AFRL-RQ-WP-TR-2013-0057

PROPULSION AND POWER RAPID RESPONSE RESEARCH AND DEVELOPMENT (R&D) SUPPORT

Task Order 0004: Advanced Propulsion Fuels R&D

Subtask: Optimization of Lipid Production and Processing of Microalgae for the Development of Biofuels

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Fuels and Energy Branch
Turbine Engine Division

FEBRUARY 2013
Interim Report

AIR FORCE RESEARCH LABORATORY
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AIR FORCE MATERIEL COMMAND
UNITED STATES AIR FORCE

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
February 2013	Interim	30 June 2010 – 29 June 2012

4. TITLE AND SUBTITLE

PROPULSION AND POWER RAPID RESPONSE RESEARCH AND DEVELOPMENT (R&D) SUPPORT
Task Order 0004: Advanced Propulsion Fuels R&D
Subtask: Optimization of Lipid Production and Processing of Microalgae for the Development of Biofuels

5a. CONTRACT NUMBER
FA8650-08-D-2806-0004

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
62203F

6. AUTHOR(S)

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Edwin Corporan (AFRL/RQTF)

5d. PROJECT NUMBER
06WP

5e. TASK NUMBER
N/A

5f. WORK UNIT NUMBER
Q0E7

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Dayton, OH 45432

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Aerospace Systems Directorate
Wright-Patterson Air Force Base, OH 45433-7542
Air Force Materiel Command
United States Air Force

10. SPONSORING/MONITORING AGENCY ACRONYM(S)
AFRL/RQTF

11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)
AFRL-RQ-WP-TR-2013-0057

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES
PA Case Number: 88ABW-2013-0424; Clearance Date: 30 Jan 2013. This report contains color.

14. ABSTRACT

Microalgae are considered a suitable feedstock to produce biofuels or bio-oils. Some species are known to naturally accumulate large amounts of lipids as storage metabolites. In the initial phases of this research, native algae were isolated. Techniques for the purification of these algae from field samples were developed, which resulted in the isolation of twenty different microalgae cultures. The major task of this phase was the evaluation and improvement of biomass and lipid production of potential microalgae strains. For this, a direct method was developed for rapid estimation of lipid content of the microalgae of interest. Several laboratory techniques to process microalgae broth were evaluated. Ultrasound at frequencies of 3 MHz, and quartz crystal microbalance (QCM) techniques were tested in terms of response as well as performance to flocculate microalgae cells. Two lipid extraction devices (Soxhlet and Gregar) were tested using different acetone-hexane combinations. Results show that hexane is an appropriate solvent based on the reproducibility and stability in both devices. An economic analysis was performed to determine the most cost-effective algae harvesting process assuming a fix pond site and supercritical extraction unit with an annual production of 222 million pounds of microalgae paste. Analyses show that a process plant, which considers the process of a pond → dewatering → freeze dryer → supercritical fluid extraction is the most attractive in terms of investment due to the lower fixed capital and annual operational costs.

15. SUBJECT TERMS
microalgae, lipids, biofuels, alternative fuels, algae strains

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT:	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON (Monitor)
a. REPORT	b. ABSTRACT	c. THIS PAGE			Edwin Corporan
Unclassified	Unclassified	Unclassified	SAR	56	19b. TELEPHONE NUMBER *(Include Area Code)* N/A

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ACKNOWLEDGEMENTS

This effort was funded by the Air Force Research Laboratory (AFRL) Aerospace Systems Directorate. Thanks to AFRL, forty students from chemistry, biotechnology, chemical engineering, mechanical engineering and agricultural sciences were able to collaborate in this initiative. Also this program allowed students and professors to present their research in different forums such as: SHPE National Conference, 2011; PR-LSAMP, 2011 and ABTech First Conference, 2012. Recently, our research group was awarded second place of Best Paper Award by the American Society of Engineering Education, presented in San Antonio Texas by Dr. Colucci. Moreover, AFRL support sponsored the Master's in Chemical Engineering project titled "Cost Analysis Of Local Bio-Products Processing Plant Using Microalgae Biomass As Feedstock" presented by Jesús García in July 12, 2012. Finally, special thanks to Nora Soto, administrative assistant of the Chemical Engineering Department, for her assistance throughout the program.

1.0 EXECUTIVE SUMMARY

There is a global need to generate alternate fuels from renewable non-fossil sources. Microalgae are considered a suitable feedstock to produce biofuels or bio-oils. Some species are known to naturally accumulate large amounts of lipids as storage metabolites. In the initial phases of this research, native algae were isolated. Techniques for the purification of these algae from field samples were developed, which resulted in the isolation of twenty different microalgae cultures. The major task of this phase was the evaluation and improvement of biomass and lipid production of potential microalgae strains. For this, a direct method was developed for rapid estimation of lipid content of the microalgae of interest. A combination of Percoll density gradient centrifugation and the Nile Red (NR) fluorescence techniques was standardized for screening and separation of microalgae with high lipid production. Microalgae with high lipid content layered cells much higher (at a lower Percoll concentration) compared to lower lipid producers. The different bands on the Percoll gradient were assayed for lipid content by NR spectroscopy, and correlation of lipid content to banding patterns was confirmed. To increase lipid productivity, a native *Chlorella* sp. (selected based on its original lipid content) was chemically mutagenized and screened. Of the 300 individual mutants screened, three that initially showed higher lipid production were selected for physiological studies. Wild type and mutant cells were grown with different nitrogen regimes. Results from Percoll and NR scans showed the highest lipid production was achieved when mutant cells were grown with minimum nitrate after reaching mid-log phase (0.294 mM). Even though the highest lipid production was achieved with mutant cells grown at a specific phase with low nitrogen levels (0.294 mM), the maximum cell growth was obtained from mutant cells with the highest nitrate treatment. Test results demonstrate that it is possible to modify the genetic background of microalgae to improve total lipid content by the use of chemical mutagents. It was estimated that a mutant (#3) produced a six-fold increase in lipids compared to the control algae cells. Two other mutants, #278 and 294, were found to have high lipids content as well. Mutant # 294 had twice the amount of lipids than control cells. More importantly, mutant #294 had high content of neutral lipids like triglycerides which can be efficiently converted to biofuels.

In the need to identify appropriate technologies to process microalgae broth, different laboratory apparatus techniques were evaluated. Ultrasound at frequencies of 3 MHz, and quartz crystal microbalance (QCM) techniques were tested in terms of response as well as performance to flocculate microalgae cells. A hydrodynamic cavitation (HyCu) ultrasound system was used to break microalgae cell walls, but was found to be ineffective due to small size of the cells, which were not affected by the osmotic pressure.

In terms of lipid extraction, two extraction devices (Soxhlet and Gregar) were tested using different acetone-hexane combinations. Results show that hexane is an appropriate solvent based on the reproducibility and stability in both devices. However, subtle differences were observed between the techniques regardless of the solvent used.

An economic analysis was performed to determine the most cost-effective algae harvesting process assuming a fix pond site and supercritical extraction unit with an annual production of 222 million pounds of microalgae paste. Analyses show that a process plant, which considers the process of: a pond → dewatering → freeze dryer → supercritical fluid extraction is the most attractive in terms of investment due to the lower fixed capital ($80 M) and annual operational

costs ($145.3 M). This results in a lower production costs for microalgae high protein paste of $0.80/lb, $0.75/lb, and $0.73/lb to produce the bulk paste equivalent to $5.99/gal, $5.65/gal and $5.50/gal of oil at a 10% of interest rate with an income tax rate of 42 percent at 5, 10 and 20 years financing, respectively.

In other related activities, a Green Laboratory initiative was implemented as part of the safety protocols, and K-12 demonstrations and workshops were held by students and professors resulting in an award winning paper from the American Society of Engineering Educators.

2.0 GROWTH, HARVESTING AND LIPID ANALYSIS OF SEVERAL MICROALGAE STRAINS INCLUDING COMMON, LOCAL AND GENETICALLY ALTERED SPECIES

Initial phases of this research focused on the isolation of native microalgal species from sub-tropical climates in the island of Puerto Rico. Techniques such as antibiotic treatment for the isolation and purification of algal species from mixed samples have been developed. This resulted in the isolation of twenty different algal strains which now constitutes the laboratory's collection. All these cultures grow well in fresh as well as brackish water. Some of the strains also grew very well in municipal waste water from a tertiary waste water treatment plant. Based on their growth characteristics and ease of cultivation, five strains were selected for further study. DNA from these five strains was extracted and the partial 18S rRNA gene was amplified and sequenced. These generated sequences allowed for the identification of these organisms to the genus level. The analysis of the oil content from the algae showed that *Chlorella sp.* (#16) was the best candidate for strain improvement.

Chemical mutagenesis has been used with industrial strains of microbial species to improve yields of desirable compounds. In the case of mutagenizing algae, our aim was the identification of strains that could do one of the following:

a. Produce more lipids as compared to wild type
b. Since most microalgae produce lipids only in the late log/stationary phase, identify mutants that could start lipid production during early log phase. In terms of lipid production this could reduce the harvest time.

With this in mind, *Chlorella sp*. #16 was mutagenized as outlined in the section below.

2.1 Chemical Mutagenesis of *Chlorella sp.* #16

A direct method for screening high lipid algae producers was developed. Low-time consuming techniques were developed for algae culturing in order to estimate lipid accumulation in a relatively short period of time. Oil production estimates are based on two main techniques: Percoll separation and NR fluorescence. Percoll™ is a well-referenced media for density gradient centrifugation of cells, viruses and subcellular particles. This colloidal silica can physically separate cells from a mixed culture by layering cells with highest lipid content at the top based on buoyant density (Eroglu et al., 2009, Morales et al., 1990). Percoll density gradient centrifugation is routinely employed in biochemical and molecular research to separate different cell types, and to fractionate sub-cellular compartments and macromolecular complexes on the basis of their differential buoyant densities, independent of particle size or shape. This method is especially useful for rapidly screening bio-oil content in strains of algae whose lipid content may vary with cultivation conditions and time. Proteins may vary greatly in mass but not in density.

In this work, a new approach is presented to generate high lipid producers by mutating and selecting oil producing strains of *Chlorella* sp. EMS (Ethyl Methanesulphonate) was used as candidate mutagen to increase lipid production in *Chlorella* sp. EMS is an alkylating agent mutagen that induces random mutation in the DNA. This mutagenesis can potentially generate strains with higher content of polyunsaturated fatty acids.

Procedure: Density Gradient Separation and Lipid Determination (DGS-LD)
Culturing Techniques - Native *Chlorella* cells were cultured to mid-exponential phase in Bristol medium. A 1 mL culture sample was centrifuged for five minutes at 7500 rpm, washed twice and suspended in 500 µL of PN buffer (0.1 M sodium phosphate pH 7.0). Cells (2 x 10^8 cells/ml) were exposed to 40 µL EMS/mL of culture. To ensure proper exposure to the alkylating agent, samples were mixed vigorously and incubated at 30˚C for 30, 40 and 60 minutes; control was also incubated for 30 minutes without EMS. EMS was inactivated by 500 µL of fresh-made sterile 10% (w/v) sodium thiosulphate. The cells were washed with 1 mL of PN buffer, collected by centrifugation (5 minutes at 7500 rpm), re-suspended in 1 mL of Bristol medium. Mortality was estimated by spreading serial dilution of untreated and mutagenized cells (approx. 100 cells) on agar plates.

Lipid determination - 1 mL sample (optical density - OD 0.5) was stained with 3 µL of Nile Red (NR) solution (250 $mg^{-1}L$) for 10 seconds to measure lipid fluorescence using a Spectrofluorophotometer Shimadzu model RF-5301. Spectra were generated by setting excitation and emission monochromators to 495 and 520 through720 nm, respectively. Samples from cultures emitting peaks for either neutral (580 nm) or polar lipids (600 nm) were prepared for isolation with Percoll.

Percoll separation - The silica colloid Percoll™ has become the density gradient medium of choice since it is harmless to the cell and no interference substance is introduced during the process. Corex® 15 mL centrifuge tubes were loaded with a high to low concentrations from 100, 80, 60, 30, and 10%. The 10 and 100% step volume were of 10 mL each. Remaining step volumes were 3 mL. One mL of algae culture (OD 1.5 to 1.6 $_{750\ nm}$) was layered on top and centrifuged at 5930G at 4˚C for 20 minutes. Samples pass through the gradient until reaching a point where their density matches the surrounding Percoll solution. Bands generated were collected by pipetting and pelleted by spinning at 2000G for 10 minutes at room temperature. Concentrated cells from each band were washed with 2 mL (for 10 and 30%) and 12 (for 60, 80, 100%) mL of deioinized water. To confirm, bands with less buoyant density were harvested and stained for NR spectroscopy.

Results:
EMS lethality - Mutant manual screening from plated cells (Figure 1) that survived EMS treatment were carried out in agar plates after dilution of 100 colonies (estimated) per plate. Survival for each treatment was estimated by counting cells grown on plates inoculated with 100 colonies. The highest cell survival was obtained on control plates followed by 30 minutes treatment with EMS (Figure 1), while the lowest survival (18 percent) was seen on cells after 60 minutes of exposure. Mutants and control cells were cultured on liquid Bristol media for further manipulation.

