to 1l of “ASW STK”

| Stock Solution | Volume added (ml) |
|-----------------------|--------------------------|
| Nitrate STK | * |
| Phosphate STK | * |
| Silicate STK | 1 |
| EDTA STK | 1 |
| Trace metal STK | 1 |
| Ferric STK | 1 |
| Vitamine STK | 1 |

Nitrate STK and Phosphate STK should be added after being diluted in to appropriate amounts to give the required final concentration (using following guidelines)

| Mixture | NO₃⁻ Concentration mg/ml | PO₄³⁻ Concentration mg/ml | Nitrate STK (ml) | Phosphate STK (ml) |
|----------------|---|--|-----------------------------|-------------------------------|
| f | 0.15 | 0.0113 | 1 | 1 |
| f/2 | 0.15 | 0.00565 | 1 | 0.5 |

All stock solutions are typically stored in refrigerated conditions after preparation.

During the exponential growth stage of algae additional nutrients nitrate and phosphate should be supplied in excess⁵⁶. For this, addition of supplemental NaNO₃ or NaPO₄ is necessary^{46, 52}. However, when the culture enters the mature stationary phase, lipid enrichment in the cells is achieved via limiting the nitrate and/or phosphate concentrations of the medium^{46, 50}.

Recycling of the culture media may be a necessity in commercial production of algae. However literature suggests against media recycle due to the presence of cell wall remains (during cell division *Nannochloropsis* releases a thick and multilayered parent cell wall that causes downstream separation issues⁶⁰ and the presences of auto-inhibitors that can reduce the growth rate significantly and cause cell coagulation⁵⁵).

Measuring Algal Concentration

Growth measurements of algal cultures are done by measuring variations of cell concentrations. Techniques such as cell counting, optical density measurement or ash free dry weight are used in this regard ^{61,62}.

Cell counts are measured using Nebular improved hemocytometer ³⁶. Counting the number of cells present in an algal sample via a hemocytometer is the most direct method to measure cell concentrations. However, the small sampling sizes that could be accommodated in counting chambers may contribute to significant sampling errors.

It is possible to use **flow cytometry** to count the number of cells in a given volume of broth. In this method, a suspension of algae is passed through a laser beam which is perpendicular to the flow. A series of detectors are used to measure forward scattered and side scattered beams. In some cases, a florescent detector is used to measure florescence from excited florescent chemicals in cells. A combination of such data is used to calculate the concentration of cells in the suspension ⁶².

Ash free dry weight is another technique that is widely used to present algal concentrations. In this case, a given volume of algal culture is separated from culture medium, washed and then dried at 105 °C followed by drying at 550 °C ^{42,63}. The algal concentration is then reported on a dry/wet weight basis.

Optical density measurements using a spectrophotometer ⁴³ at a given wavelength (540 nm) ⁵¹ is another technique widely used to present algal cell concentrations. In this

method, a calibration table is developed correlating optical density measurements with gravimetric cell contents and cell concentrations are reported as optical density measurements. In the event that optical density is reported, the calibration information is needed to make proper interpretations. However, it should be noted that there can be errors associated with optical density measurements due to interactions with chlorophyll concentrations.

Table 13 depicts various advantages and disadvantages of cell concentration measurement techniques available.

After dewatering, the algal cell walls should be disintegrated to access material within the cellular matrix^{31,64}. All cellular material, subsequent to cell-disruption, ends up in a soupy matrix^{64,65}. This complex mixture that consists of hydrophobic, hydrophilic and amphiphilic molecules is challenging to separate. Consequently, effective separation methods are needed for algae-based fuels and chemical industry to be feasible and sustainable.

A block diagram to show the possible process pathway of algal oil extraction is presented in.

Table 13. A comparison of various cell concentration measurement techniques

| Method | Advantages | Disadvantages |
|---------------------|---|--|
| Cell Counting | <ul style="list-style-type: none"> • The technique directly gives the amount of cells per a volume of broth. | <ul style="list-style-type: none"> • Tedious and time consuming. • Low reproducibility and accuracy. • Cannot distinguish dead cells from live ones. • Larger initial investment for microscope and cell counting chamber. |
| Flow cytometry | <ul style="list-style-type: none"> • Fast, accurate and reproducible. • Can give the number of different cells in a mixture separately. | <ul style="list-style-type: none"> • Cannot distinguish cells and cell aggregates. • Lower cell throughput. • Is a costly and sophisticated system. |
| Ash free dry Weight | <ul style="list-style-type: none"> • Uses a relatively larger sample- reducing the sampling error. | <ul style="list-style-type: none"> • Tedious and time consuming. • Only an indirect measurement of the cell count. |
| Optical Density | <ul style="list-style-type: none"> • Fast, accurate and reproducible. | <ul style="list-style-type: none"> • Should calibrate with cell dry weight or cell counting. • Cannot compare between samples of different conditions or species. • Needs a spectrophotometer. |

Harvesting and Concentration of Algae for Down-stream Processing

A basic challenge in processing algal biomass is the effusive presence of moisture. Microalgae in their natural environment grows to solid concentrations of only around 0.1% (wet wt.)^{66,67}. For industrial processing, the algal slurry needs to be concentrated to 300-400 g dry weight per liter⁶⁸. Consequently, algae must first be dewatered prior to almost all processing operations. Although thermal drying is the most straightforward technique for dewatering, it is energy intensive, expensive, and unsustainable for large-scale operations. Thus, the industry needs a cost effective and less energy intensive dewatering technologies⁶⁹.

Moisture and Algal Separation. Algae-in-water can be treated as a dilute system of particles suspended in an aqueous medium consisting of primarily glycoproteins and polysaccharides^{70,71}. As a result of the active sites (primarily consisting of hydroxyl groups) of cell wall constituents, we have observed that algal cell walls possess a negative surface potential of -27 mV (at neutral pH). This indicates that the cells are in incipient stability—meaning that with time, the cells will flocculate and settle to the bottom of the container (). Our observations with *N. oculata* and *N. salina* cells were that it can take 2-4 days for appreciable sedimentation to occur. Obviously, natural sedimentation is not fast enough for commercial use^{12,72,73}.

