This thesis for honors recognition has been approved for the

Department of Chemistry.

Director — Date: 5/12/17

Reader — Date: 5/3/17

Reader — Date: 5/3/17

Reader — Date: 5/5/17

To whom it may concern;

This thesis, Enhancing biofuel lipid feedstock accumulation in the microalgae *Ostreococcus tauri*, has been placed under an embargo. The research that was conducted for this study will be presented in an additional forthcoming paper. Any questions may be addressed to Emma Patello (emmapatello@gmail.com).

Enhancing biofuel lipid feedstock accumulation in the microalgae *Ostreococcus tauri*

Emma Patello, Chuck Smallwood*, William Chrisler, and James Evans*

Mentors (*): Chuck Smallwood and James Evans

Environmental and Molecular Science Laboratory, Pacific Northwest National Laboratory, 3335 Innovation Blvd, Richland, WA 99354 USA

Abstract

Microalgae produce a diverse array of biomolecules and have attracted attention as alternative sources for biofuel feedstocks. Unlike other forms of alternative energy resources, algae do not require arable land nor fresh water for production. To be a viable alternative energy source, the amount and types of lipid accumulation in microalgae needs to be characterized and optimized. Changes in lipid production and cell biomass were monitored in the microalgae *Ostreococcus tauri* under differing nutrient conditions. We discovered that partial depletion of both nitrogen and beta-glycerol phosphate with simultaneously supplementation of glycerol resulted in marked increases of neutral lipid content.

Introduction

Environmental impacts of non-renewable fossil fuels have prompted the search for alternate energy resources. Electric power-producing renewable energy sources such as solar, hydroelectric, and wind are not as convenient as fossil fuels due to the current lack of efficient methods for energy storage (Armand and Tarascon 2008). Sources such as biodiesel and advanced biofuels have the similar advantages to that of fossil fuels, as they also store energy in the form of chemical bonds (Sy Tran et al. 2012). However, biofuel production costs present challenges that must be addressed for them to be a viable energy source, such as issues with efficient release of trapped energy (Yeoman et al. 2010, Li et al. 2015). Ethanol from corn as well as other starch crops are considered the "first-generation" biofuels and have attracted much attention worldwide but exhibit major drawbacks (Dellomonaco et al. 2010). While providing large amounts of energy, starch is neither as efficient nor as energy rich as lipids. Biodiesels use the higher-energy bonds found in lipids that come from vegetable oils or animal fats (Biermann et al. 2011). However, as with both biofuel and biodiesel production, much of the biomass that is used either takes resources away from food production or is a possible food for humans or other animals (Tollefson 2008, Mata et al. 2009, Dellomonaco et al. 2010, Yeoman et al. 2010). For this reason, alternative sources for lipid production are currently a major area of research (Trentacoste et al. 2013, Fields et al. 2014, Garay et al. 2014, Talebi et al. 2015). Algae have shown significant potential for production of lipids and do not require arable land for growth, but instead utilize the noncompetitive resource of salt water (Dismukes et al. 2008, Mata et al. 2009, Medipally et al. 2015).

While multiple species of algae show promise in the production of lipid biofuel feedstocks (Hamilton et al. 2014, Sorigue et al. 2016), *O. tauri* was the focus of this study. *Ostreococcus tauri* is a microalga and currently the smallest known free-living eukaryotic organism at ~1 µm in diameter with a genome that is 1,235 mb. In addition, it lacks a cell wall, and most of the organelles appear only once (Derelle et al. 2006). These characteristics make *O. tauri* an attractive candidate for genetic modification after the nutrient conditions that enhance lipid production are defined. In other microalgae, starvation of key nutrients results in an increase in lipid production, specifically triacylglycerol (TAG), (Khozin-Goldberg and Cohen 2006, Merzlyak et al. 2007, Sharma et al. 2012). During the course of this research, *O. tauri* was grown in standard and differing nutrient depleted media conditions and then subsequently assessed for cell biomass and lipid accumulation.