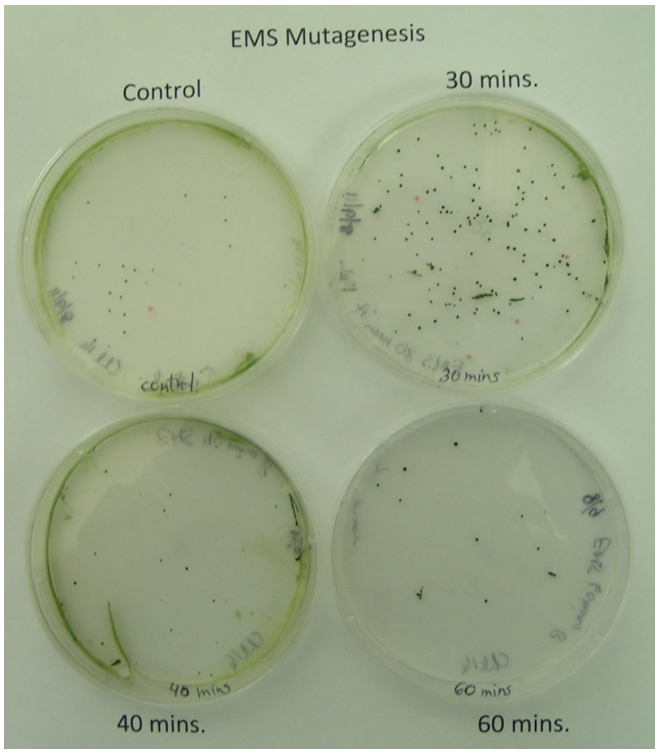


Figure 1: Cell Survival after EMS Mutagenesis

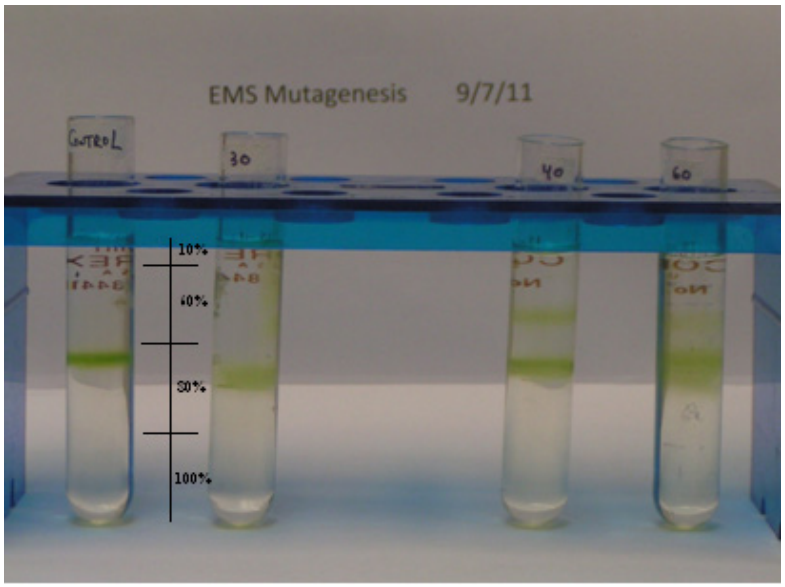


Figure 2: Percoll density gradient separation of *Chlorella* wild type (left tube) and mutated cells (30, 40, 60 minutes)

(*A 60-100% Percoll gradient was employed with a 20% increment among the gradient steps.)*

Mutant separation by Percoll gradients - As shown on Figure 2, cells were separated by step-gradient centrifugation in Percoll. Wild type cells banded as a single lower layer in all tubes, whereas mutants appeared at the top of the tube. A single band at the bottom of 80% was produced on control and 30 minute treatment, suggesting population from 30 minutes treatment

was mainly composed of wild-type cells. As expected, a different cell distribution was obtained on the longest EMS treatments (40, 60 minutes). Cultures incubated with 40 and 60 minutes incubation with EMS generated an extra band at the top of the gradient. These cells have a density equivalent of less than the 60% Percoll. Evidently, mutagenesis led to a lower density, hence higher lipid content.

Each discrete band layered in Percoll was incubated in agar plates and liquid media to further separate and isolate. It is expected that some mutants with lower density will produce storage lipids in less time or have higher oil production than wild type. Subsequently, single colony mutants from plates were incubated and examined using light fluorescent microscopy and with fluorescent spectroscopy at different life stages for qualitatively estimate storage lipid content. Cells with highest lipid content should layer at the top of the gradient.

2.2 Colony Screening by Morphological Variation

Mutated cells collected from each Percoll band were plated on Bristol media. Visual analysis from plated cells was performed to compare normal (control) colonies with atypical cells. Some colonies treated with EMS for 60 minutes exhibited changes in shape as shown in Figure 3. Two additional plate transfers were carried out to stabilize the mutation. Subsequently, mutant colonies were grown in liquid media and monitored for lipid content using NR staining and fluorescent spectrofluorophotometry. Mutated cells were also grown and monitored to estimate chlorophyll content by emitted auto fluorescence from unstained samples. As shown in Figure 4, no significant difference was observed on chlorophyll peaks emitted at 680 nm in spite of morphological variation.

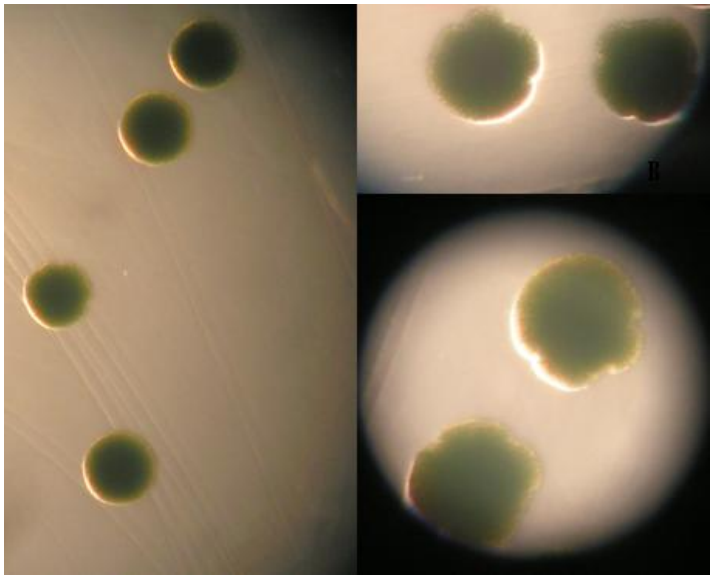


Figure 3. Colonies with normal shape (left image; 10x magnification) and atypical morphology (right image; final magnification: 20x).

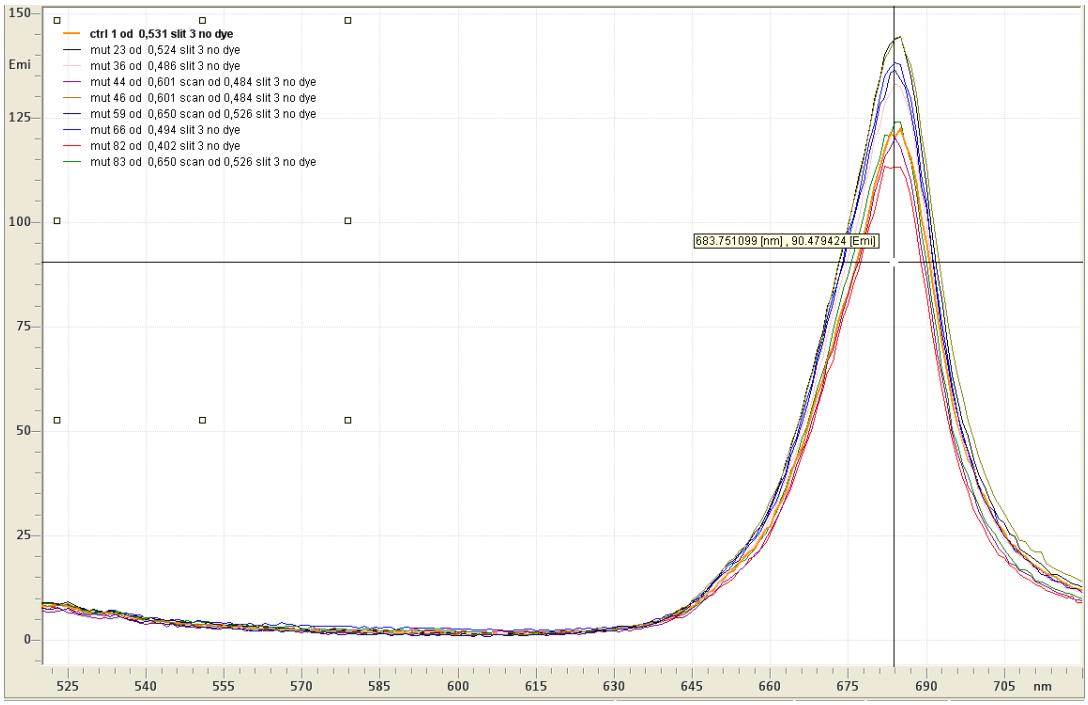


Figure 4: Chlorophyll content on unstained samples from wild type cells (orange) and mutants.

2.3. Mutant Stability: Selection of Putative Constitutively-Oil Producing Mutants

Chlorella sp. is not known to produce high amounts of lipids during lag phase. Native *Chlorella*-16 sp. was chemically mutated with EMS to generate mutants with enhanced attributes such as producing lipids constitutively or have a faster growth rate. Here we report a general screening method to stabilize mutants with putative increased lipid production levels.

Procedure:
As previously reported, *Chlorella* sp. cells that survived EMS treatment were grown in Bristol media and separated by density using Percoll centrifugation. Control (unmutated) cells were also separated on a Percoll step-gradient. Mutants with lower density, suggest a higher lipid content compared to those from untreated cells and collected for spread on master grid plates. Single colonies were transferred to Bristol plates several times to stabilize mutations and grown into patches on agar.

Results:
Single colonies were transferred by spotting 16 colonies per plate (Figure 5). A total of 300 putative mutants were streaked on agar plates (Figure 6).

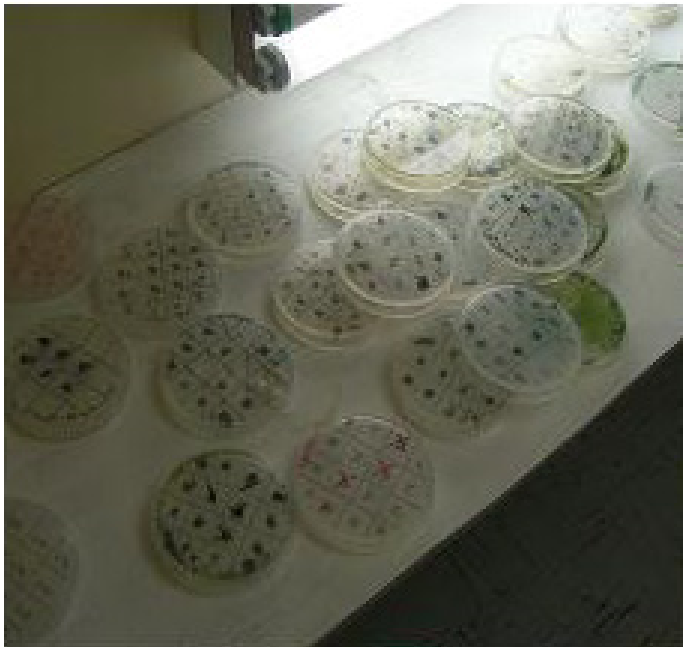


Figure 5: Mutant patch screening for chlorophyll.

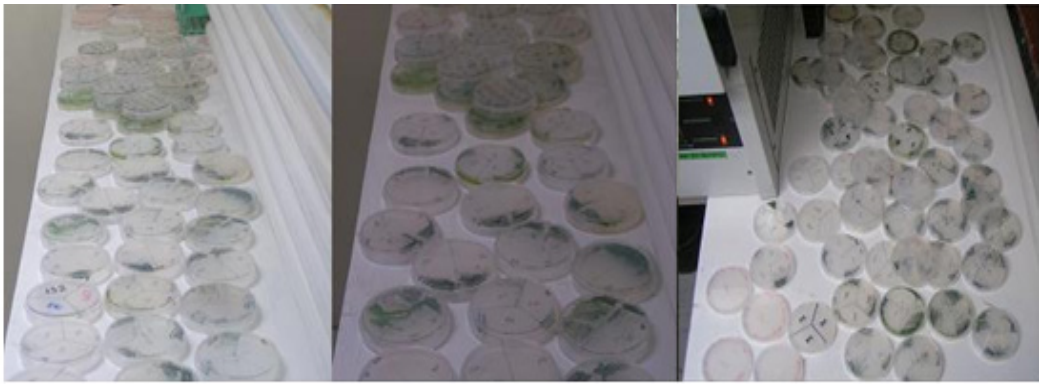


Figure 6: Mutant streak-transferring after initial isolation.

The first set of mutants were grown on 15 mL tubes with Bristol media and separated by density centrifugation using Percoll gradient. Figure 7 illustrates different discrete banding patterns on mutant and wild-type cells. Mutated culture (Tube 3) generated a band at bottom of 80% step, and a second band at 60%, whereas wild type cells (control) banded at 100%, 80% and a faint third band at 60% Percoll step. The control sample, composed by cells with different ages, layered at different Percoll steps. Bands layered at the top of the gradient have a lower density and probably higher lipid content. Cells collected from each band were cultured on Bristol media to perform optical density measurements and lipid emission scans. Each mutant was grown on liquid media until reaching 0.5 OD and specific cells within the culture with higher lipid content were again separated on a Percoll gradient. Bands of interest were collected and grown in liquid media to estimate lipid content with NR spectroscopy.

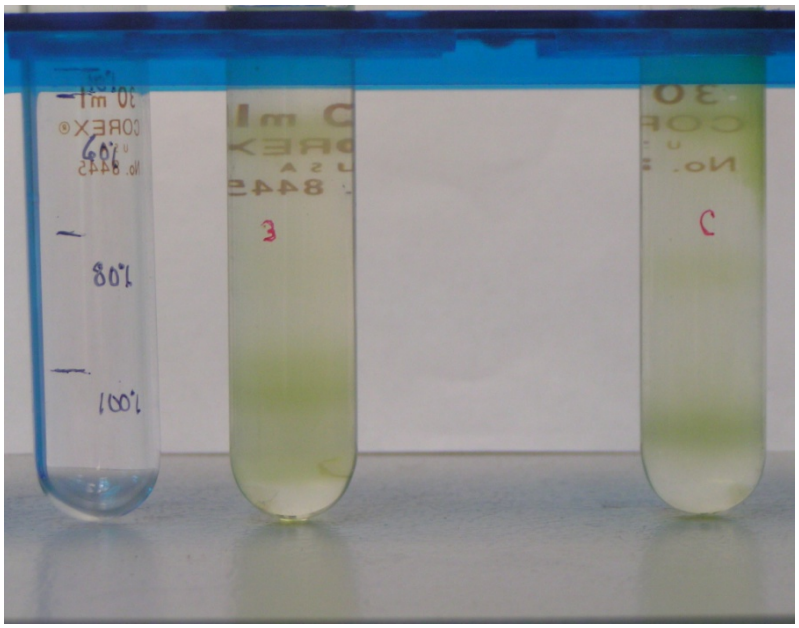


Figure 7: Cell separation on mutated *Chlorella* sp. (3) and untreated cells (C).

2.4 Mutant Separation Method Using Percoll Centrifugation and Spectrofluorescence

Procedure:
Mutated *Chlorella* cells that survived treatment with EMS were grown in single colony culture plates; hundreds of single colonies were transferred to liquid Bristol culture tubes for DGS-LD. After centrifugation, chemical mutated cells with lowest density (more lipid content) will band on the lowest % Percoll step. Higher number of mutant cells was used on Percoll centrifugation to verify no additional bands were generated as a function of cell concentration. This band was collected and transferred to liquid media and grown autotrophically until reaching optical density value needed for spectroscopy measurements. After 5 to 7 days, the culture was ready to perform NR fluorescence scan for lipid determination.