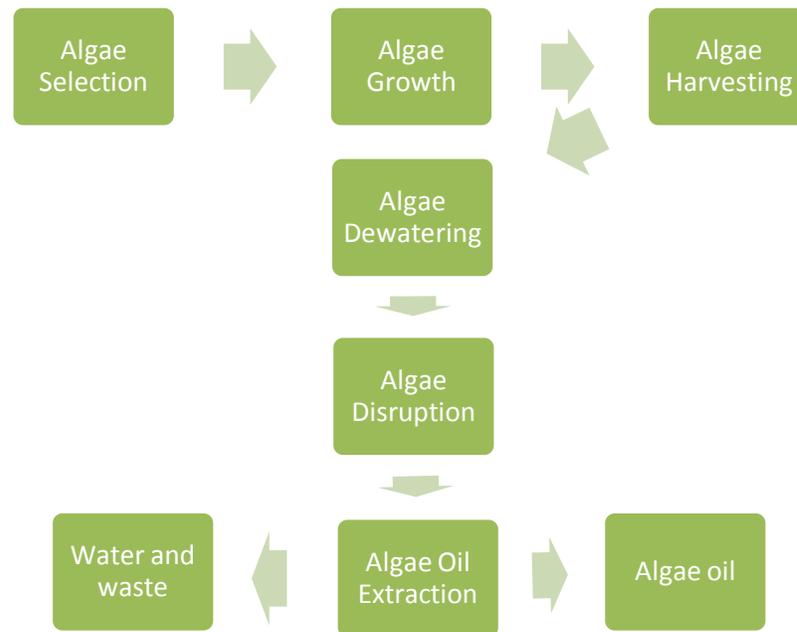


Figure 21. Process pathway of algal oil extraction

Various physical methods (filtration, sonication, and centrifugation ¹²) and chemical methods (flocculation and coagulation ^{12, 74, 75}) have been developed to rapidly separate algal cells from water.

The behavior of algae in water and how surface charges are used for harvesting algae can be well explained by the DLVO (Derjaguin, Landau, Vervey and Overbeek) theory ⁷⁶. According to this theory, the stability of a particle in solution is dependent upon its total potential energy function V_t which is the balance of competing contributions V_a (attractive-largely governed by Wan der Waals forces), V_r (repulsive-governed by electrical double layer forces) and V_s (potential energy). The theory recognizes that V_t is

the balance of competing contributions of all of these forces as shown in Equation 1. The potential energy due to the solvent makes only a marginal contribution to the total potential energy over the last few nanometers of separation. The DVLO theory suggests that the energy barrier resulting from the repulsive force prevents two particles approaching one another from adhering together—such as the case of algae suspending freely in an aqueous solution. But if the particle collides with sufficient energy to overcome that barrier, the attractive force will pull them into contact and allow the particles to adhere strongly and irreversibly. Therefore, if the particles have a sufficiently high repulsion, the dispersion will resist flocculation and the colloidal system will be stable. However, if a repulsion mechanism does not exist or is forcibly attenuated (e.g. by adding a charge neutralizer to the cell surface) then flocculation or coagulation will eventually occur.

$$V_t = V_a + V_r + V_s \quad (5)$$

An initially formed aggregate is called a floc and the process of its formation is called flocculation. If the aggregate changes to a much denser form, it is said to undergo coagulation. An aggregate usually separates out either by sedimentation (if it is more dense than the medium) or by creaming (if it less dense than the medium). Although the terms flocculation and coagulation have often been used interchangeably in literature,

coagulation is difficult to reverse whereas flocculation can be reversed relatively easily by a deflocculation process⁷⁷.

So how is it possible to speed up flocculation and coagulation process? Wastewater industries promote flocculation via surface charge neutralization by employing numerous physical and chemical techniques. Addition of positive ions such as alum, lime, and iron/aluminum salts in small concentrations has proven effective in instigating algal floc formation by neutralizing naturally occurring negative charges in the algal cell-wall surface¹². Cationic polyelectrolytes⁷⁸ such as chitosan and poly acrylamides in pure form or mixed with cheaper cation enhancing additives (such as alum and lime)⁷⁹⁻⁸² have also been successfully used. Charge neutralization by subjecting the algal broth to cation-producing electrodes, such as copper or aluminum electrodes (i.e., electro coagulation), has also been successfully demonstrated¹². Following is a more detailed discussion of the harvesting and concentration technologies that have been attempted so far.

Concentration of Algae

Generally, the water concentration of algae slurry in the bioreactor where the algae is grown is around 99.9 % (w/w)⁶⁶. However, it is necessary to concentrate algae to prevent processing of large quantities of water downstream. Due to specific gravity of algal cells, their size, and their negative cell wall charge, natural sedimentation of algae is not fast enough for commercial applications^{12, 72} (for maximum efficiency in the industry it is recommended to have a algal slurry having 300 to 400 g dry weight per

liter⁶⁸). Sedimentation, centrifugation and flocculation are methods which can be used for this purpose and are discussed in more detail below.

Sonication

One can suggest that making physical contact between cells by sonication is an effective means of increasing their settling rate. Due to the negative charge that the cell walls carry, algae in its natural environment stays in a status referred to as “incipient stability” without clumping together¹². However in sonication, the algal cells are allowed to collide among each other with sufficient energy to overcome the repulsive energy barrier. The new attractive forces will pull them into contact and allow the particles to adhere strongly and irreversibly allowing sedimentation.

Flocculation

Difference Between Coagulation, Flocculation and Precipitation

Coagulation, flocculation and precipitation are three popular methods of separation of solid particles from liquids. Even though literature uses these terms interchangeably¹², they have distinct meanings: **Flocculation** is the conglomeration of particles to increase the settling velocity of particles by increasing the particle diameter. On the other hand, in **coagulation**, larger colloidal suspensions are destabilized to improve particle settling, specially, when particle density is lower than conglomerate density. Generally coagulators are characterized by rapid mixing compared to gentle mixing used in flocculates. **Precipitation** is the formation of solids from a solution. In algal harvesting

we expect to increase the algal colloidal size by agglomerating the particle size. Therefore the correct terminology to be used for this process is flocculation.

How Flocculation Works

Terminal velocity of a particle settling in a dilute suspension like algae can be expressed by the Stokes' equation ¹⁷.

$$U_s = 2(\rho_p - \rho_f)a^2g/9\mu \quad (6)$$

Where U_s is the terminal velocity of the particle, ρ_p is the particle density, ρ_f is fluid density, a is particle diameter, g is gravimetric constant and μ is viscosity of the fluid.

For example if there is a suspension of algae ($\rho_p = 1300 \text{ kg/m}^3$, $a = 2 \text{ }\mu\text{m}$) in water ($\rho_f = 997.044 \text{ kg/m}^3$, $\mu = 0.000891 \text{ kg/m.s}$ at $25 \text{ }^\circ\text{C}$) settling velocity for a single algal cell can be calculated to be $7.413 \times 10^{-7} \text{ m/s}$ or 0.267 cm/h . In other words after one hour of standing in a settler, algae free water layer on the surface will be 0.267 cm deep.

The easiest way to increase the settling velocity of a particle is to increase the particle diameter by flocculation ¹⁷. For example if a colloidal size of $20 \text{ }\mu\text{m}$ is achieved (in the previously considered algal broth) the terminal velocity of the colloids will be $7.413 \times$

10-5 m/s or 26.7 cm/h. In other words the settler will have 26.7 cm deep layer of algae free water after one hour of settling.