Methods

1. Strains

The strain (OTH0995) of *O. tauri* used in this study was obtained from the National Center for Marine Algae (NCMA).

2. **Making of the media and *O. tauri* growth**

Keller media (K media), the media used for *O. tauri*, is a common growth media for microalgae and is composed of a variety of salts and supplemented nutrients (Keller et al. 1987) (Table 1). K media was made fresh within 4-5 days of use. All of the media from each set of depletion experiments was made with the same Artificial Sea Water (ASW) base to decrease variability and a sufficient stock of K Trace metals and F/2 Vitamins was made prior to the start of these experiments. During the course of the study, nitrogen (present in the forms of $NaNO_3$ and NH_4Cl) and phosphorus (present in form of Na_2 beta-glycerophosphate (BGP)) content in K media was varied to determine the effects of depletion. Glycerol was also supplemented into some of the media where BGP was absent or reduced as a source of additional carbon to drive lipid accumulation in *O. tauri* cells. (See Supplemental Material for a complete list of Media conditions tested)

A 12-hour diurnal cycle that consisted of blue light (470 nm) with an intensity of approximately 20 µmoles photons/m^2/s was used to grow *O. tauri* for both normal and starvation conditions. Cell cultures were spectroscopically monitored each day at absorbance readings of 680 and 750 nm for up to nine days.

Ostreococcus tauri cell stocks were grown in a culture of K media until their mid-log phase then pelleted and washed with the specific media composition that was to be assessed. Samples were run in groups of similar media alterations with K media with and without any nitrogen acting as controls. Depletion experiments were typically set up in a gradient fashion, from normal amounts of components to complete absence of those components. These included a nitrogen gradient, BGP gradient, and a combined inverse BGP/glycerol gradient. Several sets of samples were run to cross-compare the best conditions from the various gradient tests.

After the stock cultures were determined to be adequately grown, the cells were spun at 1,500 x g for six minutes. The supernatant was discarded and the pelleted cells were re-suspended in five mL of the specific media composition that was to be assessed. The cells were spun again 1,500 x g for six minutes, and the supernatant discarded. The cells were again re-suspended in their specific media composition and divided into replicates to be measured. The absorbance was recorded immediately afterwards at 445, 600, 680, and 750 nm. The absorbance at these wavelengths was then measured approximately every twenty-four hours for the following nine days.

3. Analysis with Flow Cytometry with Fluorescence-Activated Cell Sorting (FACS) analysis

During the mid-log phase of *O. tauri*, typically 72 hours, the cells in the different media types were analyzed for lipid accumulation to quantify the characteristics of the different populations by staining with nile red lipid stain. Flow cytometry was used to characterize *O. tauri* cells in terms of neutral, phospholipid, and chlorophyll content as well as cell size.

Table 1: Components in Keller Media, adapted from Keller (1987). F/2 Vitamins, K trace metals, and K nutrients were made as stock solutions. All other components were added fresh each time a solution was made.

Components	Final Concentration
Artificial Sea Water	
NaCl	4.20×10^{-1} M
KCl	1.00×10^{-2} M
$MgCl_2 \cdot 6\ H_2O$	2.00×10^{-2} M
$CaCl_2 \cdot 2\ H_2O$	1.00×10^{-2} M
$MgSO_4 \cdot 7\ H_2O$	2.45×10^{-2} M
$NaHCO_3$	2.50×10^{-3} M
K Nutrients	
$NaNO_3$	8.82×10^{-4} M
NH_4Cl	5.00×10^{-5} M
Na_2 beta-glycerophosphate $\cdot$ $6\ H_2O$	1.00×10^{-5} M
H_2SeO_3	1.00×10^{-8} M
Tris-base	1.00×10^{-3} M
K Trace Metals	
$Na_2EDTA \cdot 2\ H_2O$	1.12×10^{-4} M
$FeCl_3 \cdot 6\ H_2O$	1.17×10^{-5} M
$Na_2MoO_4 \cdot 2\ H_2O$	2.60×10^{-8} M
$ZnSO_4 \cdot 7\ H_2O$	7.65×10^{-8} M
$CoCl_2 \cdot 6\ H_2O$	4.20×10^{-8} M
$MnCl_2 \cdot 4\ H_2O$	9.10×10^{-7} M
$CuSO_4 \cdot 5\ H_2O$	3.92×10^{-8} M
F/2 Vitamins	
Vitamin B_{12} (Cyanocobalamin)	3.69×10^{-10} M
Biotin	2.05×10^{-9} M
Thiamine $\cdot$ HCl	2.96×10^{-7} M
18.2 MΩ H2O	To one liter

