Evaluating techniques of quantifying lipid yield and cell density of *Chlorella protothecoides* (Krüger) grown on waste glycerol

A thesis submitted to the graduate division of the University of Hawai‘i at Hilo in partial fulfillment for the requirements for the degree of

Master of Science

in

Tropical Conservation Biology and Environmental Science

December 2015

by

Topaz Palakika Collins

Thesis Committee:

Jason E. Adolf, Chairperson

William S. Sakai

Lisa M. Keith

Keywords: *Chlorella protothecoides*, biofuel, glycerol, heterotrophic, BODIPY 505/515, NucRed Live 647

Acknowledgments

I am grateful to L.M. Keith and the Plant Pathology Unit of the USDA-ARS-DKI-PBARC for allowing the use of their laboratory, and to J.E. Adolf for the use of the UH Hilo Marine Science BD Accuri C6 Flow Cytometer. W.P. Heller and K.R. Kissinger assisted in some laboratory analyses. T. Holitzki and E. Johnson of the UH Hilo Analytical Lab performed analysis on waste glycerol (supported in part by the National Science Foundation (NSF) award number EPS-0903833). The Connolly Lab at the Daniel K. Inouye College of Pharmacy lent support with the use of their BD C6 Flow Cytometer Plus. L. Muehlstein and A. Anapolsky reviewed an early version of the manuscript. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the NSF. This project was funded by the Hawai‘i Department of Agriculture, Agribusiness Development Corporation and the Office of Naval Research. Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the University of Hawai‘i or the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Abstract

Chlorella protothecoides (Krüger) is ubiquitous as a research organism for biofuels. The genus consists of species that are photosynthetic, heterotrophic, and mixotrophic, and cellular lipid yield can be relatively high compared to other algal species. The research presented here addressed the differences between conventional and novel methods of quantifying lipid yield and cell density of *C. protothecoides* produced on various glycerol media. Conventional methods of light microscopy, gravimetric lipid analysis, and colony forming units, were compared with BODIPY 505/515 lipid-stained cells and NucRed Live 647 nucleic acid stained cells analyzed by flow cytometer. This is the first report of using a dual stain technique to simultaneously quantify cell density and lipid yield of an algal species for biofuel production. Results indicated that there is no significant difference ($p > 0.05$) between BODIPY 505/515 and NucRed Live 647 for cell density. There is no significance between the dyes and hemocytometer counts ($p>0.05$). Lipid analysis indicated no significant difference between flow cytometer lipid per cell and %oil/vol ($p>0.05$), especially for UTEX B25. This indicates that the novel method of utilizing BODIPY 505/515 or NucRed Live 647, singularly or in concert, is comparable to conventional methods of lipid and cell density analyses. Because all three strains of *C. protothecoides* obtained cell densities of between 10^7 and 10^9 cells/mL and highest oil yield was about 45% per cell, waste glycerol has the potential to become a viable feedstock for Hawaii's growing biofuel industry.

Contents

Tables

Figures

1. Introduction

1.1 Importance of biofuel

Sustainable and alternative energies such as solar, wind, wave, and biofuel have been gaining interest in today's global society. Biofuels are among the top subjects researched on the quest for sustainability and microalgae are at the current forefront (Chisti 2007; da Silva et al. 2009; Shekh et al. 2013). Although much research is being conducted to determine sources for more and better alternative fuels (Chen et al., 2009; Minty et al., 2011, Minty et al., 2013), easy and reliable screening methods are few (i.e. Nile Red; Greenspan et al., 1985). Refining algal production techniques as well as developing rapid and easy screening methods for cell densities and lipid yields can be accomplished with a ubiquitous species, such as *Chlorella protothecoides* (Krüger), which is the most commonly used algal species in biofuel research and production.

The State of Hawaii has signed into law a Clean Energy Initiative that states Hawaii will reduce oil dependence by 70% and become 100% energy efficient by 2045 (H.R.S. §196 – 10.3; Hawaii Clean Energy Initiative website 2015). Petroleum supplies 90% of the State's energy, 51% of which is utilized by the transportation sector. In 2012, Hawaii used 277.1 trillion Btu, 9.6 trillion Btu from biomass, and 21.2 trillion Btu from renewable energies (U.S. Energy Information Administration (EIA) website, 2015). Many sectors across the State – including military, agriculture, and residential – have begun implementing alternative energies and alternative energy research, especially for biofuels.

There is an established zero-waste project at the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Daniel K. Inouye Pacific Basin Agriculture Research Center (DKI-PBARC) in Hilo, Hawaii where agricultural waste streams are redirected to produce high value products. Culled papaya were utilized as the model system. Commercial papaya production on Hawaii Island is an $8.2M industry producing in excess of 25M pounds; however, an estimated 35%-50% of the total crop is off-quality fruit that are

unsalable and wasted (USDA-NASS, 2013). The DKI-PBARC zero-waste initiative has successfully converted culled *Carica papaya* (papaya) to bio-oil (Heller et al., 2015). Taking this papaya waste and converting it to oil and downstream products such as animal feed reduces cost of importing feedstock; reduces landfill use; and may reduce pest and disease pressure in the field by encouraging better field sanitation.