Therefore it is clear that the flocculation substantially increases the settling velocity

Electrolytic Flocculation

Like most of the microorganisms, algal cells wall contains a negative charge ^{17, 83}. The charge around a suspended particle is described via Zeta potential. When the zeta potential is high, particles repel each other and dissuade flocculation. Therefore, in nature, algal cells do not flocculate spontaneously. However when an electrolyte is added to the suspension, oppositely charged ions from electrolyte neutralizes particle charge and reduces the zeta potential, increasing the rate of flocculation of the suspension ¹⁷.

In electrolytic flocculation, a flocculation agent is added to the algae water slurry. A mixing apparatus is needed in this process to mix the flocculent with algae. Then a milder level of mixing is provided to assist agglomeration of the cells together. Finally a settling stage is provided to let flocculated algae to sediment to the bottom of the container. This layer can be separated to produce concentrated algal biomass slurry containing as high as 10-15 g/l ¹². Water layer, which still contains a minimal amount of algae, can be recycled to the bioreactor to replace a large amount of feed water requirement after suitable treatment.

Polyelectrolyte Flocculation

Polyelectrolytes are polymers with repeating electrolyte groups. Because of the multiple electrolyte groups, polyelectrolytes can attach several of the oppositely charged ligands. Due to the electrolytic charge, the zeta potential of the particles reduces. The reduced repulsive forces render increased chance for collision to occur facilitating flocculation¹⁷. Additionally polymeric nature of the polyelectrolyte causes an entanglement of particles. Therefore flocculated particles from polyelectrolytic flocculation are generally much larger than flocculated particles from electrolytic flocculation¹⁷.

Since algal cells have anionic surfaces, anionic and nonionic polymers do not have a significant effect on flocculation of algal cells. Therefore, cationic surfactants should be used at optimum concentration⁷⁸. This concentration can be changed due to culture conditions and medium conditions. Therefore it is necessary to estimate the optimum amount of flocculants needed on site. Jar testing can be used in such situations.

Also, the molecular weight of the polymer used for flocculation and pH of the solution can affect the flocculation efficiency⁸². Presence of oxidation agents like chlorine in solution decreases the amount of flocculation needed for the flocculation of algae⁸¹.

At this time, polyelectrolytes are much expensive than electrolytes. Therefore, polyelectrolytes alone are generally not used for flocculation. Instead, polyelectrolytes are added to the mixture after electrolytic flocculation to increase the flocculation

characteristics. Cationic polyelectrolytes have demonstrated to be effective when used with alum⁸⁰.

Effect of pH on Chemical Flocculation

pH adjustment plays a major role in flocculation of algae. Golukes and Oswalds have reported that they were able to remove most of the algae by increasing pH to the range of 10.6 to 10.8. However they noted that additional removal of algae by further increasing the pH was negligible¹².

Addition of ferrous/ferric sulfate with calcium hydroxide to form ferrous/ferric hydroxide, which is a flocculent, is reported to be a quite effective and economical flocculation method, which is frequently used in industry¹². Golukes and Oswalds have performed a series of experiments to obtain same supernatant clarity (85% to 87% light transmission) by using combinations of initial pH (By addition of CaOH) and FeSO₄ as shown in the Table 14.

Table 14. pH and FeSO₄ concentrations for 85 to 87% light transmission ¹²

| Initial pH | FeSO ₄ mg/l |
|------------|------------------------|
| 10.5 | 120 |
| 10.9 | 100 |
| 10.10 | 60 to 80 |
| 11 | 40 |
| 11.1 | 0 |

Alternative Flocculation Methods

Cationic exchangers. Goluke and Oswald have conducted experiments to observe the effect of pH variation on algal settling for algae suspended in distilled water. They observed the clarity of the supernatant with the change of the pH ¹². According to their observations, pH level of 3.0 gives the maximum separation rate. Settled cells are reported to be in compact clumps which indicate that the clump formation is due to surface attraction among cells. However, when pH of the cell suspension, (Where the cells have grown) was increased, no effect on settling rate was observed. They also reported that Ca⁺⁺, Mg⁺⁺ and Na⁺ concentrations up to 4 mM with the combination of pH changes displayed no effect on flocculation.

Passing algae through a weak or strong cationic exchanger effectively decreases negative charges on algal surface to increase algae flocculation. However cationic exchange

should be regenerated frequently for this-making the operation intensive and costly. Detailed work has been done on usage of cationic exchangers for concentrating algae. However, this technique still does not seem to be cost/energy effective ¹².

Electro coagulation. An electro coagulation (EC) chamber is made up of an electrolytic cell with an anode and a cathode. When connected to a power source, the anode material electrochemically corrodes due to oxidation, while the cathode will be subjected to passivation.

An EC system consists of pairs of conductive metal plates commonly known as "sacrificial electrodes." The sacrificial anodes and cathodes can be of the same or of different materials. For neutralizing negatively charged algae, iron or aluminum, are the generally used sacrificial anodes. The positively charged ions that are continuously released into the solution neutralize the surface charges of algal cells initiating Flocculation.

Auto flocculation. One of the most interesting concentration techniques observed by researchers working in algal harvesting is called auto-flocculation. Actively photosynthesizing algae in shallow ponds tend to flocculate to the bottom of the pond during relatively warm days with plenty of sunshine. This can be a mechanism used by algae to limit sunlight exposure. During days which have a favorable weather conditions, it is possible to settle algae to the bottom of the pond with a depth less than 5 cm and obtain concentrated algal slurry by removing excess water in the pond. During unfavorable weather conditions for the mechanisms, it is possible to use chemical

flocculation agents to harvest algae. The downside of this cheap method is the increased amount of land requirement for settling ponds ¹². This phenomena has been also observed in sea water ⁸⁴.

Seawater and clays. Usage of seawater for flocculating algae has been investigated for the removal of algal biomass from oxidation ponds ⁷⁹. Also similar research has shown increased coagulation rates when algal biomass was flocculated with clay or silica. Even though research have not been done on these areas with algal harvesting from photo bioreactors, they remain as an interesting areas for low cost alternatives for algal flocculation.

pH change. Goluke and Oswald have conducted experiments to observe the effect of pH variation on algal settling for algae suspended in distilled water. On their studies they observed the clarity of the supernatant with the change of the pH level ¹². According to their observations pH level of 3.0 gives the maximum separation rate. Settled cells are reported to be in compact clumps which indicate that the clump formation is due to surface attraction among cells. However, when pH of the cell suspension (Where the cells have grown) was increased no effect on settling rate was observed. It should be noted that this method, no chemical flocculent is used.

Problems of Flocculation:

Flocculation chemicals are costly and contribute to significant amount of operational costs. Also, post treatment is required when the flocculants need to be removed for downstream processing¹².

Flocculation rate and the optimum requirement of flocculants depend on many factors like medium concentration and cell properties. Therefore there is an element of unpredictability associated with flocculation. Normally many trials are needed to determine the optimum flocculent amount that is necessary for effective flocculation.