Results

Nutrient survey of Keller standard growth media for lipid accumulation in *O. tauri*

Growth of *O. tauri* was monitored using visible spectroscopy absorbance readings of each culture at 680 nm and 750 nm as a rough indicator of both chlorophyll accumulation (680

nm) as well as cell dispersion (750 nm) in the accumulated *O. tauri* population. Growth started with a lag phase as the *O. tauri* recovered from the process of transferring to its new growth medium. This was followed by rapid growth (the "semi-log phase") which plateaued then decreased as the available nutrients became depleted. Figures 1, 2, and 3 plot the growth of nitrogen and BGP depleted conditions as well as conditions where glycerol was added.

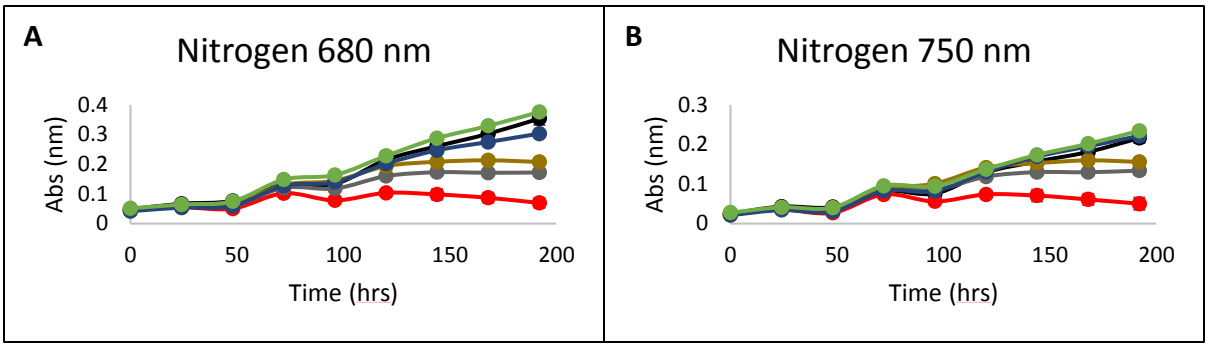

Figure 1: (8/11). Effects of nitrogen depletion on *O. tauri* over 8 days with samples collected every 24 hours. Measurements were taken at 680 nm (**A**) and 750 nm (**B**). K (black) and K–100%N (red) were used as controls. K–95%N (gray), K–90%N (gold), K–80%N (blue), and K–20%N (green) indicate the results of removing that percentage of nitrogen.