Results:
All cells from Mutant #3 (mutant 16-3) banded at 60% Percoll, the lowest density step. Wild-type cells generated light bands at 60 and 80% layers (Figure 8). Even though the number of cells from mutant #3 increased up to 6X (OD 0.366) compared to the control (0.067), all mutated cells reached equilibrium at 60% Percoll. Mutated cells from this top band were collected and grown on liquid Bristol as well as control. At 5 days, lipid measurements were performed using a spectrofluorophotometer with the NR staining technique.

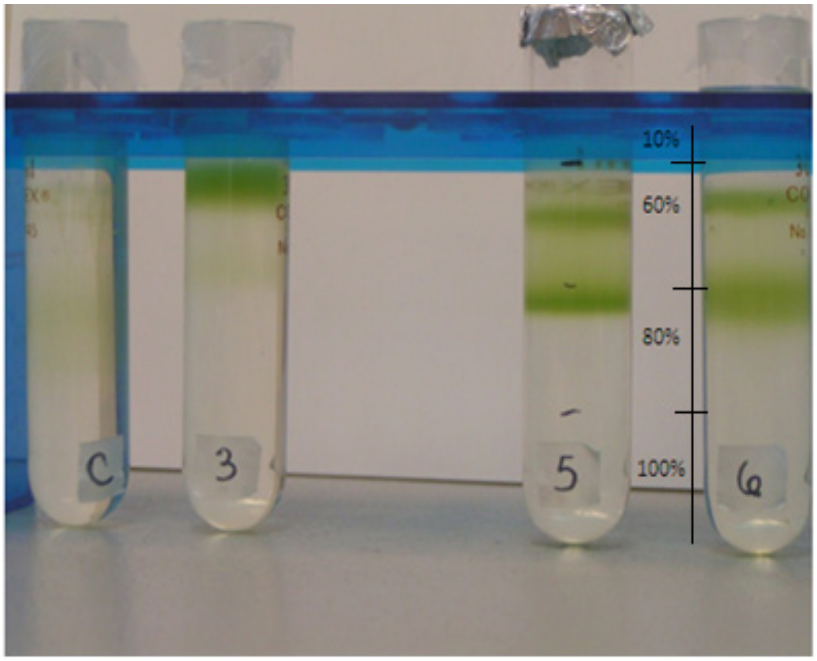


Figure 8: Bands generated in Percoll from control (left) and different mutants #3, 5 and 6 (right).

Oil-producing mutants can be screened and selected from among hundreds of single-colony cultures by performing a rapid technique like Percoll separation. Mutant #3 is estimated to produce six-fold increase of lipids compared to control, as shown on Figure 9 (both cultures with equal cell concentration for fluorescent scan). This was the first mutant found with this technique to yield high oil content. This enhanced culture was not only producing high amounts of oil but is also rich in neutral lipids (580 nm). The mutation causing high lipid production was, however; found to be unstable. Further characterization of this mutant is described in the following section.

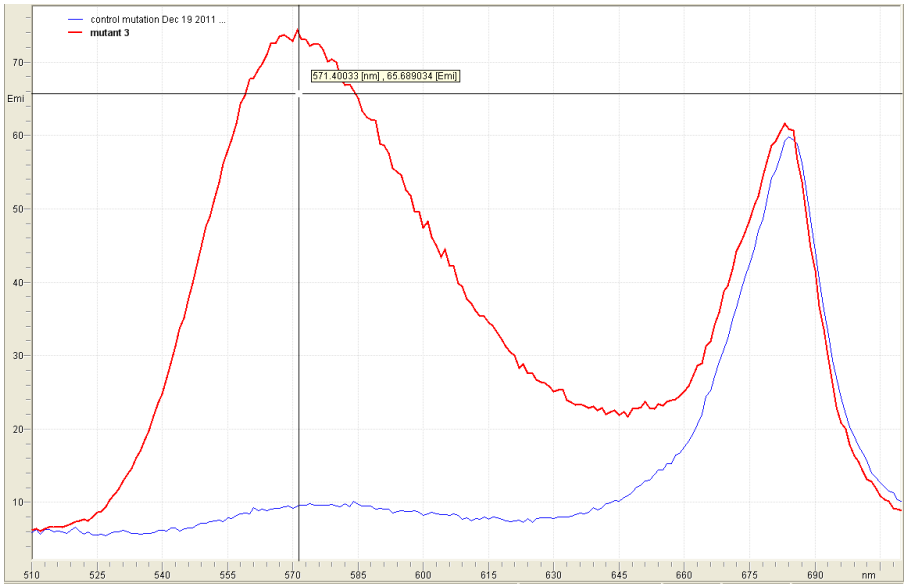


Figure 9: Neutral lipid accumulation generated by mutant (red) and wild-type (blue) *Chlorella* cells.

2.5 Screening for High Lipid Production Mutants

Procedure:
A selection of 84 single colony cultures was analyzed in this phase using the technique described above. Only the positive mutants with significant oil content are reported here. Also Mutant #3 (16-3) was cultured autotrophically at room temperature with replete nutrient supply: pH, optical density and lipid content were constantly monitored.

Results:
Mutants grown on single colony culture were screened for lipid production. As previously reported, mutant 16-3 is considered a high-lipid producer. From a total of 300 screened mutants, #278 and 294 were found to produce lipids of interest. As illustrated on Figure 10, wild type cells produces less lipids at 600 nm (polar lipids, mostly phospholipids), which are normally produced by the cell. Mutants #278 and 294 produced significantly more lipids than control cells. Both scans reached highest intensity at a shorter wavelength (570 to 575 nm). Mutant # 294 seems to have twice the amount of lipids than wild type cells. More importantly is the fact that lipids produced by this mutant peaked at 574 nm corresponding to the wavelength for neutral lipids like triglycerides. This indicates a higher storage lipid content of non-polar lipids. Mutant #278 also produced more neutral lipids but to a lesser extent than mutant #294. Even though mutant #278 samples had a lower optical density than control, there was a higher production of neutral lipids as illustrated on Figure 10.

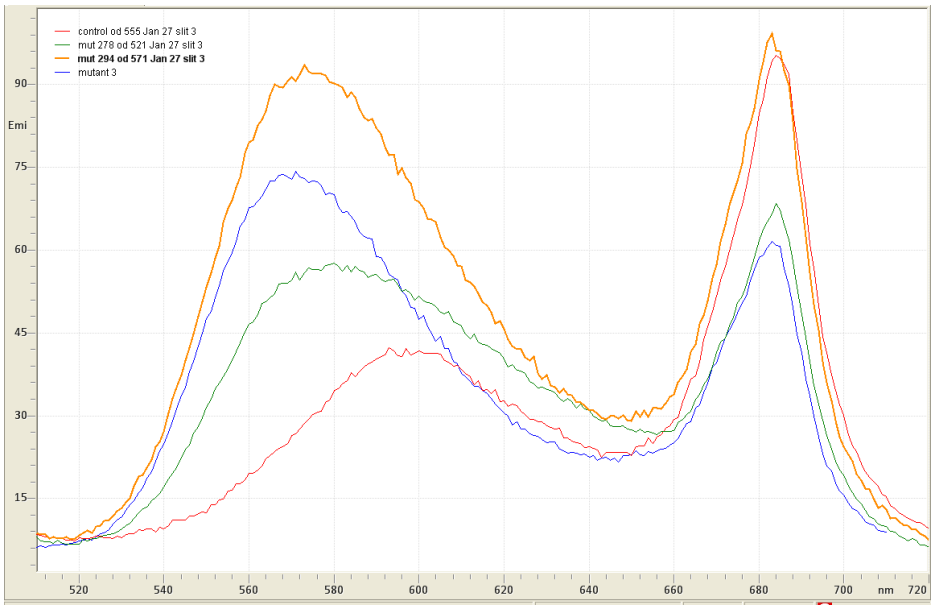


Figure 10: Mutant lipid content estimated with NR spectroscopy: control (red) OD 0.55; mutant #278 (green) OD 0.52; mutant #3 (blue), OD 0.50; and mutant #294 (orange) OD 0.57.

Mutants #278 and 294 yielded different banding patterns when separating with Percoll centrifugation. Separation for these cultures yielded two diffused bands at 60 and 80% step as shown on Figure 11. NR scan also indicated higher lipid content for these mutants compared to

wild type. The higher banding pattern for the controls in this experiment was surprising, but the NR scans indicated that the wild type was producing lipids. Since none of these cultures were synchronized, there were some experiments where the controls produced lipids. However, the mutants under those conditions produced much more lipids and showed significantly different banding pattern. It should be noted here that this technique is very useful in separating high lipid producers in a mixed population. The NR scans also indicate a shift in the peak towards neutral lipids.

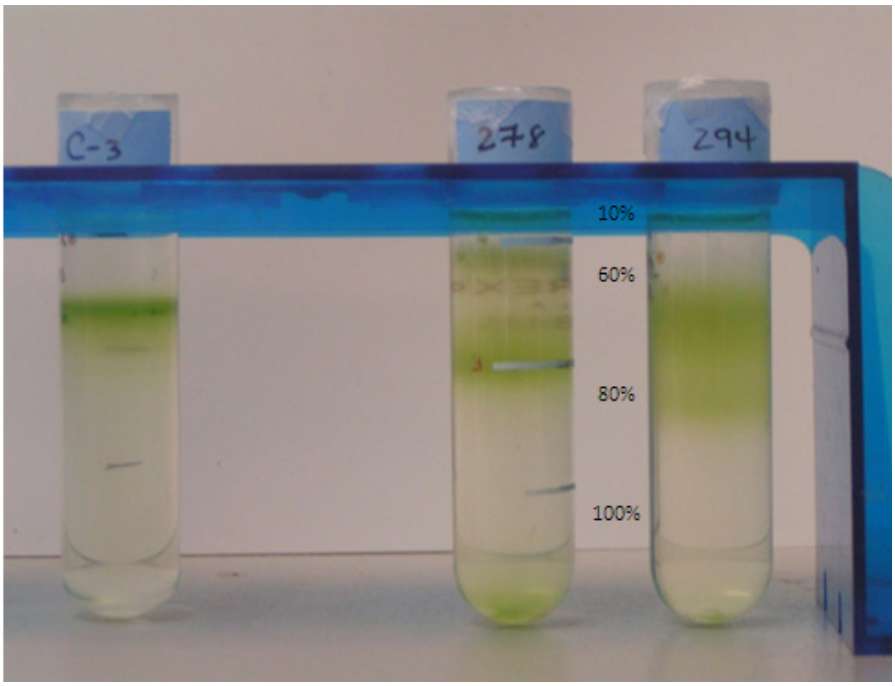


Figure 11: Percoll separation for control (C-3) and mutants #278 and 294.

Mutant 16-3: After repeated transfers in liquid and solid media, no lipid accumulation was observed for this mutant on NR scans or Percoll centrifugation under typical growth autotrophic conditions (Figure 12). This mutant and its growth patterns were subsequently studied in additional experiments with the aim of evaluating its stability.

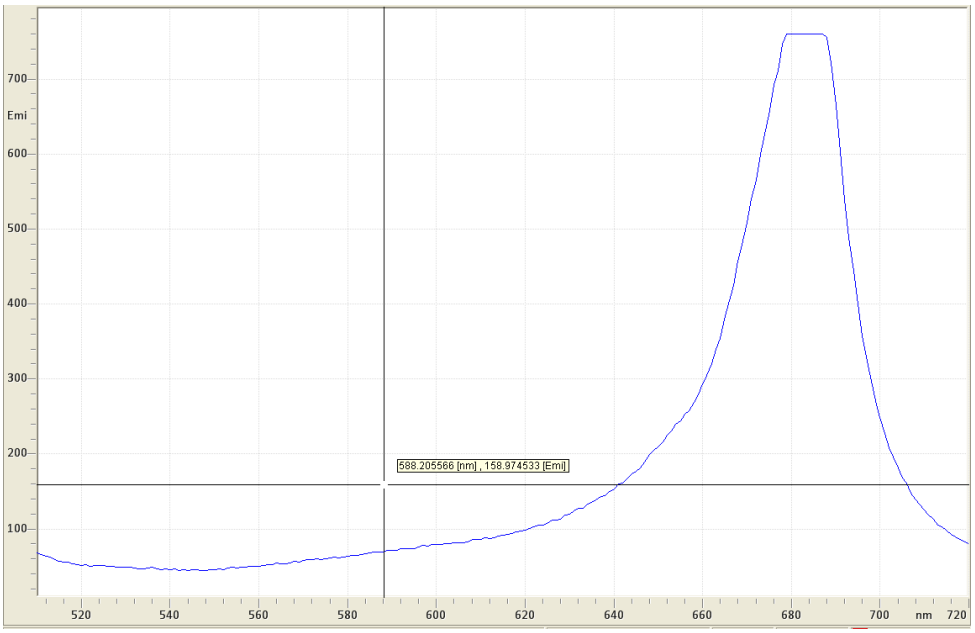


Figure 12: Fluorescence scan from *Chlorella* 16-3 mutant.

2.6 Microalgae Separation by Lipid Density - Nutrient Stress

Procedure:
Chlorella-16 sp. wild type was grown at room temperature under normal nutrient supply (2.94 mM Bristol media) and under nitrogen stress (0.29 mM) as control and experimental treatments respectively. A Percoll gradient was done after culturing for 22 days. Subsequent NR spectroscopy was performed on collected layered cells grown in Bristol medium.

Results:
As shown in Figure 13, treatments were resolved by gradient separation. Wild type control cells layered at 80% while nitrogen deprived cultures layered at 10% Percoll. Cells grown with maximum nitrogen layered at the bottom of the Percoll gradient, demonstrating low buoyancy characteristics due to its low lipid content. Fluorescent scans confirmed low lipid content on this sample. Cells with high buoyancy (low density) (Figure 11, tubes 2, 3 and 4) emitted high lipid peaks (Figure 12) when stained with NR and measuring fluorescence.

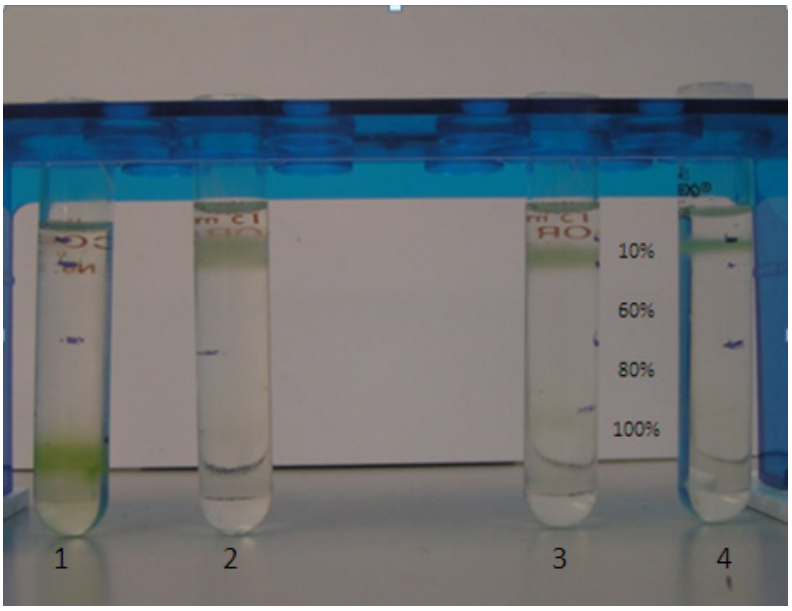


Figure 13: Percoll separation for cells with (1) and without nitrogen (2, 3 and 4).