Concentrated wet algal concentrate obtained from flocculation operation has a high amount of flocculent in it. Such extraneous material can interfere with downstream processes. For example ferric ions present in flocculated algal slurry can cause disintegration of the cells. Even though this is a problem for problem for feedstock preparation, this can be an advantageous for oil extraction. Even though Fe^{++} does have less deteriorative effects compared to Fe^{+++} , it is believed to promote wear and tear of process equipment such as those used in homogenization, oil extraction and pumping¹².

Therefore depending on the downstream process requirements it can be necessary to remove any flocculent from the algal biomass. Removal of flocculants can be challenging. One proven method for removing flocculants from algal surface is by increasing pH. It has been reported that increase of algal concentrate pH up to 2.5 has caused flocculants to detach from the cell surface.

Centrifugation

Centrifugation is one of the most popular options for concentration of algal biomass. The amount of water removal is the major attraction for this. Throughput or centrifuging time of the centrifuge, rpm, filter-aid, and the angle of disks are the potential variables that should be considered for the evaluation of centrifuging as a means of concentrating algae¹².

The amount of centrifugal force (G) required is calculated based on rotational speed and centrifuge rotor diameter according to the following relationship:

$$G = \frac{\omega^2 R}{g} \quad (7)$$

By examining the equation it is evident that the amount of force required is a function of rotational speed and this is quite an energy intensive technique⁸⁵. To reduce the energy requirements and enhance the algae separation efficiencies, hybrid techniques such as addition of flocculants (like alum or alum combined with carboxy methyl cellulose or with benzoate and lime or anionic flocculants) have been attempted. Also, alternative to disk centrifugation, decanter centrifugation has been suggested as a lesser energy intensive technique for algae concentration.

Filtration

Filtration is another technique that has been employed for dewatering and concentrating algae. Common filtration techniques that have been attempted include:

1. Filter press
2. Vacuum drum filtration
3. Micro-filtration.

In a **filter-press**, batches of algal broth would be forced via a filtration media using a simple mechanical press/piston. Although simple, the extremely small diameter of algae and the compressibility warrants usage of filter aids (like diatomaceous earth) in such operations. It has been reported that in general 7/8 parts of filter aid is required to be mixed with 1/8 parts of algae for effective separation. High viscosity of downstream broth and associated costs are major challenges when using filtration for algae concentration and dewatering ¹²

Vacuum drum filtration is similar to filter pressing except the fact that the forcing action is provide via a vacuum instead of a positive pressure.

Micro-straining is a specific form of filtration where the broth is forced through a filtration media with pores larger than those in vacuum filters (around 50 μ m). Filamentous bacteria have been successfully removed by micro-strainers ^{74, 86}.

High energy costs, slow throughputs and the difficulty of scale-up are the challenges associated with using filtration techniques for microalgae concentration^{12, 74}.

Floatation

Froth-formation can be used as a harvesting mechanism for algae. In this method changes in algal surface is induced by lowering broth pH to form foams via mechanical agitation which floats to the surface. The cells become trapped in the foam layer that in turn is harvested. In this method, cell harvest concentration is a function of a pH, aeration rate, aerator porosity, feed concentration and height of the foam.

Disadvantage of this method is the chemical costs associated with lowering pH (typically below 4.0) combined with the cost of flotation agents¹². However some algal species, like chlorella, have shown to form froth without the need for any forming agents⁸⁷. Exploiting this kind of phenomena can reduce the harvesting cost for algae.

Pre-extraction Treatment

After harvesting and dewatering, algal cell walls are required to be disintegrated to access material within the cellular matrix^{31, 64}. This is necessary for effective lipid extraction as well as separating higher value chemical constituents that are present in the cellular matrix⁴.

Due to strength of algal cell wall and size of the algal cell, this can be one of the most difficult challenges in the algal oil based fuel production process. Various techniques have been attempted for this process such as: 1) Mechanical cell disruption (including

high pressure homogenization and Sonication); 2) Chemical methods like extreme pH treatment; 3) Biological treatment methods like enzymatic degradation; and 4) Heat treatment.

Mechanical Disruption

Due to the fluid nature of algal biomass, homogenization is one of the most practical techniques to disrupt algae or other cellular matter. In homogenization, the algal broth is forced through an orifice (or a nozzle). Due to the pressure differential coupled with the shear forces exerted on the cell wall by the nozzle walls, the cells rupture ¹⁷.

Most of the homogenizers use countering streams of cell broth to increase the shearing of the cells. Extent of disruption of yeast cells (R) have been found to have the following relationship with number of passes (N), pressure (P) and a temperature dependent constant (K) ¹⁵. This equation has been tested for Manton-Gaulin valve type homogenizers.

$$\ln(1 - R) = -kNP^a \quad (8)$$

The constant a depends on the species or culture condition ¹⁶. However the type of homogenizer valve used is also a significant factor in the high pressure homogenization.

Among different types of homogenization valves, effectiveness varies and the most effective is the knife edge valve ¹⁶.

In the case of *E. coli* bacteria, the correlation changes as follows.

$$\ln(1 - R) = -kN^b P^a \quad (9)$$

Where ***b*** is a constant which depend on the cell type.¹⁸

Behavior of this equation for algae has not been tested yet.

Although factors such as the pressure differential across the nozzle, the nozzle diameter, the number of passes, concentration of algae in the broth, and the composition of the cell wall all affect the degree of cell rupture, the primary factors associated with the homogenization process are the pressure differential across the nozzle and the number of passes. It has been reported that the a pressure differential of 700 bars can result up to 80~90 % algal cell breakage ⁸⁸.

Homogenizers can be either piston type or blender type. While piston type homogenizers can exert a large amount of pressure and turbulence upon the cells, it has be observed to be difficult to process algal samples through these homogenizers due to clogging of the homogenization nozzle. Alternatively blender type cell homogenizers have been

proposed. Moderately tough cells like *Tetrahymenapyriformis*, has been successfully homogenized up to 99.9% disintegration using homogenization ⁸⁹.

Unfortunately, it has been observed that it is difficult to process algae with most kinds of homogenizers due to the presence of an intransigent cell wall. Piston type homogenizers are observed to be more effective for handling algal biomass ¹⁴. A significant drawback of homogenization is the high capital and operational cost. However, a significant advantage is the ability to handle larger payloads without drying algal suspension.

Sonication

Algal cells can also be disrupted via sonication ⁷². There are two primary types of sonicators that have been attempted for algal cell disruption: probe sonicators and bath sonicators. Studies have shown that probe sonicators are more effective than bath sonicators due to their higher energy flux through the algal sample ⁹⁰. According to work on algae-based chlorophyll extraction, by David Simon and Stuart Hilliwell probe sonicators having $19\text{W}/\text{cm}^2$ were able to render higher breakage efficiencies as compared to bath sonicators having $0.3\text{ W}/\text{cm}^2$ (the study also included a cell grinder having a Teflon grinding head rotating 500 rpm for 2 min (APHA standard method) and hand grinding with motor and pestle) ⁹⁰. They observed that the solvent medium that the algae situate can be a major factor that affects disruption efficiency.