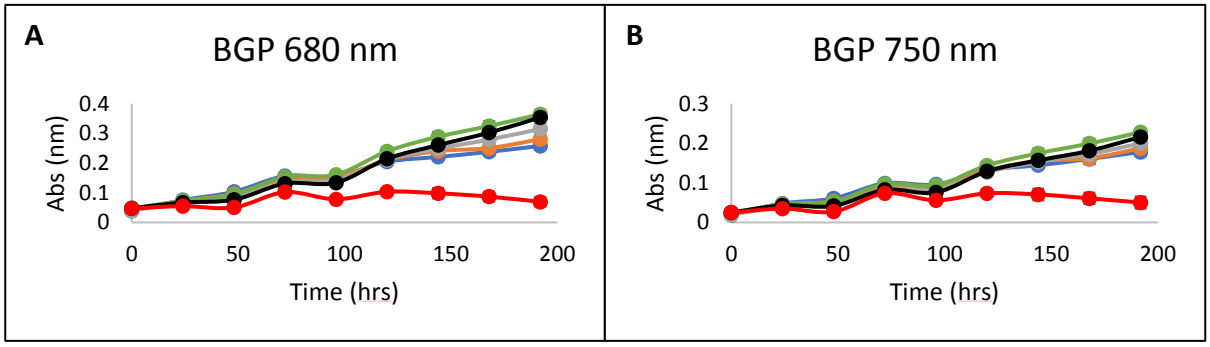

Figure 2: (8/11). Effects of BGP depletion on *O. tauri* over 8 days with samples collected every 24 hours. Measurements were taken at 680 nm (**A**) and 750 nm (**B**). K (black) and K–100%N (red) were used as controls. K–100%BGP (blue), K–90%BGP (orange), K–60%BGP (gray), and K–20%BGP (green) indicate the results of removing that percentage of BGP.

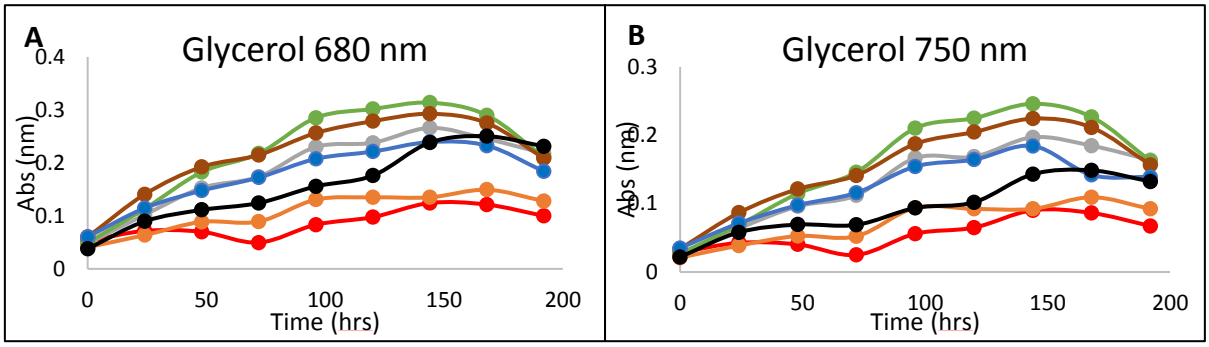

Figure 3: (7/12). Effects of glycerol depletion on *O. tauri* over 8 days with samples collected every 24 hours. Measurements were taken at 680 nm (**A**) and 750 nm (**B**). Base media was K–90%N–100%BGP. K (black) and base media without glycerol added (red) were used as controls. To the base media glycerol was added to the following final concentrations: 0.05mM (orange), 0.5mM (gray), 10mM (green), 50mM (brown), and 100mM (blue).

After cultures were tested for growth with various levels of nutrient depletion, several possible candidates were combined to test growth under multiple conditions. The absorbance graphs of the most promising results (which use all three nutrient conditions, partially depleted nitrogen, partially depleted BGP, and increased glycerol) are shown in Figure 4. A list of all the conditions tested is located in the supplemental material.