Figure 14 illustrates high neutral lipid content (575 nm) for cells banding at 10% (red scan) from nitrogen stressed culture (vials 2, 3 and 4). This represents an 8-fold increase in lipids compared to the control culture, whereas control cells (vial 1) banding at 80% did not accumulate lipids, emitting a flat line (blue scan). This experiment demonstrated that cells under nutrient deficiency induced a metabolic response in order to compensate for its survival by producing storage molecules like lipids. Hence the screening method described in above is also useful to response of cells to nutrient stress.

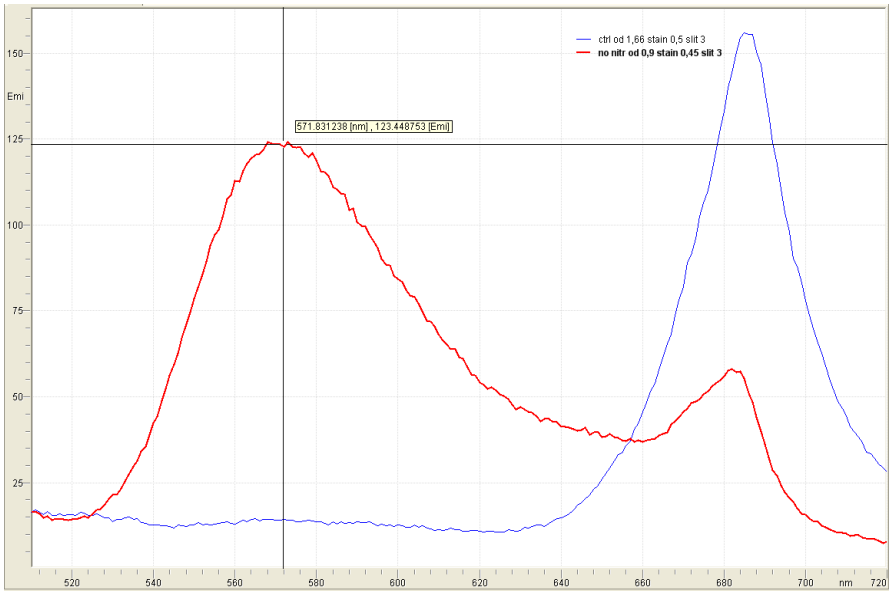


Figure 14: Microalgae lipid accumulation (575nm) on wild type cells with low density grown under nitrogen stress (red) and high density in presence of nitrate (blue).

2.7 Nutrient Effect on Mutant *Chlorella* 16 #3

The mutated native *Chlorella* sp. (mutant 16 #3) was further evaluated as a potential strain for biofuel production. Critical elements like nitrogen play a key role on cell biomass and oil production, therefore, an investigation into the effects of nitrogen on mutated cells growth was conducted.

Procedure:
Native *Chlorella* sp., wild type and mutant 16 #3 cultures were monitored in a time course to evaluate growth and lipid productivity. The experimental design was set up as follows for each cell type: Bristol media with nitrogen, low nitrogen and with nitrogen followed by no nitrogen. For the latter treatment, wild type and mutant cells were transferred from Bristol media with nitrogen to media without nitrogen after 17 days when at least one of the cultures reached mid log phase ($0.5_{\text{od 750nm}}$) for a total of six treatments as illustrated on Table 1. Growth monitoring (pH, OD and lipid production) was continuously performed for all tests. Lipid production was assessed via NR fluorescence.

Table 1 Treatments for evaluation of *Chlorella* 16 #3 mutant and wild type

Treatment	Culture	Bristol Media	Life Stage
A	Wild type	Replete nitrogen (2.94 mM nitrate)	Lag Phase
A-1 (F)	Wild type	Replete nitrogen followed by no nitrogen	Mid Log Phase
B	Wild type	Low nitrogen (0.294 mM)	Lag Phase
C (D)	Mutant	Replete nitrogen	Lag Phase
C-1 (C)	Mutant	Replete nitrogen followed by no nitrogen	Mid Log Phase
D (E)	Mutant	Low nitrogen	Lag Phase

Results:
Figure 15 illustrates cells growth from lag to early-mid log phase. Mutant 16 #3 exhibited a faster growth with an OD of 0.525 when cultured with nitrogen compared to the same mutated cells having an OD of 0.197 grown with low nitrogen and wild type culture treatments with an OD of 0.18.

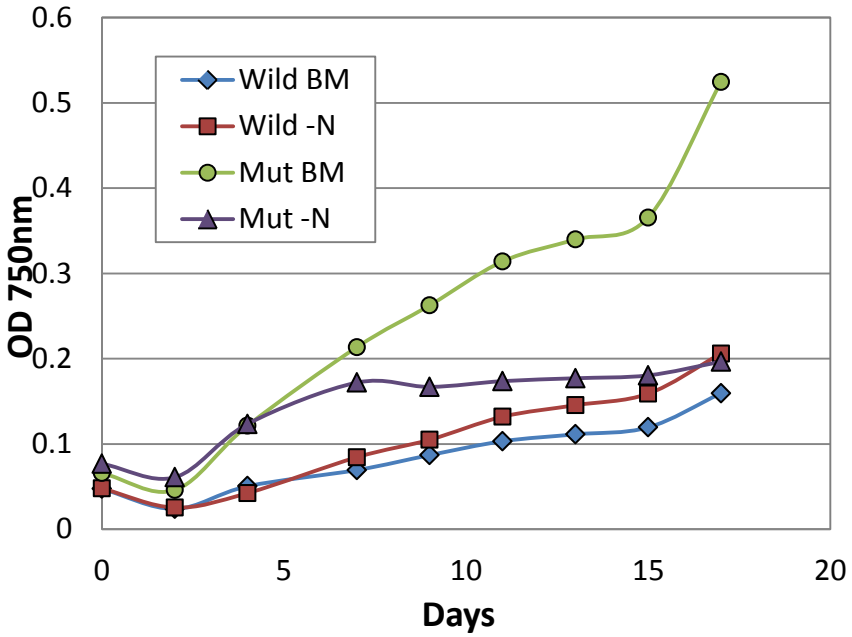


Figure 15: Mutant and Wild Type *Chlorella* cells grown with low and replete nitrogen. (Wild, wild type; BM, Bristol media (2.94 mM); -N, low nitrogen (0.294 mM); Mut, Mutant 16-3.)

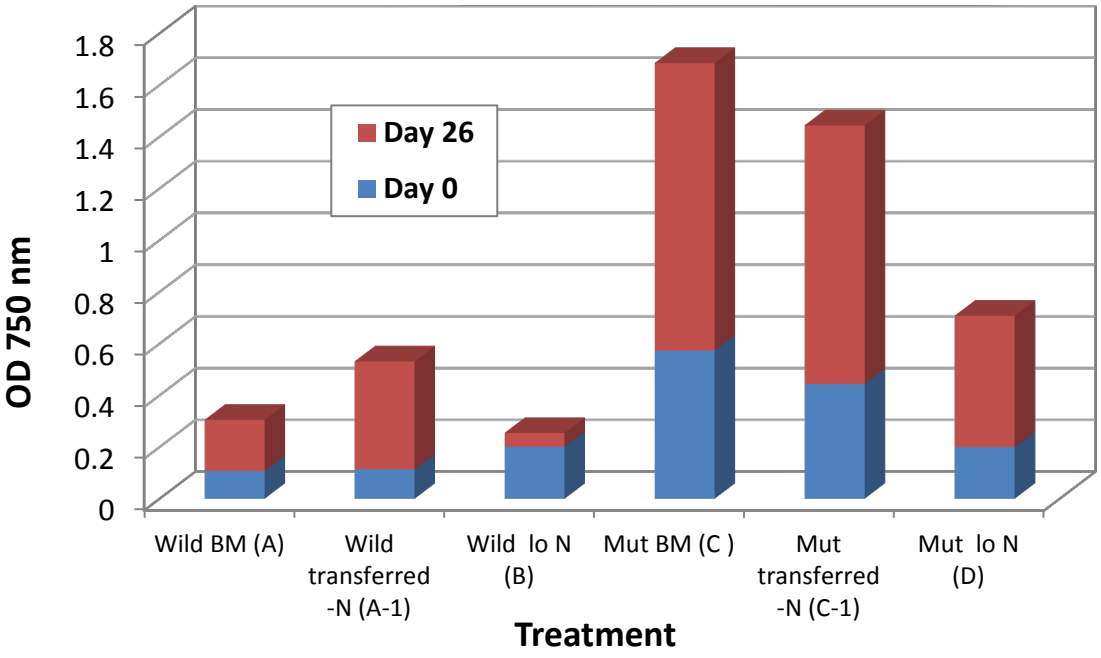


Figure 16: Effect of nitrogen stress induced in the log phase. Mutant 16-3 and wild type cells grown with replete nitrogen followed by media without nitrogen.

Growth rates on both mutants cultured with nitrogen (C) and transferred mutant cells to media without nitrate (C-1) were approx. 2.5 times higher than the other treatments (Figure 16). On the other hand, wild type cells did not follow a growth increase as a response to unlimited nitrogen supply. Growth of wild type cultures in all treatments was slower than the mutant cultures even

with maximum nitrogen availability. Lipid production at the end of the experiment is shown in Figure 17.

The highest optical density was obtained from mutant cells when cultured either with replete (C) or low nitrate concentration (C-1). However, lipid production was increased by reduced nitrogen levels (Figure 17). Maximum lipid production was estimated on samples from mutated cells grown with low nitrogen (pink) having a 4-fold increase on lipid production followed by wild type cells cultured with low nitrogen (orange) with a 3-fold increase compared to wild type cells grown with replete nitrogen.

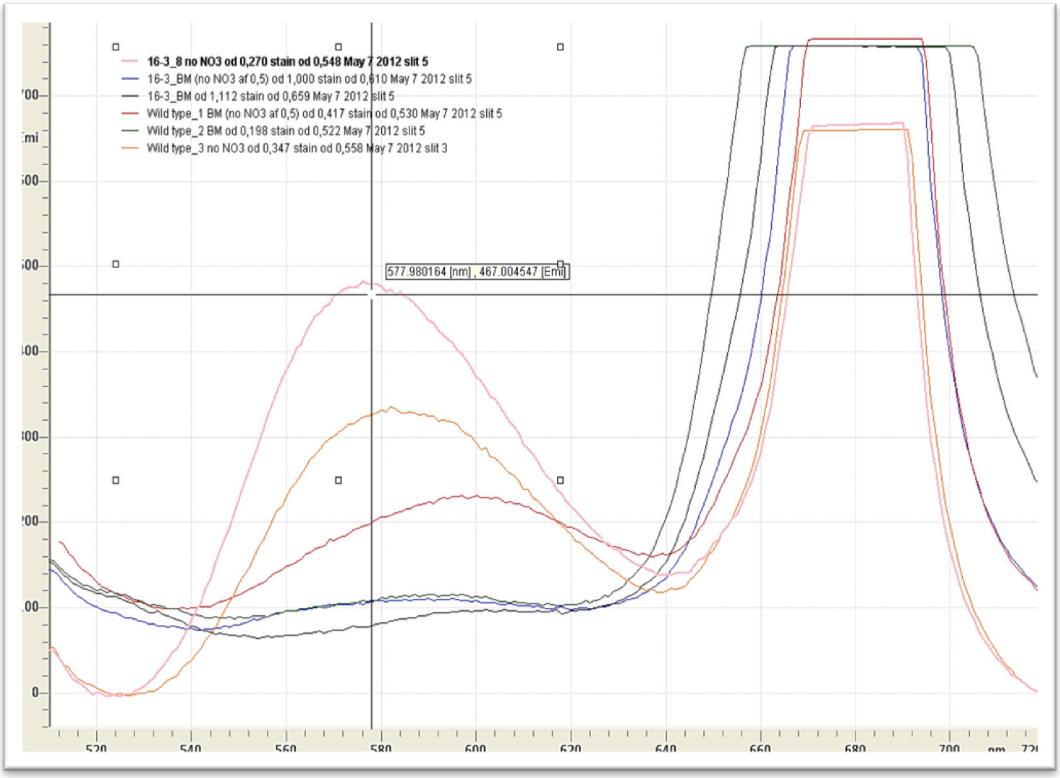


Figure 17: Effect of nitrogen on lipid production on mutant 16 #3 and wild type cells. (Wild type cultures- green: grown with replete nitrogen (trt A), orange: low nitrogen (B), red: replete nitrogen followed by no nitrogen (A-1). Mutant cultures- black: with replete nitrogen (C), blue: replete nitrogen followed by no nitrogen (C-1), pink: low nitrogen (D).

Wild type cells grown on low nitrogen (A-1 red) had twice the lipid production compared to wild type control. Even though the highest lipid production was achieved with mutant cells grown (D) with low nitrogen levels (pink), the maximum number of cells was obtained from mutant cells by maximum nitrate treatment (C). These results follow the hypothesis that nitrogen depletion promotes lipid production. However, maximum biomass production was obtained with increased nitrogen levels. Optimal nitrogen levels for high biomass and lipid yields vary among genera, even among species. *In general, higher nitrogen supply increases biomass yield and decreases lipid productivity.*

Mutant cells reached log phase in a shorter period of incubation compared to any treatment of wild type cells, which resulted in higher biomass yield and faster growth rate. The highest lipid production on mutant 16 #3 was observed when grown with lowest nitrate concentration. Findings suggest that mutant 16 #3 seems to be a conditional mutant that grows faster than wild-type and increases lipid production when limited nitrogen environment is provided. These results also suggest the mutant is better able to assimilate nitrogen as compared to wild type. This mutant appears to be effective in increasing cell growth rate, which will result in decreased harvest time.

2.8 Biomass Production as Response to Phosphorus Levels

Microalgae require specific nutrients for their biomass production. The aim of this study was to determine minimal phosphorus consumption in microalgae and effects of phosphorous concentration on growth by evaluating different phosphate levels for biomass production.