Enzymatic Degradation

Polysaccharide cell walls of Algae can be digested using enzymes to open up algal cells to extract lipids, proteins or pigments inside the cell. For example, enzymes produced by *Aspergillus* fungi has been used to degrade backbone polysaccharides like Cellulose, Xyloglucan, Xylan, Galacto(gluco)mannan and Pectin³⁰.

Usage of enzymatic degradation for degradation of cell wall of Algae has been tested for the extraction of proteins from rhodophyts. Temperature, pH and the type of the enzyme used in the reaction was found to be critical factors that determine the degree of cell-wall digestibility. The combination of enzymes used also proved to be significant. For example, simultaneous usage of cellulose and carrageens has shown to dramatically increase the protein extraction efficiencies in the work done by Joel Fleurences. This is probably due to the need of facilitation of the access to carrageenan by cellulose destruction⁹¹.

Some experiments to degrade algal cells with enzymes have reported an unfeasible increase in extraction efficiency. For example dewatered astaxanthin rich *Hematococcus* algal biomass when treated with an enzymatic mixture of 0.1% proteases K and 0.5% diesel in phosphate buffer pH 5.8 and 30 °C by Mendes-Pinto et el have resulted only in mediocre degradation yields¹⁴.

Quantification of Lipids

There are several techniques available for qualitative and quantitative analysis of lipids.

These techniques include⁹²:

1. Gravimetric methods
2. Gas/liquid chromatography
3. Fluoro-spectroscopy

Some of these techniques are well developed whereas some are still at the developmental stage. More details on the specific technologies are discussed below.

Gravimetric Methods

One of the established methods to quantify fatty acid and lipid quantity of algae is gravimetric analysis of lipids via solvent extraction. A widely used solvent system to extract algal lipids is the chloroform-methanol combination⁹³⁻⁹⁵.

In this technique, a chloroform– methanol (2:1) mixture is mixed with algal biomass to achieve 20 fold dilution (w/v) and then homogenized in a Potter-Elvehjem type homogenizer (or in a suitable high-shear blender). Floch⁹⁴ used a Potter-Elvehjem type homogenizer for algal biomass weights up to 1g with 20 times dilution and a blender for amounts greater than 1gm of the biomass with 17 times dilution. In this experiment, it was observed that after filtering the homogenate through a fat free filter paper, 1 ml resulting extract corresponded to 0.05 g of the biomass. The mixture was separated in to

two phases after addition of 20% water to obtain a upper phase with 60% of total volume and a lower phase with 40% of the total volume. The upper phase which contains non-lipid compounds was removed while the interface was washed several times using pure solvents (chloroform, methanol, water 3: 48: 47). Finally lower phase and the remaining rinsing fluids were dissolved in to one phase using methanol. A 2:1 chloroform-methanol mixture is typically used for diluting the sample to desired levels. When gravimetric is used, the solvents are evaporated and the weight of the remnants is considered as the approximate content of lipids. More accurate lipid content measurements could be made with the same sample by subjecting the lipids to transesterification and analyzing the fatty acid alkyl esters content in a gas/liquid chromatography or a mass spectrometer.

Many variations of the standard procedure can be found in literature ^{95,96}. For example, Molina Grima *et al.* ²⁸ uses Bligh and Dyer solvent system ⁹⁶ (CHCl₃/MeOH/H₂O, 1:2:0.8, vol/vol/vol) and Ahlgren *et al.* ⁹³ uses the procedure developed by Boberg, M., *et al.* ⁹⁵. It is important to note that the solvent system used can affect the measurement of lipids, especially, if gravimetry is used ²⁸.

Chromatographic Methods

Gas/liquid chromatography is the most widely used technique to accurately quantify the lipid content of algal biomass ⁹⁷. In this technique, the lipid are extracted into a solvent and triglycerides are transesterified to fatty acid alkyl esters before sending for chromatography analysis.

It is obvious that the accuracy of the measurement heavily depends on the effectiveness of the solvent extraction step and the efficiency of the transesterification step. The solvent system that is widely accepted for algal lipid extraction is the chloroform – methanol (2:1) mixture or the Bligh and Dyer solvent system.

It should be noted that Bligh and Dyer method is advantageous compared to Floch method because it uses a lower amount of solvents. However, if the amount of lipids in the sample is more than 2%, the amount of lipids extracted from Bligh and Dyer method is significantly lower than the amount of lipids extracted from Floch method. It is also possible to maximize the amount of lipid extracted by using a non-polar solvent to extract majority of lipids from a lipid rich sample before using Bligh and Dyer method to extract remaining lipids.

Several techniques have been developed to increase the effectiveness of the extraction and transesterification steps. For example it is possible to use 0.005% butylatedhydroxytoluene (BHT) in chloroform as an antioxidant to prevent oxidation of triglycerides⁹⁵. Usage of 2, 2- Dimethoxypropane can increase the acid catalysis process by removing glycerol and decreasing the reverse reaction rate of the transesterification process. Isopropanol can be used instead of methanol to counteract the possibility of solvent activated lipases .

Due to reactions of chloroform with bases and possibility of existence higher amounts of free fatty acids, it is necessary to use an acid catalyst such as 5% sulfuric acid in alcohol with prolonged reaction times for transesterification⁹⁵.

Although Extraction of lipids is not necessary for transesterification and could be done in situ, transesterification of extracted lipids gives more accurate results. When performing in situ transesterification, methanol is generally used as the transesterification reagent. H_2SO_4 is used as the catalyst due to its ability to tolerate moisture compared to base catalysts⁹⁸. The high acid value of microalgal oils makes base catalyzed reaction infeasible^{6, 99}. It should be noted that various variables like alcohol volume, reaction temperature, and duration of the reaction, Moisture content and stirring can affect the transesterification efficiency. In order to assess the transesterification efficiency, a known quantity of an extraneous fatty acid is added to the reaction mixture and run through the transesterification reaction. The efficiency obtained from this reaction is transferred to the overall reaction to accurately measure the original lipid content.

Following the transesterification, fatty acid (FA) alkyl esters can be extracted to a known amount of an organic solvent such as hexane or petroleum ether mixed with hexane and injected to the chromatograph for analyzing¹⁰⁰.

In case of gas chromatography (GC), capillary columns are normally preferred for the analysis of transesterified lipids over packed columns due to various advantages like improved speed, ability to handle sample to sample variability, and increased sensitivity. Also, liquid chromatography can be used for separating individual components of transesterified lipid mixture⁹⁹.