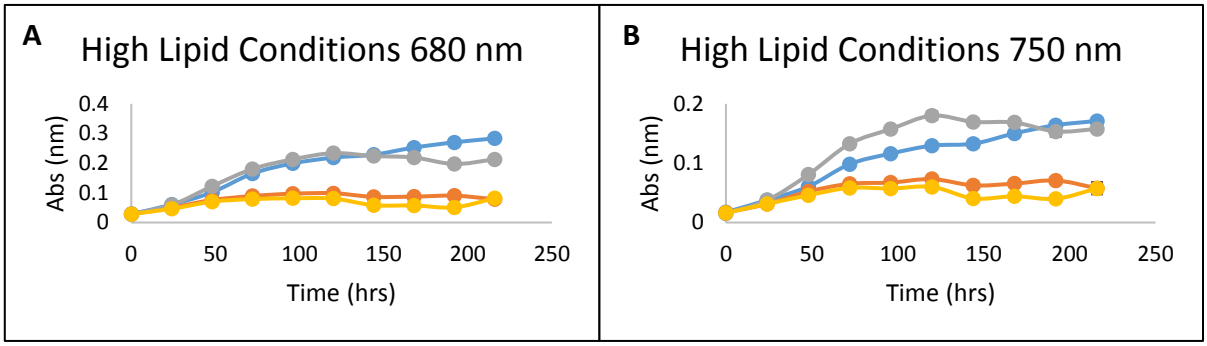

Figure 4: High Lipid Conditions. The effect of different nutrient conditions over 9 days sampled every 24 hours at 680 nm (**A**) and 750 nm (**B**). K media with no alterations (blue) and with the complete absence of nitrogen (orange) were used as controls. K media with a complete absence of nitrogen and BGP, containing 50mM glycerol (yellow) and K media minus 90% of nitrogen, minus 60% BGP, and containing 50mM glycerol (gray) were the conditions tested.

Evidence of significant increases of lipid accumulation due to an increase in glycerol is shown in the increased 750nm absorbance graphs in Figures 3-4. In addition, cell population dynamics using the same cell cultures in FACS data showed distinct differences in lipid content when in the presence of increased glycerol (Figures 5a and 5b). However, when

looking at both cellular chlorophyll content and size (Figures 5c and 5d) of only nitrogen depleted conditions there was a lack of these marked phenotypic differences. In fact, in the K-90N-60BGP+50mMGly sample, there was only slight decreases in chlorophyll content and cell size. Taken together, this leads to the conclusion that increasing the glycerol content while decreasing both the nitrogen and BGP leads to a greater accumulation of lipids without greatly affecting the amount of chlorophyll produced.

FACS analysis of high content lipid accumulation in *O. tauri*

The results of these absorption experiments were utilized for further analysis under these novel conditions that induce lipids. Ideal cell culture conditions as determined by the absorbance graphs were chosen for further analysis using FACS. The most relevant results are shown in Figure 5.

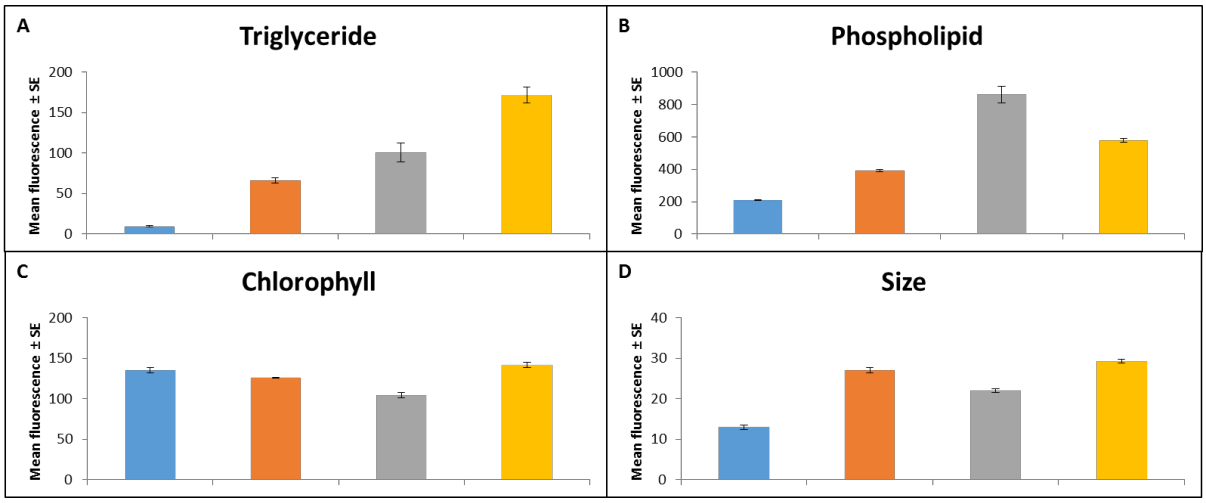

Figure 5: FACS data of High Lipid Conditions cultures revealed triglyceride (**A**), phospholipid (**B**), chlorophyll (**C**), and mean cell size (**D**) of *O. tauri* obtained after 72 hours of growth in specified media. K media with no alterations (blue) and with the complete absence of nitrogen (orange) were used as controls. K media with a complete absence of nitrogen and BGP with the addition of 50mM glycerol (yellow) and K media minus 90% of nitrogen, minus 60% BGP, and the addition of 50mM glycerol (gray) were the conditions tested. Used with permission from C. Smallwood and W. Chrisler.