Procedure:
A growth curve was performed on *S. dimorphus* using Bristol media at room temperature. Triplicate batch cultivation modes were used: Bristol media with 1.72 mM as phosphate high concentration, 15 μM for medium and 7.5 μM for low phosphate concentration. Phosphate concentrations were determined based upon findings suggested by L. Xin (et al. 2010). Microalgal cell population was estimated by optical density measurements (750nm) and pH values continuously monitored on each nutrient condition to estimated growth.

Results:
Figure 18 illustrates growth from lag to mid log phase. Cultures were monitored for 31 days. All cultures grew at a slower rate than expected. No significant difference was observed among treatments even though maximum tested concentration had approximately 230 times more phosphate than the lowest level. These findings did not reveal any effects on cell biomass production as a function of phosphate uptake.

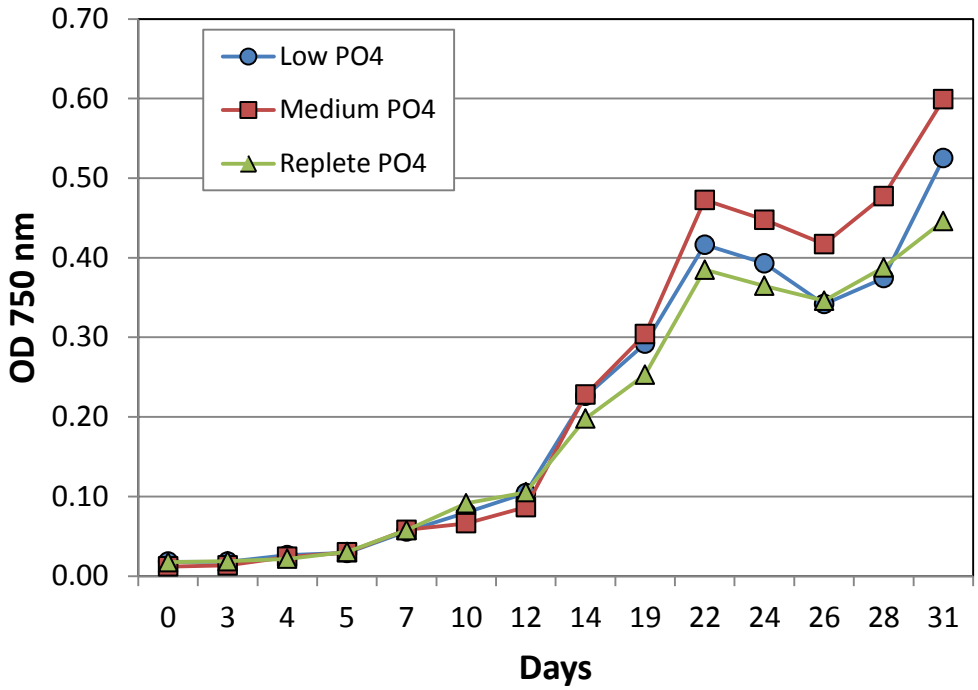


Figure 18: Phosphorous effect on *S. dimorphus* cell growth. Tests at low (7.5 µM), medium (15 µM) and high (1.72mM) phosphate concentrations

2.9 Biomass Production as Response to Light Cycle Duration

As autotrophic microorganisms, microalgae can harvest light from the sun as their main energy source and convert it into storage molecules like oil. Light/dark cycles have been proven to determine productivity of autotrophic cultures. Normally, microalgae are grown on 12:12 hr cycles but they can grow on other alternated intervals. Experiments were carried out to evaluate effect of light/dark cycle duration and frequency of intervals for the same overall light-time on cell growth.

Procedure:
Triplicates of *Chlorella* sp. wild type were grown on Bristol media with the following light/dark regimes: 18:6; 12:12 (control) and 9:3 hr twice/day. Light was supplied by LED lamps placed on top of each chamber with light intensity of 27 µmol photons/m^{-2}/s^{-1}. Cultures were grown on a shaker (100 rpm) at room temperature.

Results:
Fastest growth was achieved on 18-hr light treatment having twice the growth rate (0.880 OD) than the control (0.449 OD) as shown on Figure 19. However, treatment with 18 hr light duration divided into two intervals resulted in a slower cell growth rate (0.476 OD) than the single 18-hr interval. In this study, long-duration light/dark cycle led to a 200% biomass production increase resulting in a reduced cultivation time to generate same biomass as the other treatments. Cultures grown at 18:6 light regime reached a 0.445 OD value at 15 days, while control required 11 additional days to reach the same cell population.

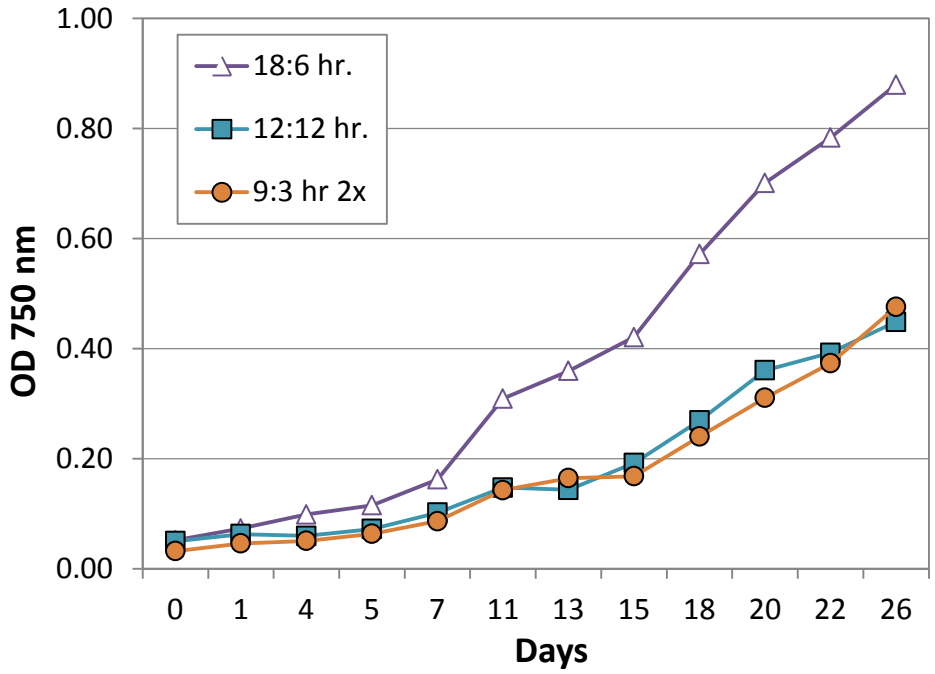


Figure 19: Effect of light cycles on the growth of native strain *Chlorella* sp.

2.10 Optimizing algae purification, maintenance and lipid production techniques

Sample Collection and Culturing:
Field samples collected from different sites are stored in an illuminated cold room at 15˚C to reduce growth rate of contaminants. Samples are filtered according to the size of species of interest ranging from 10 to 60 µm filtering membranes. Polycarbonate filtering membranes are grown in agar plates. Colonies are transferred to liquid media (CB2). Cultures are grown in liquid media with agitation; revolutions are customized by species. Erlenmeyer flasks are covered with sterile abdominal pads or silicone stoppers to prevent contamination. Purified cultures are tested for optimized production under heterotrophic conditions with several organic carbon sources like beet and sorghum juice using 2-L fermenters (Figure 20). Algae strains with fast growing rates are acclimated mixotrophically (light and carbon sources) before growing heterotrophically. Fundamental knowledge acquired on heterotrophic growth will facilitate the process of designing a fermentation medium and choosing the best strain for such process.

Figure 20: Bioreactor used for heterotrophic growth with beet juice as carbon source.

Purification:

An antibiotic matrix was designed to purify each strain; a total of twelve treatments with different combinations of antibiotics were tested for each strain to reduce bacterial and fungal population. Samples were treated for 48 hrs up to one week with antibiotics and grown in media for bacteria (B-V, FTM). Cultures were then screened under a computerized (Imager 21) electronic Zeiss microscope using specific filter sets to detect bacteria. Contaminants like cyanobacteria were observed under the microscope using filter 43HE; other bacteria were detected with SYBR green with filter set 38HE and differential interface contrast (DIC) as illustrated on Figure 21.

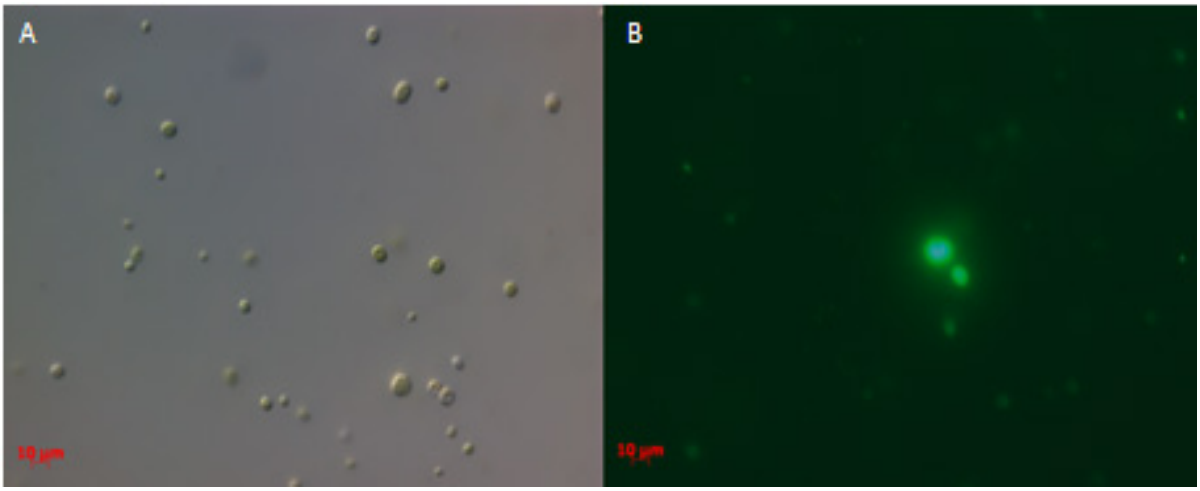


Figure 21: Algae sample with light (A) and DIC with SYBR green (B), notice bacterial fluorescence on (B).

Lipid determination:
As part of the training, a demonstration on techniques used by the General Atomics lab was provided by the GA lab staff. Lipids are typically measured using gas chromatography, flame ionization detector and mass spectroscopy in the chemical analysis laboratory. Monosaccharaides are measured using dinitrosaIicyIic (DNS) acid reagent for determination of reduced sugars with a multi-mode microplate reader. Also proteins, chlorophyll and lipids can be quantified in samples incubated in these micro titer plates.

2.11 Highlights and Achievements

1. Methods for the isolation and purification of microalgae from field samples using antibiotics to culture unialgal cultures have been successfully developed. This has resulted in the isolation of 20 microalgal cultures. Identification by DNA sequencing was also accomplished.
2. Laboratory techniques for the separation and identification (using Percoll density gradients and Nile Red fluorescence respectively) of high lipid producing algae have been developed and demonstrated. These techniques have been successfully employed in:
    a. Separation and identification of mutants with higher lipid content
    b. Identification of cells with higher lipids within a given population
    c. Identification of physiological conditions that affect lipid content.
3. Mutant agents that alter lipid content in algae have been identified. Screening of 300 potential mutants has resulted in the isolation of two mutants with increased lipid levels and one mutant with an increased growth rate and improved nitrogen assimilation. Further characterization of the mutants is warranted.
4. Research technician Vilmaris Bracero trained at General Atomics Inc. for the heterotrophic cultivation of microalgae.
5. A preliminary draft of a manuscript on these findings has been written is being edited for publication in the Journal of Phytochemistry (see Appendix).

3.0 DEVELOPMENT OF EFFICIENT ALGAE DEWATERING AND ALGAE/OIL SEPARATION TECHNIQUES

3.1 Quartz Microbalance

The QCM200 QCM Digital Controller with a QCM 25 5MHZ Crystal Oscillator was used to investigate the behavior of the microalgae during flocculation on an adsorption or deposition surface. In theory, in case of improved flocculation the adsorbed microalgae would decrease the oscillating frequency of the crystal. In addition, in certain cases such as biofouling, monolayers are formed that could be measured with the QCM. Several experiments were performed with the QCM system, and as observed previous years, the results were non-reproducible. Therefore, the QCM is not considered a reliable device for the flocculation of microalgae.

3.2 Lab scale ultrasound and flocculation experiments

Literature research was conducted on how to increase the ultrasound efficiency for faster flocculation. Flocculation effects take place in the liquid when applying ultrasound waves due to external frequency forces provided to medium. For these experiments, the goal was to achieve flocculation by applying ultrasonic waves to the algae broth. Since the original broth contained materials such as salts, cell waste and microalgae that contribute to flocculation at high frequencies, it was proposed to use distilled water with low amounts of microalgae to study its flocculation behavior under ultrasound. The images in Figures 22 through 24 show how microalgae are being flocculated using high frequency ultrasonic waves. Based on these, it is estimated that 70 to 90 percent of flocculation per dry mass of microalgae should be expected with this method.

Figure 22: Ultrasound over Petri dish

Figure 23. Chlorella after ultrasound

Figure 24: Diluted solution (left), normal broth (middle), concentrated broth (right).

A combination of ultrasound flocculation with freeze-dried cells was also investigated. The freeze dryer uses a standardized lyophilization process, which consists of using low pressure (0.1 bar) and low temperatures (-40°C) to dry the samples. Samples were processed for approximately 24 hours. After the lyophilization process was completed, the fully dried microalgae (See Figures 25 through 27) were weighted. An average of 0.0213 g was calculated from the concentrated broth and an average of 0.0154 g for the normal broth. It was observed that employing ultrasound combined with the freeze dryer for flocculation, yielded larger amounts of dry microalgae in comparison with using only ultrasound.

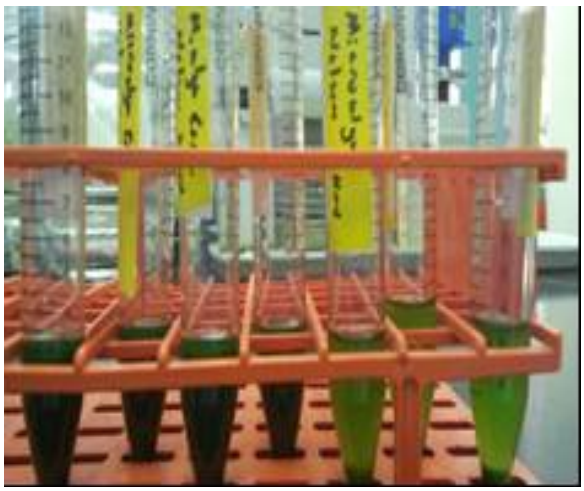


Figure 25: Test Tubes prior to freeze. Concentrated (left), Normal (*right*).