The GC analysis procedure is discussed in more detail below.

Prior to analyzing the fatty acid alkyl ester sample in a GC, the system should be calibrated to capture fatty acid esters that are generally present in microalgae. depicts the FA profile of *N. Oculata* according to various sources. FA distribution of *N. Oculata* changes with the medium, growth phase and age⁵³. However it is possible to come up with an expected profile of FAMES for *N. oculata* by considering data available in literature.

According to there are 29 FAs that are potential candidates to be present in *N. oculata* algae. However depending on the degree of accuracy needed, it is possible to omit some FAs that are not present in significant quantities. For example, the maximum % of FAs expected is compiled in (using information from). This analysis shows that it is possible to account for 90% of the total lipids just by considering only 7 FAs from the group of 29 FAs.

Individual FA alkyl ester standards or mixtures of FA alkyl ester standards can be purchased from chemical suppliers (E.g. NU-Check Prep, MN, USA) for calibration of GC. Calibration standards should closely match the FAME profile of the expected sample.

It could be noted that C 13:0 and C 19:0 FA alkyl esters are not present in the ester profile of algal lipids. Therefore it is possible to use these as internal standards. Also, since C 15:0 FA is available only in minute quantities it is possible to use C 15 FA as an internal standard to evaluate the efficiency of the transesterification process.

Table 15. FA profile of *N. oculata* grown in different conditions

| Medium | | <i>F/2</i> | <i>F/2</i> | <i>F/2</i> | <i>F/2</i> | <i>F/2</i> | <i>F/2</i> | S 88 |
|-----------------------|--------------|----------------------------|--------------|-------------|--------------|--------------|-------------|--------------|
| Growth stage | | <i>Log.</i> | <i>Log.</i> | <i>Sta.</i> | <i>Stat.</i> | <i>Stat.</i> | <i>NA</i> | <i>Stat.</i> |
| Growth Phase | | <i>Bat.</i> | <i>Semi.</i> | <i>Bat.</i> | <i>Bat.</i> | <i>Bat.</i> | <i>Bat.</i> | <i>Bat.</i> |
| Age | | 9 | 15 | 31 | 17 | 23 | NA | 9 |
| | | % weight from total lipids | | | | | | |
| Saturate FA | 14:0 | 5 | 5.4 | 4.8 | 5.8 | 5.4 | 5.7 | 3.6 |
| | 15:0 | 0.3 | 0.5 | 0.5 | 0.1 | - | - | - |
| | 15:0 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | - | - |
| | 16:0 | 16.4 | 22.7 | 20.3 | 27.7 | 33.3 | 29.5 | 17.5 |
| | 18:0 | 0.3 | 0.4 | 0.3 | 0.4 | 0.6 | 2.1 | 0.8 |
| Monoenoic Fatty Acid | 16:1 (n-9) | - | - | - | - | - | 2 | - |
| | 16:1 (n-7) | 21.8 | 19.7 | 21.2 | 19.6 | 24.4 | 23.7 | 26 |
| | 16:1 (n-5) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | - | - |
| | 16:1 (n-13)t | 1 | 0.8 | 0.9 | 0.4 | 0.2 | - | 1 |
| | 17:1 (n-8) | 0.2 | 0.1 | 0.2 | 0.1 | 0.2 | - | - |
| | 18:1 (n-9) | 2.1 | 5.3 | 4.1 | 9 | 11.9 | 4.8 | 2.7 |
| | 18:1 (n-7) | 0.6 | 0.6 | 0.5 | 0.4 | 0.5 | 0.3 | - |
| | 20:1 (n-9) | - | - | - | - | - | 0.3 | - |
| Polyenoic Fatty Acids | 16:2 (n-7) | 1 | 0.5 | 0.4 | 0.2 | 0.1 | - | - |
| | 16:2 (n-6) | - | - | - | - | - | 0.2 | - |
| | 16:2 (n-4) | 0.5 | 0.2 | 0.1 | 0.1 | 0.1 | - | - |
| | 18:2 (n-6) | 1.7 | 1.7 | 1.8 | 1.3 | 0.9 | - | 1.8 |
| | 18:3 (n-6) | 0.9 | 0.5 | 1 | 0.4 | 0.4 | 1.4 | - |
| | 18:3 (n-3) | 0.1 | 0.1 | - | 0.1 | - | 0.1 | - |
| | 18:4 (n-3) | - | - | - | - | - | 0.1 | - |
| | 20:3 (n-6) | - | - | - | - | - | 1 | - |
| | 20:3 (n-3) | - | - | - | - | - | 0.3 | 0.7 |
| | 20:4 (n-6) | 6.1 | 6.7 | 7.4 | 6.3 | 3.9 | 3.9 | 4 |
| | 20:4 (n-3) | - | - | - | - | - | 0.1 | - |
| | 20:5 (n-3) | 39.8 | 33.4 | 34.7 | 25.9 | 16.4 | 17.8 | 31 |
| | 22:5 (n-6) | - | - | - | - | - | 0.4 | - |
| 22:5 (n-3) | - | - | - | - | - | 0.1 | - | |

Log.: Logarithmic, Stat. Stationary, Bat.: Batch, Semi.: Semi Continuous.

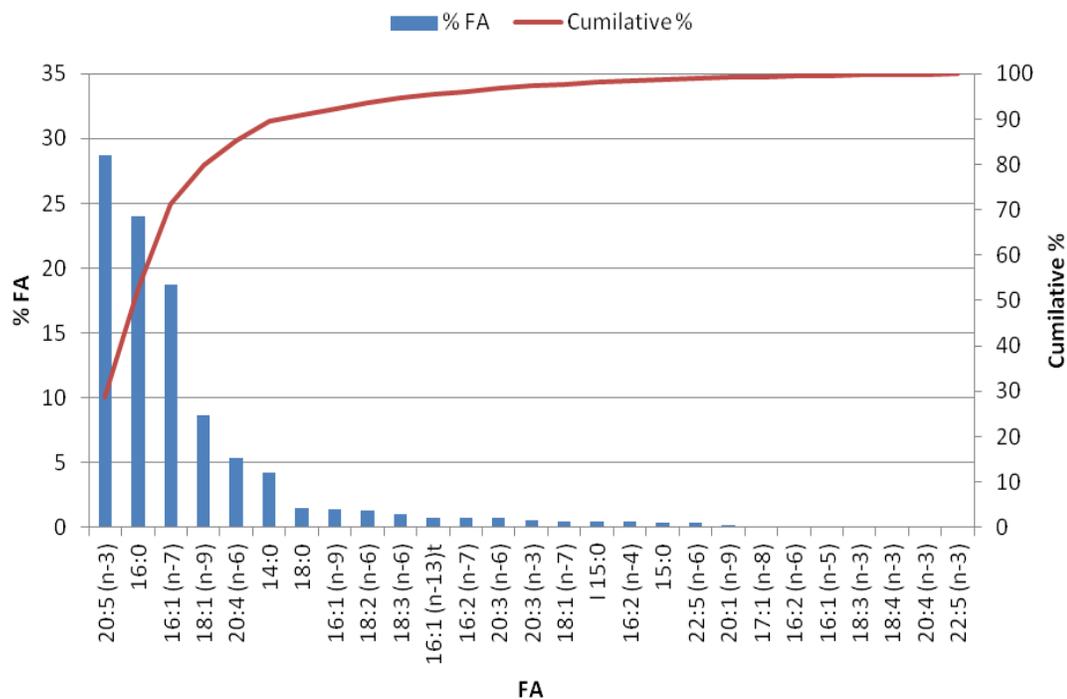


Figure 22. Fatty acid profile of *N. oculata* algae

Considering above information it is possible to give following guidelines for performing GC analysis of algal lipids.