In summary, monitoring absorbance at 680 nm and 750 nm allowed selection of *O. tauri* cultures for further analysis by fluorescence activated cell sorting (FACS) flow cytometry. FACS results found that cells grown in K media with both 90% of nitrogen sources and 60% of the BGP removed and 50mM of glycerol added resulted in both enhanced TAG production and comparable growth rates when compared to cells grown in standard K media.

Discussion

The goal of this study was to identify conditions that stimulated significant TAG lipid accumulation in *O. tauri*. The 750nm readings could be a result of cell growth, increase in cell number, or efflux of lipid droplets into media. Currently, we were unable to ascertain the primary contribution to biomass increases. Although K-100N-100BGP+50mMGly had the highest overall TAG, as indicated in the FACS data (Figure 5a), it also had the lowest biomass by 750nm (Figure 4). K-90N-60BGP+50mMGly had the best TAG and 750nm combination. It is interesting to note that glycerol appears to have a profound effect on the accumulation of lipids considering no genes for glycerol uptake were identified in *O. tauri*. Combined depletion effects while supplementing excess carbon in the form of glycerol in concentrations greater than 0.05mM (Figure 3), showed markedly increased effects on cell growth capacity well beyond that of either nitrogen (Figure 1) or BGP (Figure 2) depletion.

Overall, the effect of changing nutrient conditions was meant to manipulate the lipid metabolic pathway of the cell to increase lipid production. We observed increases in both neutral lipid as well as phospholipid content of our test cultures. Phospholipids and other charged lipid species are typically integrated into membranes and therefore inaccessible for mass extraction unless the entire cell is destroyed. Neutral lipids are ideal for biofuel feedstocks and typically appear as triglycerides which consist of three fatty acids and one glycerol. The carbons in the fatty acid come from acetyl CoA, the main substrate in the citric acid cycle. Glycerol can be made from dihydroxyacetone phosphate (DHAP), a component of glycolysis, and the carbon for this process is either fixed in the chloroplast from CO_2 in the atmosphere or from nutrient carbon sources (Berg et. al., 2012). By stressing *O. tauri* as we did here, the cell could switch metabolism from making carbohydrates to making lipids (is a more efficient energy storage option) or they could both continue to accumulate simultaneously. Further work will be needed to tease out the interplay between carbohydrate and lipid accumulation.

Our study of nutrient growth conditions to produce high lipid phenotypes has provided validation and insights into the metabolism of *O. tauri*. Using the nutrient conditions found to increase lipids without sacrificing biomass reveals that additional unmapped proteins or pathways may be involved in utilizing glycerol as an organic carbon source. Comparing the media conditions in which lipid accumulation is increased with normal media conditions using proteomics would identify proteins in *O. tauri* are up- or down-regulated. From this data, essential metabolic steps could be genetically targeted for genetic engineer *O. tauri* for further optimization.

Acknowledgements

First and foremost, I would like to thank James Evans and Chuck Smallwood for allowing me to participate in their research and for being amazing mentors. In addition, I would like to thank William Chrisler for collecting and analyzing the FACS data. Finally, thanks goes to Daniel Gretch and Jeffery Morris at Carroll College for their help editing this thesis.