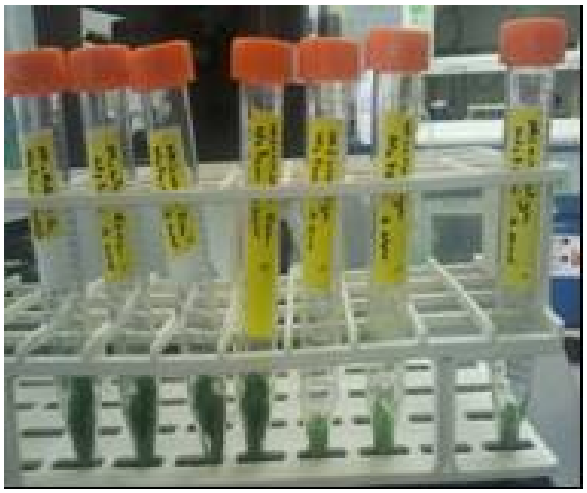


Figure 26: Test Tubes after lyophilization process. Concentrated (left), Normal (right).

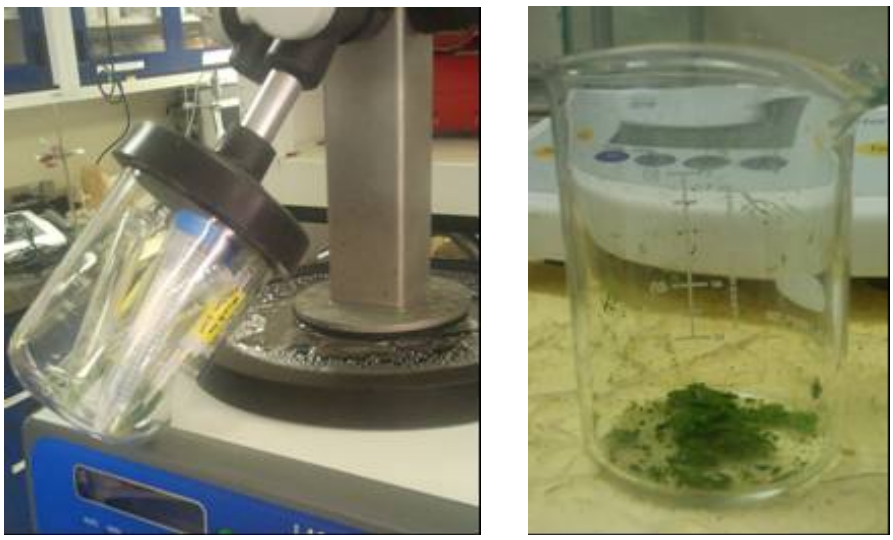


Figure 27: Freeze Dryer (left) *Chlorella sp*. test tubes, (right) dry *Chlorella sp*.

Other flocculation experiments at high frequencies (3 MW) studied the effect of the pH level of the culture. The pH levels were adjusted from 7 to 11 to investigate its effect on flocculation rate. It was observed that for culture pH levels of 7 to 10, similar flocculation behavior was obtained. The concentration ratios (final/initial) were very similar, as shown in Table 2 and Figure 28. At a pH 11, the ratio decreased considerably. This behavior is not well understood, and further research at high pH levels is warranted.

Table 2 Flocculation rates at varying pH levels

pH	Concentration (cells/mm^2)	Ratio after treatment
Original broth	4,187.50	1
7.0	63,906.25	15.26
8.0	56,906.25	13.59
9.0	55,000	13.13
10.0	64,031.25	15.29
11.0	12,625	3.01

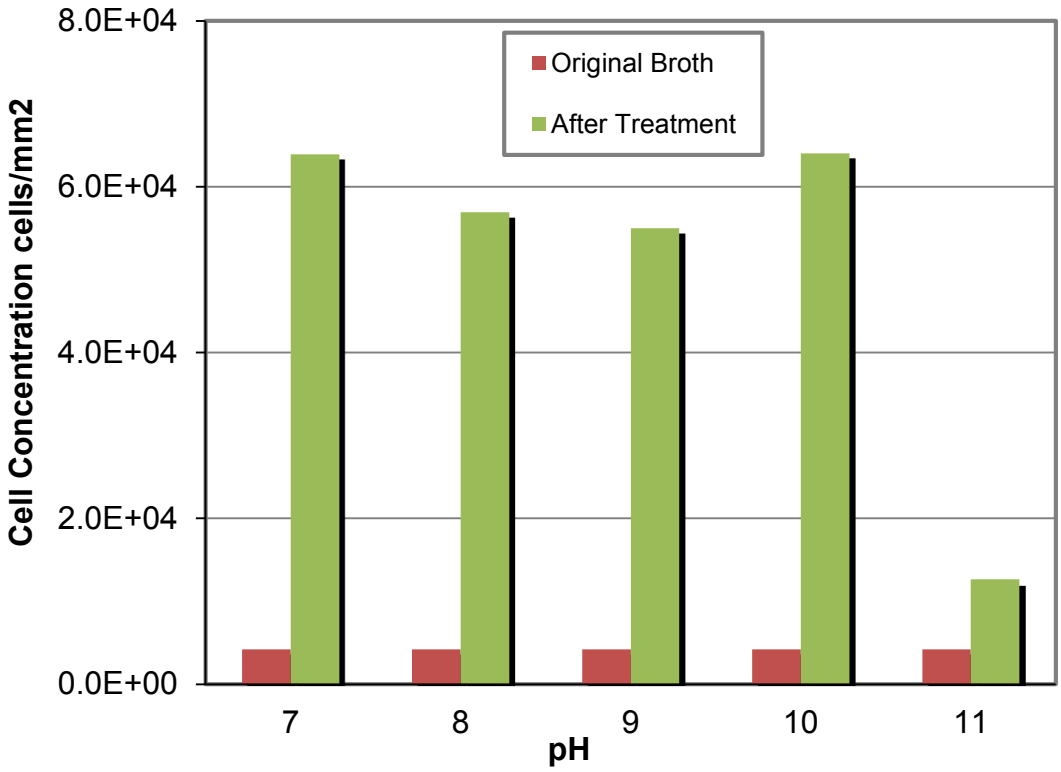


Figure 28: Comparison of cell concentration at given pH after ultrasonic treatment

3.3 Implementation/Operation of a Hydrodynamic Cavitation Ultrasound (HyCU) Harvesting/Extraction system

A Hydrodynamic Cavitation Ultrasound (HyCU) system was used to assess its effects on the cell breakage due to high pressure induction in microalgae. Hydrodynamic cavitation systems operate similar to ultrasonic cavitation, producing extreme turbulence in the solution. Suppliers (www.arisdyne.com/vp/cavitation.htm) claim that the cavities are smaller than their ultrasonic counterparts, which in some cases will benefit the application. In addition, continuous flow nature of these systems makes them easier for scale-up. The system consisted of two Triplex Direct Drive Plunger pumps (Model 2SF20ES, ½ and ¾ horsepower WEG motors) connected in parallel. The flow streams were combined and passed through a specially engineered orifice where the fluid was subjected to shear and pressure. The fluid was then projected over a knifelike blade at high velocity, where a strong cavitational field was formed. The pressure and cavitational energy created by the orifice and blade efficiently and effectively created tightly uniform emulsions and dispersions with particle sizes as small as 1 μm. The unit also had two tanks that were operated in recycle mode.

Experimental Method:
Unit sterilization – The HyCu unit was sterilized mixing two tablespoons of dish soap, three cups of commercial chlorine and three gallons of water into the container. The solution was re-circulated for 20 minutes after which the container was washed three times with fresh water. Water filled containers were added to the HyCu container to occupy volume. These were inside the container during the sterilization. Ten liters of algae broth were added to the container. The first 15 mL (0 minutes) sample was taken before turning on the unit. The unit was turned on and 15 mL samples were collected every 15 minutes for 180 minutes. Each sample was poured in a 15-mL centrifuge tube taken directly from the system's hose. Samples were treated with trypan blue and placed in a light microscope using a hemacytometer to count the amount of live cells.

Results and Discussion:
The first experiments showed the differences in cell content before and after the treatment were not significant. It was assumed that there was not enough cell concentration for the HyCu to be effective. The experiment was performed three times with increased cell concentrations, yielding the same results. Light microscope observations, Figure 29, show no wall breakage of microalgae cells. This suggests that cavitation by pressure differential does not provide sufficient energy to break microalgae cells when they are too small, which is the case for *Chlorella sp*. Figure 30 shows a small cell concentration decrease trend with treatment time, but the effect was not significant. In conclusion, the HyCU system was ineffective in rupturing microalgae cells for the separation of algae and lipids.

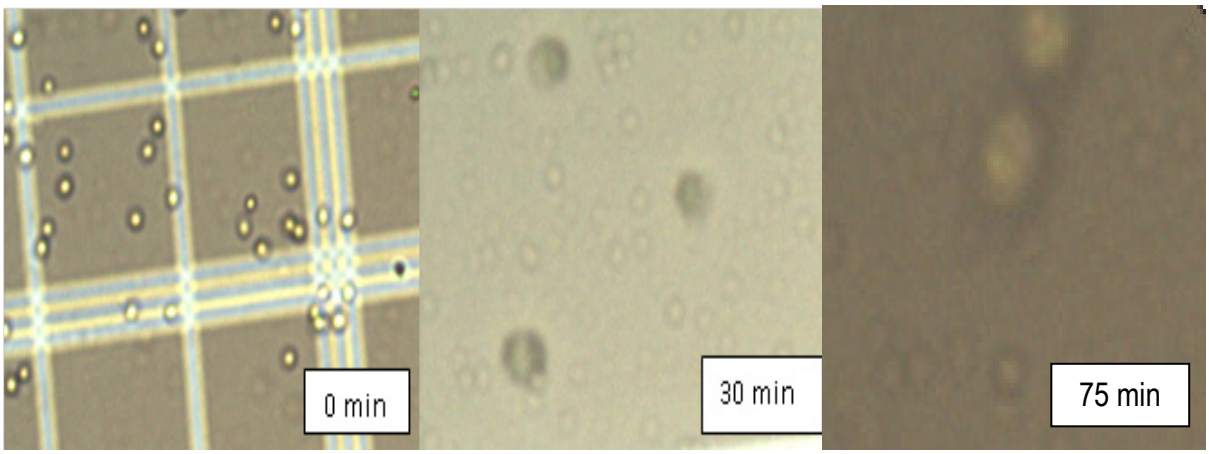


Figure 29: Comparison of cell content and form at 0, 30 and 75 minutes of HyCu treatment with 400X of total magnification

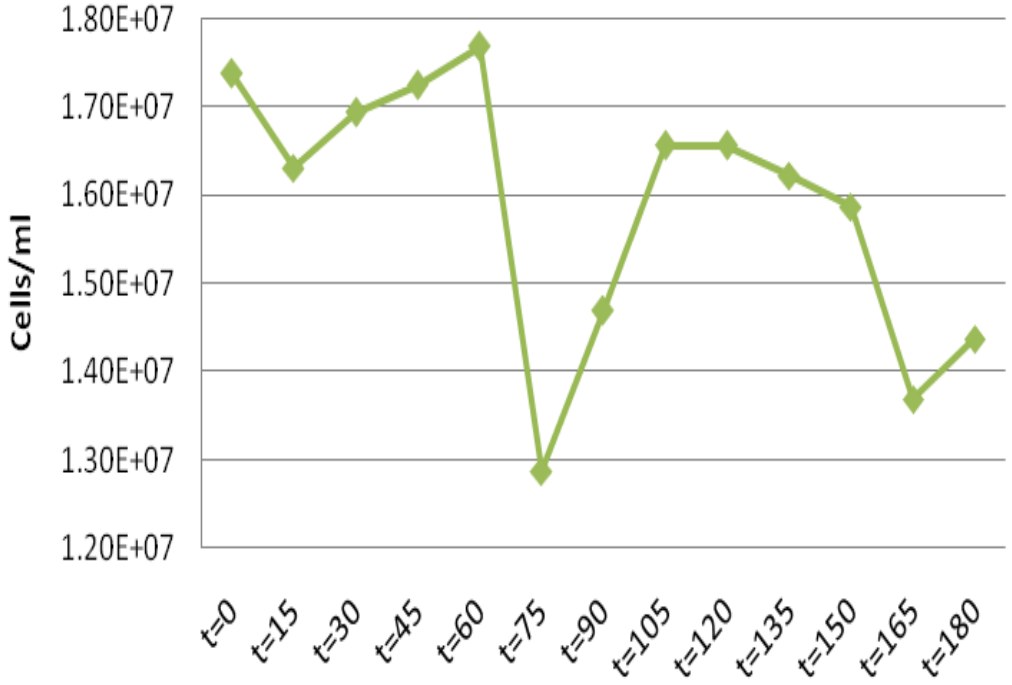


Figure 30. Cell concentration change with time (in minutes).

4.0 OIL EXTRACTION METHOD DEVELOPMENT

4.1 Oil Extraction Methods

The extraction methods considered were the semi-continuous Soxhlet and continuous Gregar using acetone:hexane in a 2:1 ratio as solvent. Fish food was used as sample for oil extraction experiments as it contains microalgae with reported value 4% of oil as main ingredient. Test runs of 15, 30, 45, and 60 minutes were performed. Approximately 200 mg of fish food where weighted per test run. After each run was completed, a rotary evaporator was used to remove all the solvent from the sample, the sample was dried and the percentage of organic extracted was calculated. The oil/organics content of the microalgae was determined by weighing the empty clean flask before extraction various times, until a constant weight was achieved; then weighing the flask with the obtained extraction in the same manner, and finally subtracting both values. Over the months the process has been continuously modified and improved in order to enhance precision and accuracy of the results. Furthermore, additional solvents have been used such as acetone, hexane, isopropanol and blends of these.

The MicroGregar extraction method showed better reproducibility (lower standard deviation) in the 15, 30 and 60 minute run times as compared to the MicroSoxhlet extraction method. Meanwhile, MicroSoxhlet showed a tendency to require longer extraction times than MicroGregar, where 30 minutes runs showed better extraction efficiency. Table 3 and Figure 31 illustrate the results for each run.