Calibration of GC

1. Identify major fatty acids in the algal species in the consideration.
2. Decide internal standards to be used. In the case of *N. oculata*, C 13, C 15 and C 19 FAs could be used as internal standards. Concentration of

the standards should be close enough to the values of FA alkyl ester concentrations of the sample.

3. Prepare a standard mixture of FA alkyl esters which closely resemble the expected ester profile.
4. Add internal standards to the calibration sample and use it to calibrate the GC.

Sample preparation

1. Prepare sample for transesterification by freeze drying or extracting lipids using Bligh and Dyer extraction process.
2. Transesterify the lipid sample. Using C15 FA as internal standard with 4% sulfuric acid in methanol.
3. Extract FAMES in to Hexane.
4. Add internal standard C 13 and C 19.
5. Inject in to GC and obtain the peak areas.
6. Compare with calibration standard, make adjustments using the internal standard to correct for inefficiencies associated with the transesterification process, and decide the FA ester profile of the sample.

7. Relate this to the original lipid content.

Fluoro-Spectroscopy

Determination of the amount of algal lipids using gravimetric methods or chromatographic methods can be tedious and time consuming. As an alternative and rapid method of algal lipid determination, Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) or BODIPY 505/515 staining of the sample can be done followed by spectrofluorometric analysis. Nile Red will fluoresce in a non polar environment and could serve as a probe to detect non polar lipids in cells. Nile Red permeates all structures within a cell, but the characteristic yellow fluorescence (approximately 575 nm) only occurs when the dye is in a non polar environment, primarily neutral storage lipid droplets⁸. It was observed that when Nile Red fluorescence was measured in algal cultures over time, the fluorescence increased as the culture became N deficient. The fluorescence level was linearly correlated with an increase in the total lipid content, determined gravimetrically, in a growing culture of algal cells.

Nile red stained algae gives two peaks in Fluorescence spectroscopy. First peak, which represents lipids, is observed at 580 nm and the second peak, which represents chlorophyll fluorescence, is observed at 680 nm. Fluorescence profile studies performed in our laboratory with *N. oculata* algal samples shows these dual peaks with non-stressed and stressed samples (). A clear increase in area under the peak at 580 nm is observed for the stressed sample representing higher lipid content.

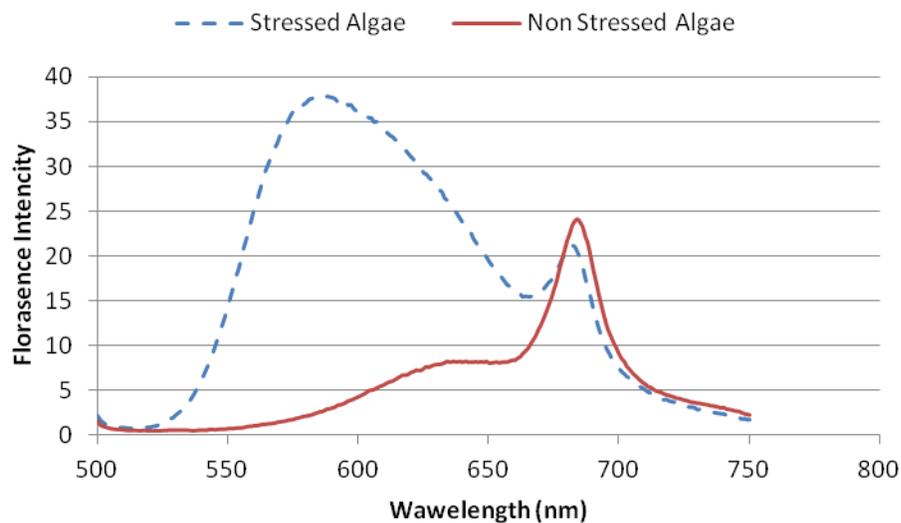


Figure 23. Florescence intensity (in arbitrary units) of Stressed algae and Non Stressed algae.

There are several procedures developed for staining algal samples. In Cooksey *et al.* (1987)'s procedure¹⁰¹, the dye was dissolved in acetone and used at a concentration of 1 mg/ml of cell suspension. In *Amphora coffeiformis* diatom species the fluorescence of the dye in live stained cells was stable for only 2-7 minutes and rapid measurements had to be done to ensure consistent results. However, it has been reported that adequate staining time (10 minutes) should be allowed for consistent results by incubating the sample in room temperature⁹². Experiments in our laboratory suggest the need for even longer times.

In a more developed protocol ⁸, a fixed volume of a diluted algal culture (typically 4 ml) mixed with 0.04 ml of a Nile Red solution (0.1 mg/ml in acetone) allowed determining the fluorescence after 5 min using a fluorometer equipped with the appropriate excitation and emission filters. 450~490 nm narrow band excitation filter and 570~590 nm emission filter is typically used in the analysis of the Fluorospectrometer ¹⁰¹⁻¹⁰³. In other reports Nile red in acetone (250 µg/ml) ¹⁰¹ with or without enhancement agent like glycerol (0.05 to .12 g/ml) or Dimethyl Sulfoxide (0.5×10^{-3} ~ 4×10^{-3} mg/ml) have been used ¹⁰³.

Limitations of the Nile Red Based Lipid Measurement Techniques:

Interspecies comparisons may be subject to misinterpretation due to the species-specific staining differences. Nile red staining of *N. oculata* does not show a higher consistency within the species (to a lesser degree) and inter species (to a greater degree). The reason for this can be inconsistent staining, which may occur result of the relative impermeability of microalgal cell wall and smaller size of intracellular lipid globules inside the cell. For example flow cytometry have shown that only 25% of the cells of a *Nannochloropsis sp.* culture can be stained by direct Nile Red staining ⁹². The differences in the kinetics of fluorescence in stained cells varied in different species, presumably due to differences in the permeability of cell walls to the stain, and differences in how the lipid is stored in the cells, i.e., as large or small droplets, are attributed to difficulties in making interspecies lipid comparisons.