Supplemental Material

List of Medias tested

Sample #	Media Type
6/21/2016	
1	K-90%N-100%BGP+Gly
2	K-90%N-95%BGP+Gly
3	K-90%N-90%BGP+Gly
4	K-90%N-80%BGP+Gly
5	K-90%N-60%BGP+Gly
6	K-90%N-40%BGP+Gly
7	K-90%N-20%BGP+Gly
8	K-90%N-0%BGP+Gly
7/1/2016	
1	K-90%N-100%BGP+0%Gly
2	K-90%N-95%BGP-5%Gly
3	K-90%N-90%BGP-10%Gly
4	K-90%N-80%BGP-20%Gly
5	K-90%N-60%BGP-40%Gly
6	K-90%N-40%BGP-60%Gly
7	K-90%N-20%BGP-80%Gly
8	K-90%N-0%BGP-100%Gly
9	K
10	K–100%N
7/12/2016	
1	K-90%N-BGP+0mMGly
2	K-90%N-BGP+0.05mMGly
3	K-90%N-BGP+0.5mMGly
4	K-90%N-BGP+2.5mMGly
5	K-90%N-BGP+5mMGly
6	K-90%N-BGP+10mMGly
7	K-90%N-BGP+20mMGly
8	K-90%N-BGP+50mMGly
9	K-90%N-BGP+100mMGly

10	K
7/18/2016	
1	K-90%N
2	K-100%N
3	K-100%BGP
4	K-100%BGP+20mMGly
5	K-90%N-100%BGP+20mMGly
6	K-100%N-100%BGP+20mMGly
7	K
7/23/2016	Best conditions
1	K
2	K-100%N
3	K-90%N
4	K-60%BGP
5	K-60%BGP+50%Gly
6	K-100%BGP+50mMGly
7	K-90%N-100%BGP+50mMGly
8	K-90%N-60%BGP+50mMGly
9	K-90%N-60%BGP+20mMGly
10	K-90%N-100%BGP+20mMGly
7/30/2016	Best conditions final
1	K
2	K-100%N
3	K-90%N-60%BGP+50mMGly
4	K-100%N-100%BGP+50mMGly
8/4/2016	
1	K
2	K-20%N
3	K-80%N
4	K-90%N
5	K–95%NN
6	K-100%N
7	K-90%N+6mMBicarb
8	K-90%N-60%BGP+6mMBicarb
9	K-90%N-100%BGP+6mMBicarb
10	K-90%N+20mMGly
11	K-90%N-60%BGP+20mMGly
12	K-90%N-100%BGP+20mMGly
8/11/2016	
1	K-100%BGP
2	K-90%BGP
3	K-80%BGP

4	K-60%BGP
5	K-40%BGP
6	K-20%BGP
7	K
8	K–100%N
9	K–95%NN
10	K-90%N
11	K-80%N
12	K-20%N

LITERATURE CITED

Armand, M. and J. M. Tarascon (2008). "Building better batteries." Nature **451**(7179): 652-657.

Berg, J.M., Tymoczko, J. L., Stryer, L. (2012). *Biochemistry*. New York, NY: W. H. Freeman and Company.

Biermann, U., U. Bornscheuer, M. A. Meier, J. O. Metzger and H. J. Schafer (2011). "Oils and fats as renewable raw materials in chemistry." Angew Chem Int Ed Engl **50**(17): 3854-3871.

Dellomonaco, C., F. Fava and R. Gonzalez (2010). "The path to next generation biofuels: successes and challenges in the era of synthetic biology." Microb Cell Fact **9**: 3.

Derelle, E., C. Ferraz, S. Rombauts, P. Rouze, A. Z. Worden, S. Robbens, F. Partensky, S. Degroeve, S. Echeynie, R. Cooke, Y. Saeys, J. Wuyts, K. Jabbari, C. Bowler, O. Panaud, B. Piegu, S. G. Ball, J. P. Ral, F. Y. Bouget, G. Piganeau, B. De Baets, A. Picard, M. Delseny, J. Demaille, Y. Van de Peer and H. Moreau (2006). "Genome analysis of the smallest free-living eukaryote Ostreococcus tauri unveils many unique features." Proc Natl Acad Sci U S A **103**(31): 11647-11652.