Table 3. Results for 15, 30, 45 and 60 Minute Oil Extraction Runs

Fish food	**Soxhlet**	**Gregar**	**Soxhlet**	**Gregar**	**Soxhlet**	**Gregar**	**Soxlet**	**Gregar**
Run #	15 min	15 min	30 min	30 min	45 min	45 min	60 min	60 min
1	8.98	9.25	9.40	9.50	10.50	9.97	9.95	9.78
2	8.78	10.16	10.14	9.43	10.60	10.28	10.56	9.57
3	8.29	10.09	7.03	10.41	10.44	10.32	8.52	8.70
4	7.09	8.4	8.81	8.67	10.10	9.66	8.34	8.29
5	7.05	10.57	9.25	9.07	10.10	10.33	9.14	9.08
AVG	8.04	**9.69**	8.93	**9.42**	10.35	10.11	9.30	9.08
STDEV	**0.92**	**0.87**	**1.04**	**0.58**	**0.21**	**0.26**	**0.94**	**0.61**

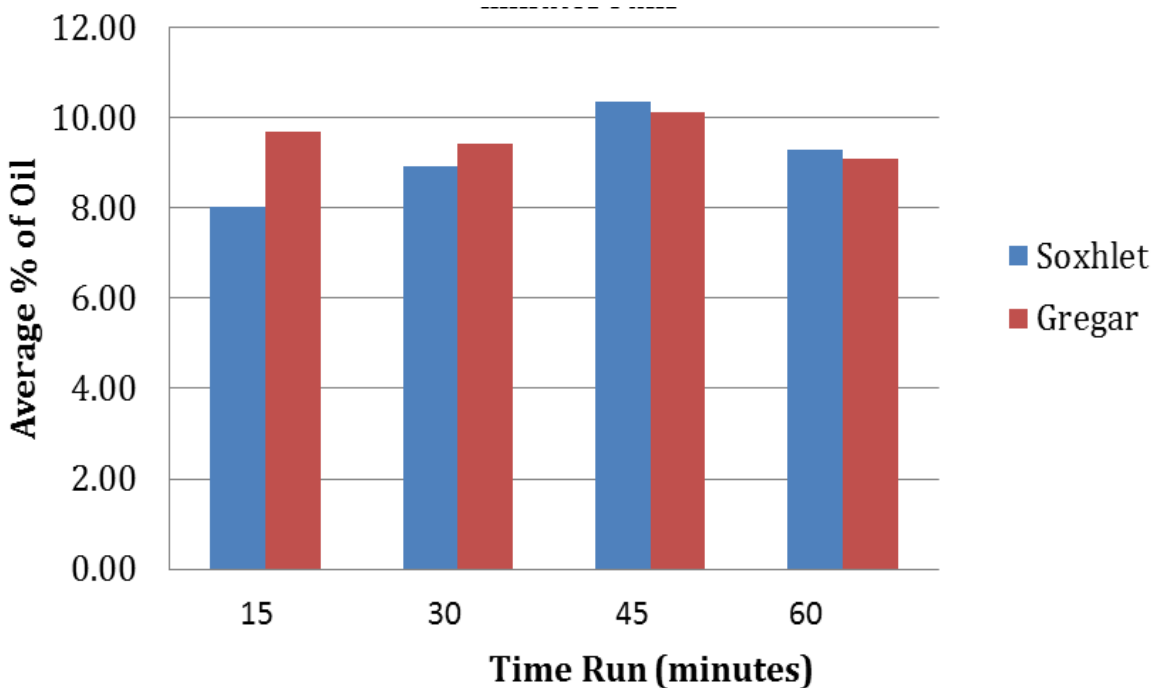


Figure 31: Results obtained for the average organic extracted percent as a function of the two extraction methods for times of 15, 30, 45 and 60 minutes runs.

The data show that the highest organic extracted percentage was obtained with Gregar using 15 and 30 minutes of extraction time and for Soxhlet using 45 minutes of extraction time. For the 15-, 30- and 45-minute runs, the standard deviation of Gregar was lowest in comparison with Soxhlet. Only at 45 minutes, Soxhlet showed a lower standard deviation. Finally, for the 45-minute runs Soxhlet gave the highest extracted oil value in comparison with Gregar. The 60-minute run time is a special case, because a batch of new fish food was used.

In summary, both the Soxhlet and Gregar devices performed similarly with relatively good reproducibility for oil extraction from microalgae. Detailed procedures for oil extraction from microalgae using both the Soxhlet and Gregar devices will be covered in a technical article prepared by Miriam Fontalvo as part of her PhD dissertation.

5.0 DESIGN OF A PRELIMINARY PROCESS FLOW DIAGRAM FOR MICROALGAE GROWTH, HARVESTING AND PROCESSING

5.1 Process Flow Diagram and Cost Analysis

A key component of this project was the study of different process flow configurations and respective capital and operating costs for growing, harvesting and processing microalgae feedstock for lipid production. The details are provided in the Master's thesis of Jesús García, which will be submitted in December 2012. The following is a summary of the main results. Table 4 describes each case scenario. Table 5 shows the required investment to build the facilities for each case. These estimates include the fixed capital cost, the working capital needed to run the plant during the first month, and the subsidized cost of land. Case 1 contains more operational units to harvest the microalgae broth than other cases.

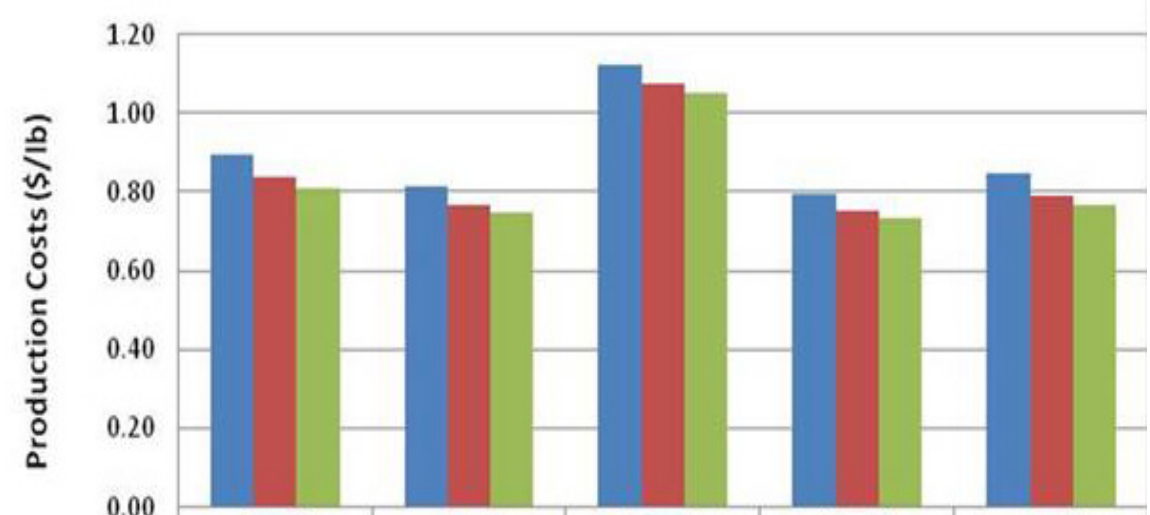
Table 4 Summary of Studied Scenarios for Microalgae Lipid Production

Case #	Description
1	Pond →Flocculation →Dewatering →Centrifugation →Drying →SC Extraction
2	Pond →Flocculation →Dewatering →Freeze Drying →SC Extraction
3	Pond →Freeze Drying →SC Extraction
4	Pond →Dewatering →Freeze Drying →SC Extraction
5	Pond →Flocculation →Membrane Filtration →Drying →SC Extraction

Table 5 Investment Information for Each Case Scenario (In Million Dollars)

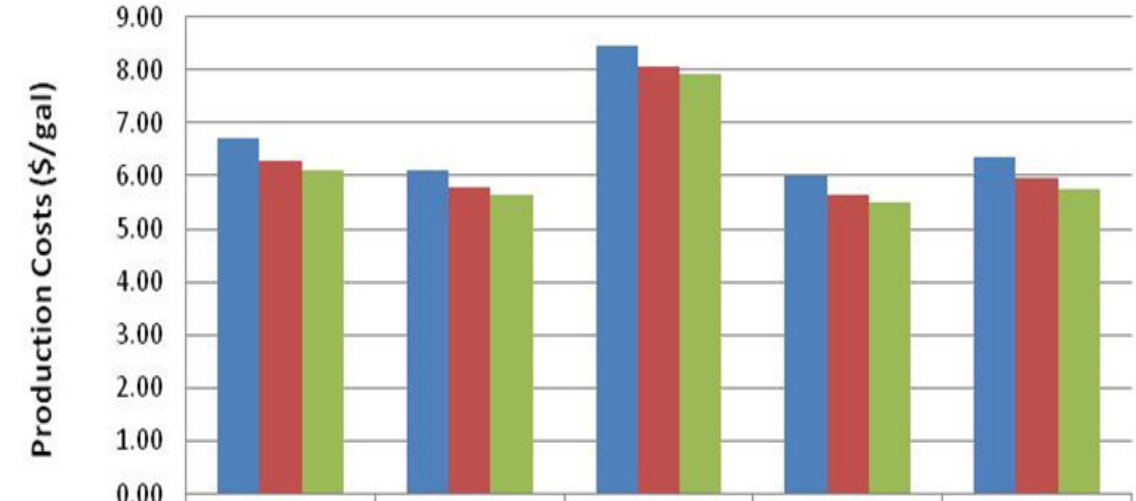
Item	Case 1	Case 2	Case 3	Case 4	Case 5
FCI ($M)	**101.8**	**80.7**	**88.5**	**80.8**	**96.8**
Land ($M)	**9.8**	**9.8**	**9.8**	**9.8**	**9.76**
Working Capital ($M)	**23.6**	**23.0**	**28.3**	**22.7**	**22.92**
Total Investment ($M)	**135.1**	**113.5**	**126.6**	**113.2**	**129.47**

Unit production costs of bulk paste that includes lipids, proteins and carbohydrates were estimated (Figure 32). It includes both capital and operation expenses. The equivalent of the unit production cost for lipids (oils) in $/gallon is shown in Figure 33. Most of the production costs are driven by operational expenses or cost of manufacturing as shown in Table 6**.** These are driven by the operation of the freeze dryer and supercritical extraction (SC) system (all cases). In case 3, the effect of freeze dryer costs is increased given that a dewatering step before the dryer is not present. In the other freeze drying cases (2 & 4) a pre-dewatering step was provided. In addition, notice that the costs of cases 1, 2 and 4, 5 are very similar and driven mainly by the SC extraction operation costs. Table 7 shows that the cost of manufacturing depends strongly on the utility costs where the electricity was assumed at approximately $0.24/kW-hr and water at $0.00063/lb. For purpose of this analysis, a breakeven criterion was assumed for each case scenario with overall microalgae-to-bulk paste conversion of 50 percent. This is equivalent to 222.21 million pounds per year of bulk paste, and an annual production of 20 million gallons of lipid. This analysis was based on a mass balance and operational conditions to obtain equipment size and overall costs at preliminary design level. Detailed analysis of the different process units is required to perform a system energy balance, which is beyond the scope of this project.



	Case 1	Case 2	Case 3	Case 4	Case 5
Life - 5 years	0.89	0.81	1.12	0.80	0.85
Life - 10 years	0.84	0.77	1.08	0.75	0.79
Life - 20 years	0.81	0.75	1.05	0.73	0.77

Figure 32: Bulk paste unit production cost, $/lb at different financing periods.



	Case 1	Case 2	Case 3	Case 4	Case 5
Life - 5 years	6.71	6.12	8.45	5.99	6.35
Life - 10 years	6.28	5.78	8.08	5.65	5.95
Life - 20 years	6.09	5.63	7.91	5.50	5.77

Figure 33: Lipids unit production cost for lipid (oils), $/gallon, at different financing periods.

Table 6 Breakdown of Total Lipid Production Costs For 10 Years Financing in $/Lb

Expense/Case Scenario	Case 1	Case 2	Case 3	Case 4	Case 5
Total Production Cost	**0.84**	**0.77**	**1.08**	**0.75**	**0.79**
Cost of Manufacturing (COM)	0.72	0.67	0.96	0.65	0.68
Land Cost	0.01	0.01	0.01	0.01	0.01
Fix Capital Cost	0.11	0.09	0.11	0.09	0.10
FCI/PC (%)	13.1	11.7	10.1	12.0	12.7

Table 7 Summary of Cost of Manufacturing Including Contingencies, Operating Labor, Raw Materials, Waste Treatment and Utilities

Case Scenario	Case 1	Case 2	Case 3	Case 4	Case 5
COM ($M/yr)	159.9	149.2	214.6	145.3	151.0
0.18FCI ($M/yr)	18.3	14.5	15.9	14.5	17.4
2.73C_OL ($M/yr)	38.3	35.3	30.0	32.9	35.3
1.23C_RM ($M/yr)	26.2	26.2	23,.8	23.8	26.2
1.23C_WT ($M/yr)	6.5	6.5	6.5	6.5	6.5
1.23C_UT ($M/yr)	70.6	66.6	138.4	67.6	65.6

5.2 Project Summary Conclusions

1. An economic analysis was performed for five different case scenarios maintaining a fix pond site and SC extraction unit with variation in the harvesting technologies for each case for an annual production of 222 million pounds of microalgae paste.
2. Case 4, which considers the use of pond → dewatering → freeze dryer → supercritical fluid extraction shows to be the more attractive in terms of investment due to the lower value of fixed capital ($80 M), as well as lower annual operational costs ($145.3 M).
3. This results in a production cost of $0.80/lb, $0.75/lb and $0.73/lb to produce the bulk paste equivalent to $5.99/gal, $5.65/gal and $5.50/gal of oil at a 10% of interest rate with an income tax rate of 42% at 5, 10 and 20 years financing, respectively.
4. In all the cases, operational costs were the predominant factor due to the high cost of utilities required by the SC extraction unit.
5. It is highly recommended to perform a detailed analysis to:
   a. Find strategies to reduce the associated operational cost to these units,
   b. Analyze other extraction systems that also preserve the paste integrity.
6. Selling remaining biomass as bio-fertilizer versus disposal has a positive effect in minimum production cost of $0.30/lb
7. In summary, these results indicate that a microalgae-based production facility requires a biorefinery operation philosophy. Specially, a variety of products must be produced efficiently and sold in a wide variety of markets such as commodities (biofuels) and value added specialties (proteins). A single product approach will not justify neither the investment nor the operational costs required to operate the plant.

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APPENDIX

A.1 Manuscript for Scientific Journal - Rapid Procedure for Separating High-Lipid Containing Microalgae

Introduction:

There's a worldwide demand to develop and expand alternative supplies to fossil fuels. Biomass feedstock is needed to produce adequate supply for biodiesel production. Microalgae, with more than 30,000 known species are a promising class of feedstock known for its higher productivity over terrestrial crops. These photosynthetic microorganisms do not compete with food production since the can be grown in marginal lands that are unsuitable for agricultural applications. Depending on environmental conditions where they are grown, such as nutrient stress (e.g., Nitrogen depletion) some algae species can produce large amounts of oil as a storage product, achieving 50 to 60% lipids of dry weight (Sheehan et al., 1998).