It has been noticed that pretreatments used prior to the staining can also dramatically affect Fluorospectrascopy results. To illustrate this, non-stressed *N. oculata* samples was

stained for different time periods after two different homogenization conditions. The results are shown in.

According to, it is possible to see that the fluorescence peak at 680 nm decreases with progressive homogenization treatments. This may occur as a result of the reduction of cell wall material during homogenization. The analysis also shows that the importance of staining time for fluorescence studies-the observations show a required minimum staining time of 20 minutes.

In order to circumvent issues associated with staining material penetration, several techniques have been suggested. It has been observed that, fixing the stained cells with formaldehyde or ethanol preserved the Nile red fluorescence for 2 hours, but cells that were chemically fixed before Nile red staining did not exhibit the characteristic yellow fluorescence⁸. Elevation of sample temperature^{103, 104}, usage of microwaves¹⁰⁵, changing the polarity of the solvent¹⁰¹ and lyophilization are other techniques that have shown to enhance Nile red penetration into algal cells. In most cases a combination of techniques has been demonstrated to work best.

For example, lyophilized (freeze dried) algal samples have shown a higher fluorescence consistently and accuracy (0.1% difference) when compared to gravimetric methods¹⁰². In other studies homogenizing followed by using DMSO (dimethyl sulfoxide) or glycerol has shown to increase Nile Red penetration. Both glycerol and DMSO are believed to enhance neutral lipid staining by decreasing hydrophobicity of the suspension¹⁰³. However, it should be noted that higher DMSO concentrations can lyse

cells and can lead to inaccurate results ^{106, 107}. Also, unlike glycerol, further growth of cells is severely suppressed by DMSO ⁹². Therefore DMSO cannot be used for cell sorting using fluorescence.

Some authors have reported that high quantum yield dyes like BODIPY 505/515 ((4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) provide better cell penetration and high permeation rates of the dye in to the cell when used in combination with a low concentration of DMSO. Also it is reported that the BODIPY 505/515 has a lower emission spectrum which makes it suitable for some applications like confocal imaging ¹⁰⁴.

Measurement bias on cell concentrations. It has been reported that the luminescence measurements can vary depending on the concentration of the algal cells. Accordingly, it is necessary to have a consistent cell concentration in algal cell culture that is subjected to fluorospectroscopic measurement. This can be done by using counting chambers or by measuring the optical density of algal cells. For example, diluting algal culture to attain a given optical density (c.a. 0.8 absorbance or cell count of 38×10^6 cells ml^{-1}) at a given wavelength (c.a. 750 nm) has been successfully used for comparing fluorospectroscopic measurements ⁹².

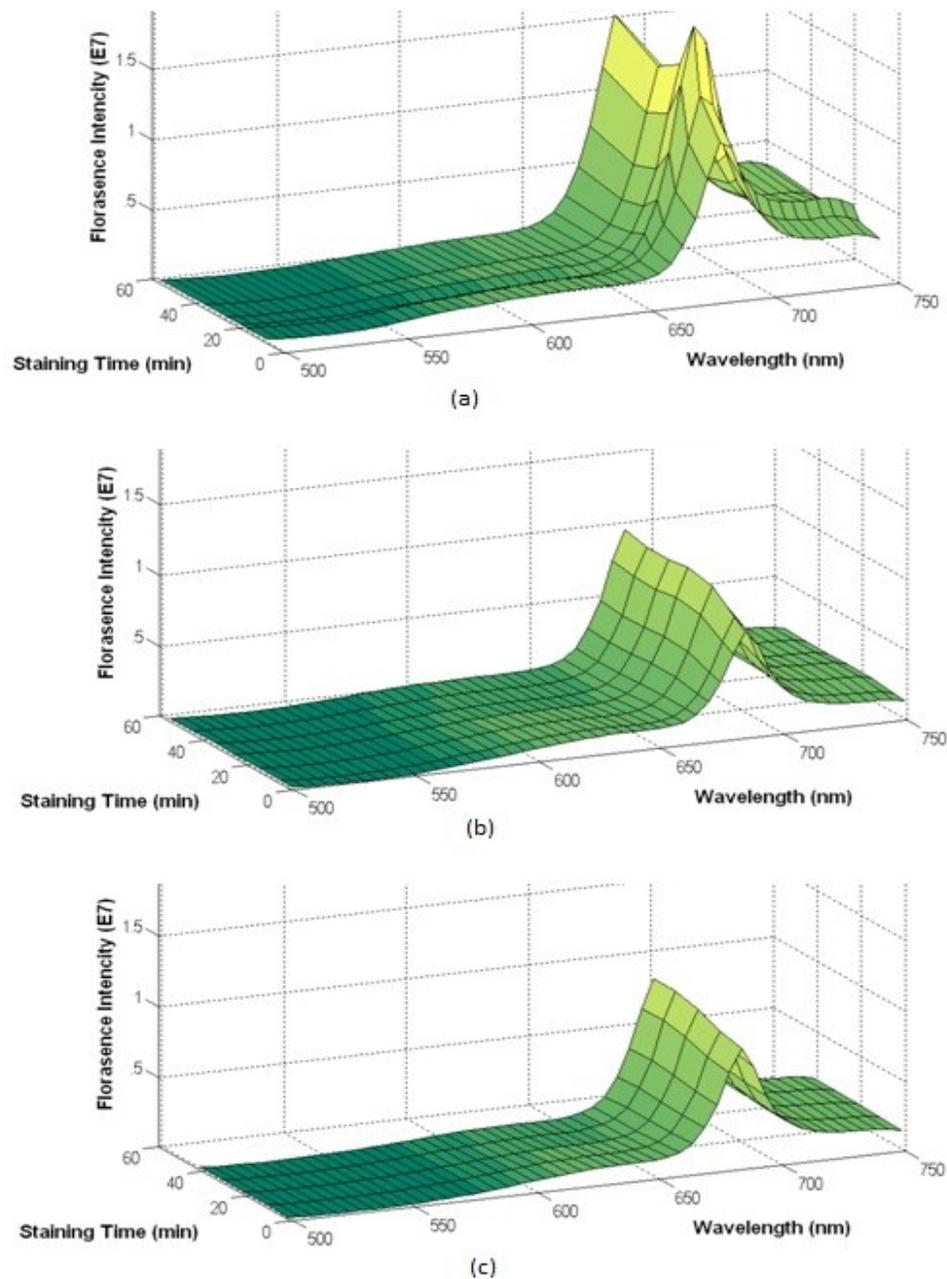


Figure 24. Fluorescence intensity Vs. Staining time

(a; Non homogenized *N. oculata*, b; Homogenized with 2 passes through 100 μm (z4) Nozzle with 25 00 PSI, c; Homogenized with 2 passes through 100 μm (z4) Nozzle with 25 00 PSI.)

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