Dismukes, G. C., D. Carrieri, N. Bennette, G. M. Ananyev and M. C. Posewitz (2008). "Aquatic phototrophs: efficient alternatives to land-based crops for biofuels." Curr Opin Biotechnol **19**(3): 235-240.

Fields, M. W., A. Hise, E. J. Lohman, T. Bell, R. D. Gardner, L. Corredor, K. Moll, B. M. Peyton, G. W. Characklis and R. Gerlach (2014). "Sources and resources: importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation." Appl Microbiol Biotechnol **98**(11): 4805-4816.

Garay, L. A., K. L. Boundy-Mills and J. B. German (2014). "Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives." J Agric Food Chem **62**(13): 2709-2727.

Hamilton, M. L., R. P. Haslam, J. A. Napier and O. Sayanova (2014). "Metabolic engineering of Phaeodactylum tricornutum for the enhanced accumulation of omega-3 long chain polyunsaturated fatty acids." Metab Eng **22**: 3-9.

Keller, M. D., R. C. Selvin, W. Claus and R. R. L. Guillard (1987). "Media for the Culture of Oceanic Ultraphytoplankton." Journal of Phycology **23**(4): 633-638.

Khozin-Goldberg, I. and Z. Cohen (2006). "The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte Monodus subterraneus." Phytochemistry **67**(7): 696-701.

Li, X., V. Y. Yu, Y. Lin, K. Chomvong, R. Estrela, A. Park, J. M. Liang, E. A. Znameroski, J. Feehan, S. R. Kim, Y. S. Jin, N. L. Glass and J. H. Cate (2015). "Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels." Elife **4**.

Mata, T. M., A. A. Martins and N. S. Caetano (2009). "Microalgae for biodiesel production and other applications: A review." Renewable & Sustainable Energy Reviews(14): 217-232.

Medipally, S. R., F. M. Yusoff, S. Banerjee and M. Shariff (2015). "Microalgae as sustainable renewable energy feedstock for biofuel production." Biomed Res Int **2015**: 519513.

Merzlyak, M. N., O. B. Chivkunova, O. A. Gorelova, I. V. Reshetnikova, A. E. Solovchenko, I. Khozin-Goldberg and Z. Cohen (2007). "Effect of nitrogen starvation on optical properties, pigments, and arachidonic acid content of the unicellular green alga Parietochloris incisa (Trebouxiophyceae, Chlorophyta)." Journal of Phycology **43**(4): 833-843.

Sharma, K. K., H. Schuhmann and P. M. Schenk (2012). "High Lipid Induction in Microalgae for Biodiesel Production." Energies **5**(5): 1532-1553.

Sorigue, D., B. Legeret, S. Cuine, P. Morales, B. Mirabella, G. Guedeney, Y. Li-Beisson, R. Jetter, G. Peltier and F. Beisson (2016). "Microalgae synthesize hydrocarbons from long-chain fatty acids via a light-dependent pathway." Plant Physiol.

Sy Tran, L., B. Sirjean, P. A. Glaude, R. Fournet and F. Battin-Leclerc (2012). "Progress in Detailed Kinetic Modeling of the Combustion of Oxygenated Components of Biofuels." Energy (Oxf) **43**(1): 4-18.

Talebi, A. F., M. Tohidfar, S. M. Mousavi Derazmahalleh, A. Sulaiman, A. S. Baharuddin and M. Tabatabaei (2015). "Biochemical Modulation of Lipid Pathway in Microalgae Dunaliella sp. for Biodiesel Production." Biomed Res Int **2015**: 597198.

Tollefson, J. (2008). "Energy: Not your father's biofuels." Nature **451**(7181): 880-883.

Trentacoste, E. M., R. P. Shrestha, S. R. Smith, C. Gle, A. C. Hartmann, M. Hildebrand and W. H. Gerwick (2013). "Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth." Proc Natl Acad Sci U S A **110**(49): 19748-19753.

Yeoman, C. J., Y. Han, D. Dodd, C. M. Schroeder, R. I. Mackie and I. K. Cann (2010). "Thermostable enzymes as biocatalysts in the biofuel industry." Adv Appl Microbiol **70**: 1-55.