Developing new strategies to screen for prospective microalgae species producing suitable compounds for the biofuel industry is essential for commercial success. The combination of known monitoring techniques can provide a broader overview of key parameters including biomass production and intracellular oil content. Conventional testing of lipid content in biological samples such as solvent extraction and gravimetric determination are time consuming and generate high amounts of waste (Han et al., 2011). Some practical methods have been previously tested for screening lipids such as rapid colorimetric quantification of lipids from algae (Wawrick and Harriman, 2010) and Nile red fluorescence scans using microplates to monitor neutral lipids in *Chlorella vulgaris* (Held and Raymond, 2010). Percoll ™Gradient centrifugation has been consistently used to fractionate cells on the basis of their buoyant density, (Whitelam et al., 1983). Utilizing a suspension of micro algal cells, it should thus be possible to separate high lipid producing cells from low or non-lipid producing cells utilizing such a gradient.

To test this hypothesis, we present here a combination of Percoll gradients and the NR fluorescence technique (Deng et al., 2011) for the isolation of microalgae with high lipid producing capabilities.

Materials and Methods:

Sample collection and Isolation

Chlorella sp. was obtained from a 30 ml freshwater sample obtained from a rice field channel at the Agricultural Experimental Station in Lajas municipality, University of Puerto Rico in February 2012 at coordinates $18^0 00' 46.85''$ N and $67^0\ 04'\ 21.37''$. The 30 ml water sample was first filtered through a nylon sterile 60 micron mesh then with a 5 micron polycarbonate filter (Millipore). Filtered sample was subsequently grown in Bristol medium (HaiYing, et al., 2012) and isolated using a step antibiotic treatment with cycloserine (100 mg/L^{-1}) bacitracin (100 mg/L^{-1}) and tetracycline (40 mg/L^{-1}) for 48 hr each followed by a combined treatment of tetracycline and cycloserine.

DNA Purification Procedure:
Genomic DNA was isolated using the technique described by Fowley et al. 2004 with one modification: our extraction procedure used 10% detergent hexadecyltrimethylammoni-um bromide (CTAB) instead of dodeyltrimethylammonium (DTAB).

Polymerase chain reaction:
Approximately 100 ng of Genomic DNA from algal isolate were used for PCR amplification. ~ 681 base pairs(bp) of the 5' end of the 18S rRNA gene were successfully amplified using highly conserved primers (s) 5' GTCAGAGGTGAAATTCTTGGATTTA3') and (as) 5'AGGGCAGGGACGTAATCAACG-3' Ferris et al.(2005)**.** Cycling conditions 95°C for 5 min, followed by 30 cycles of 94°C for 45 seconds, 53°C for 45 seconds, 72° 1 min. and a final extension for 7 minutes at 72°C.

PCR, cloning, sequencing, and taxonomic analysis:
Cloning of PCR-amplified rRNA gene sequences was performed using a pGEM-T Easy® vector (Promega Corporation, Milwaukee, WI)according to the manufaturer's instructions. Plasmid isolation was performed using a QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.) and were sequenced using the universal M13 forward and reverse primers with an ABI Prism® Big Dye ™ Terminator reaction and the ABI 310 Genetic Analyzer (Applied Biosystems, CA. USA) Preliminary sequence data was edited using the online tool Biology Workbench 3.2 (San Diego Super-computer Center, http://biowb.sdsc.edu) and compared and analyzed using BlASTN (Altschul and Lipman 1990). The isolated sequence had 99% homology with *Chlorella*sp 18s rRNA gene (GenBank database no. 224487723.

Culturing Techniques:
Native *Chlorella* sp. (Chlorophyceae) batch cultures were grown in 200 ml Bristol media ($NaNO_3$ 2.94 mM, $CaCl_2$ 0.17mM, $MgSO_4$ 0.3mM, K_2HPO_4 0.43mM, KH_2PO_4 1.29 mM and NaCl 0.43mM). Control triplicates were supplied with 2.94 mM nitrogen and experimental replicas were grown with minimal nitrogen source 1mM.for 21 days under conditions of nitrogen-sufficiency and nitrogen depletion. Cultures were maintained under a photoperiod of 16/8 hr of light/dark cycle with cool-white fluorescent lights with 27 $\mu mol/m^{-2}/s^{-1}$ and shaken continuously @ 110 rpm at 26°C.

Percoll Gradient for Algal Separation:
A discontinuous density gradient from 100, 80, 60, 30 and 10% v/v Percoll™ was prepared in 15 ml glass centrifuge tubes. Percoll step concentrations were determined based on previous results using a continuous gradient ranging from 0 to 100% (data not shown). A one ml cushion layer of 100 % Percoll was loaded, subsequently each 3 mL aliquots of 80, 60 and 30% steps volumes were carefully layered and labeled on the centrifuge tube. Finally 1 ml layering platform of 10% percoll was added. Samples were taken from triplicate cultures that were incubated for 21 days under conditions of nitrogen-sufficiency and nitrogen depletion. To visualize banding 1 ml of algae culture (1.5-1.6 $_{od\ 750nm}$) was loaded and centrifuged at 5930 *g* at 4˚C for 20 min (J2-MC Beckman). Cell band positions were estimated by optical inspection. To remove the colloidal silica solution and concentrate cells bands were rinsed with deoinized water using 2 volumes for lower gradients (10 and 30%) and 6 volumes of cell suspension for higher concentrations (60, 80

and 100%). Washed cells were then resuspended in deionized water at an optical density (750 nm)of 0.5 for lipid determination.

NR Fluorescence:
To confirm lipid content on generated cell layers, different bands collected from Percoll separation were stained with 3 µl of NR stock solution (250 $mg^{-1}L$ in acetone) for 10 sec. The fluorescence intensity of Nile red in sample was measured then excited at 495 nm before measuring the emission between 520 and 720 nm, setting both excitation and emission lits at 5nm using an RF-5301 PC Spectrofluorophotometer (Shimadzu). Neutral lipids can be detected at 580 nm (Guan et al., 2009). One ml per sample of algal suspension was stained with 3 µl of 250 $mg^{-1}L$ Nile Red dissolved in acetone.

Results

Cell Separation:
To evaluate whether gradient centrifugation can separate high lipid-content cells from non-synchronized culture, treatments were resolved on Percoll. There was a 1.7, 3.9, 8.5 and 10.3% difference in density for 80, 60, 30 and 10% respectively, compared to 100% Percoll concentrations. Figure A-1 shows the results of the density gradients. Control cells (nitrogen replete) layered at 80% (tube A) while nitrogen deprived cultures equilibrated at the 10% Percoll (tubes B, C and D) with an estimated buoyant density of 1.0 and 1.10 g/ml respectively. Cells separated by Percoll gradients were viable (data not shown) and subsequently their lipid content was measured using NR spectroscopy.

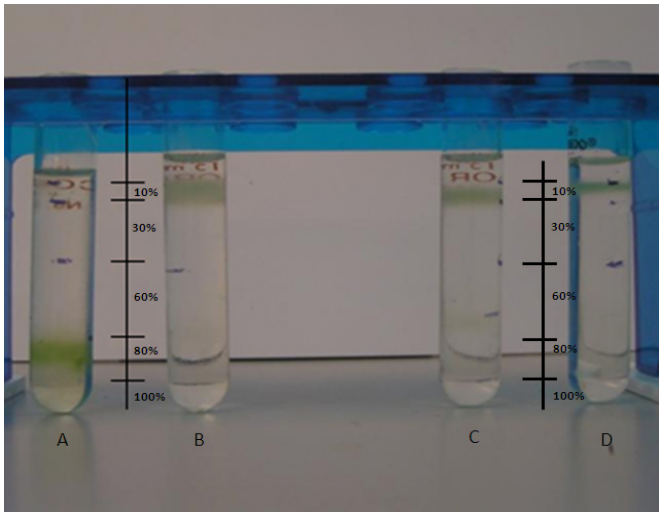

Figure A-1. Banding position for cultures with nitrogen (one control tube from triplicates: A) and without nitrogen (B, C and D).

Nile Red Fluorescence:
Collected bands layered from 10% and 80% Percoll were stained with Nile red solution and lipid content was measured by a fluorescent emission spectrum. Cultures with significant neutral and polar lipid production peaked at 580 to 620 nm respectively and chlorophyll auto fluorescence emission was detected at 680 nm. Fluorescent spectral scans in Figure A-2 demonstrates a high

neutral lipid content for cells banding at 10% (red) whereas control cells banding at 80% did not show a marked peak (blue). The 10% band sampled from nitrogen deprived culture showed a lipid peak of an 8-fold increase. This indicated that cells with higher lipid content banded at lower Percoll concentrations proving the validity of this technique.

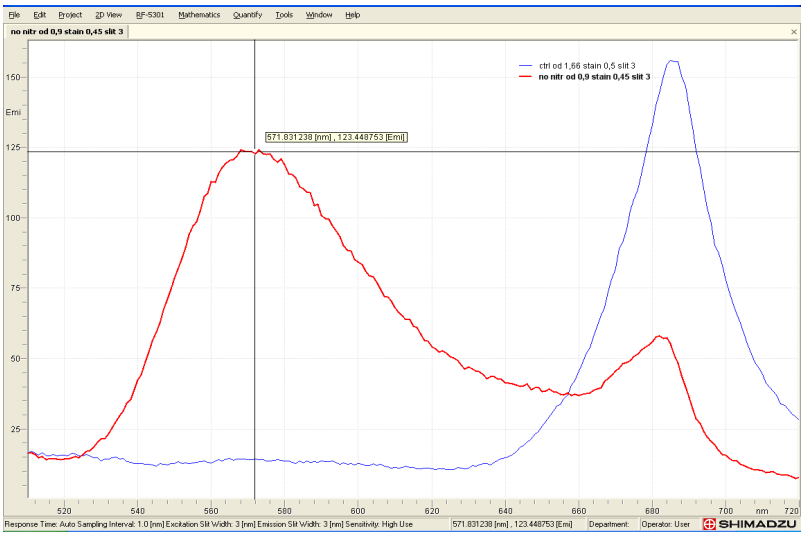

Figure A-2. Microalgae lipid accumulation (575nm) cells with low density (10% band, nitrogen stress) (red spectrum) and high density (80%, control) Percoll band (blue).

Discussion

Native *Chlorella* sp. cultures grown under different nitrogen conditions exhibited variable lipid content. Reports describing lipid accumulation as a result of nutritional deficiency prompted us to grow microalgae in nitrogen deficiency media. Cho et al. (2011) induced lipid productivity in *Chlorella* sp. 277 by modifying nitrogen and carbon concentrations. Morales et al. (1990) isolated mutants in a density gradient centrifugation with altered glycerol content relying on the fact that *Debaryomyceshansenii* under salt stress accumulated glycerol, thus changing the cell buoyant density.

In this study, cells exhibiting different buoyant densities as a result of their lipid content were evaluated using Percoll gradient and Nile red scan. These combined techniques are suitable as a direct method for isolating and screening cells respectively. Eroglu et al. (2009) estimated biopolymers content using a density gradient separation protocol with live single cells of microalgae and other microorganisms under in situ conditions; Guan and other (2009) successfully used Nile red fluorescent spectra to compare cellular lipid accumulation in *Chlorella*. In our experiments cells reaching density equilibrium at 80% (Figure A-1) Percoll step, with less buoyancy seem to lack storage lipids, as indicated by a flat line on the spectra. Figure A-2 confirmed that different banding positions can be directly attributed to intracellular lipid content. Spectral emission scans demonstrated the highest lipid content in cells peaking at 575 nm; these fluorescence spectra confirmed that highest lipid producing cells band much higher (10% Percoll) than cells that have a lower lipid content (80% Percoll).

Results proved the utility of lipid fluorescence and buoyant density separation method for quick estimation of lipid content in live cells and isolating high lipid producing algae. It can be

performed in few hours without the need of using laborious procedures. This technique can be applied for constant monitoring of oil-production variability for prospecting rich-oil strains. It is a rapid method suitable for screening of environmental samples or genetically altered cells that are metabolically different in terms of lipid content.

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A.2 Related activities

General/Administrative
New equipment was acquired under this program to measure cell growth: turbidimeter and hemacytometer. Miriam Fontalvo finalized her thesis experiments and will defend her Ph.D. thesis later this year. Jesús García defended his thesis project on Thursday, July 12, 2012 as his last requirement towards a Ms. Eng. degree in Chemical Engineering. Cecilia Díaz is working her thesis field experiments and preparing to defend her Ms. Sc. thesis in Spring 2013.

Student Visit to General Atomics:
September 2011 - Vilmaris Bracero visited General Atomics facilities in San Diego, California to be trained on algal culturing techniques for biodiesel production. This company is worldwide recognized for high-technology systems development ranging from the nuclear fuel cycle to remotely operated surveillance aircraft, airborne sensors, and advanced electric, electronic, wireless and laser technologies. Vilmaris Bracero learned new techniques at the Advanced Process Systems Division for algal biodiesel production including algae isolation and purification, culture maintenance, and lipid optimization.

A.3 Educational Activities

Table A-1: Posters presented during the last year.

Poster	Presenter
Extraction of bioproducts from microalgae	Luis Colon
Application of green technologies for the production of bio-oil from microalgae biomass as a feedstock	Adrian Lopez
Development of procedure to obtain biofuels using microalgae biomass as a feed stock	Miriam Fontalvo
Cost analysis of local bio-products processing plant using microalgae biomass as feedstock	Jesús Garcia
Sustainable Energy Initiative Laboratory	José Colucci

Workshops:
Another contribution of this project was that K-12 student workshops and science fair projects were held in our laboratory co-sponsored by the Department of Education CIVIS Initiative. These workshops were documented in a paper that won the second best paper award from the American Society of Engineering Education. The following is the abstract of this awarded document.

CHEM E Sustainable Energy Demos, Workshops, Town Hall Meetings and Other Stakeholder Engagement: Working the Pipeline

José A. Colucci-Ríos, Miriam Fontalvo, Efraín O'Neill-Carrillo
University of Puerto Rico-Mayagüez

Abstract – A Sustainable Energy Laboratory in the Chemical Engineering Department has been instrumental in the effective incorporation of sustainability into chemical education targeting audiences (hundreds per year) from the whole spectrum: K-12, undergraduate students, graduate students and the general public. The latter includes strong alliances with various stakeholders that led to several awards such as the *2009 EPA Region II Environmental Quality Award.* The laboratory's cultural transformation started in 1995 sponsored by the Department of Energy with environmentally friendly topics related to renewable energy such as Fuel Cell applications, Solar Detoxification, Biomass Conversion processes, etc. Workshops and Demonstrations were integral components of those efforts. At the turn of the century the focus increased to include sustainability principles such as social equity, ethics and community participation but with energy as the central theme. This focus has been primarily supported by a Sustainable Energy Initiative implemented in the College of Engineering. These activities are effective in providing information to various stakeholders and increasing interest in sustainable energy.