1.2 Autotrophic and heterotrophic production

Most of the focus for biofuels production has been on photosynthetic, autotrophic growth. This requires large areas of land for agricultural crops for second-generation fuels or shallow-water ponds and raceways (Chen et al., 2009; Al-Haj 2012). Growth in autotrophic organisms is usually stimulated by sunlight, nutrients, and physical-chemical interactions of temperature, salinity, turbidity, and dissolved oxygen; heterotrophic organism growth can be stimulated by similar parameters, but oil production is usually expected by a reduction in nutrients (Shekh et al. 2013). Some species of algae are mixotrophic, and simultaneously may be photosynthetic and heterotrophic. One such example is *C. protothecoides*, isolated from a freshwater environment (Sheehan et al., 1998).

Heterotrophic algae, when produced with alternative energy sources, can yield more lipid per gram of dry weight compared to autotrophic production (Chen & Walker 2011; Chisti 2007). Heterotrophic and mixotrophic production may not require as many hectares as photosynthetic production because of the utilization of enclosed bioreactors. These bioreactors can be continually fed with culture and harvested without shutting down production units; most units have internal sterilization procedure, which may potentially lead to an overall cost benefit (Vasudevan & Briggs 2008). Open ponds and raceways may require harvesting of the entire production unit; along with the sterilization process, this can lead to additional expenses and loss of production time (Chen et al., 2009; Al-Haj 2012).

In more recent years, there has been a growing interest in heterotrophic microalgae for biofuel production (third-generation fuels; Kun 2012). Heterotrophic microalgae are produced on alternative carbon sources, may be grown in the dark, and can be found in vertical production units rather than open ponds or raceways that are required for photosynthetic microalgae production. There are numerous publications depicting growth and lipid production of heterotrophic microalgae, especially for *Chlorella* species (Chisti 2007; Heredia-Arroyo et al., 2010); however, most carbon sources consist of glucose, urea and others (Sheehan et al., 1998; Hsieh & Wu, 2009; Liang et al., 2009).

1.3 Alternative carbon sources for production of biofuel

Global production of biofuel, mostly biodiesel, has been increasing especially in the European Union, where more than 20 countries now produce more than 22 million metric tons of biofuel annually (European Biodiesel Board, EBB, 2011). In the United States, biofuels research was first explored in the 1950s through the Aquatic Species Program; however, in 1995, the Department of Energy closed down the program and began focusing on bioethanol production (Sheehan et al., 1998). Most, if not all, of the current alternative fuels use agriculture crops [e.g., soy (*Glycine max*), corn (*Zea mays*)] as their source (da Silva et al., 2009), while fewer commercial successes have been documented for microalgae. There are eleven biodiesel fueling stations in the state of Hawaii, produced by Pacific Biodiesel from vegetable oils and animal fats [Pacific Biofuel 2013, 2015]).

Utilizing waste products as carbon sources for microalgal culture has gained popularity and could be considered as the future source for energy (Shen et al., 2008; Lu et al., 2010; Kun 2012). Waste glycerol is a byproduct of the biofuels industry and has been investigated as a carbon feedstock for heterotrophic microalgae production. Studies have shown that *C. protothecoides* can obtain high densities and lipid accumulation on waste glycerol (as high as 57.9% lipid yield per cell) (Chen & Walker 2011; Heredia-Arroyo 2010; Miao et al., 2004; Perez-

Garcia et al., 2011). Although waste glycerol is currently an inexpensive feedstock for heterotrophic algal growth due to the increased production of biofuels worldwide, not all waste glycerol are produced from the same feedstock. Vegetable oils, poultry and other animal fats all contribute to biofuels production and thus each batch of waste glycerol varies in nutrient content (Chen & Walker 2011; Sivakaminathan 2012).

Lipids accumulate in microalgal cells at different rates depending on species and culturing methods. In most species studied, triaceylglycerols (TAGs) are synthesized by the endoplasmic reticulum (ER) and stored in the cytoplasm (Chen et al., 2009). Much of the fatty acids found in algal cells are produced in and for the chloroplast. In their review of biofuel, Chen et al. (2009) summarize the formation of TAGs as involving new fatty acids being synthesized in the chloroplasts and then used in the glycerol backbone and acyl transfers from acyl coenzyme A (CoA) in the ER. Limiting the rate of formation is conversion to malonyl-CoA of acetyl-CoA, producing the 16- or 18-carbon fatty acids. These are then the precursors for TAGs usually through the third position of fatty acids on glycerol-3-phosphate (Chen et al., 2009).

1.4 Culture and harvest methods

Raceways (an outdoor pond constructed to be long, narrow, and shallow), shallow ponds, and photobioreactors (closed reactor unit in which cell growth occurs in sterile media) appear to be the best ways to obtain large quantities of algal biomass (Sheehan et al., 1998; Vasudevan & Briggs 2008). Most production is still laboratory scale due to the high cost of building large-scale production units (Sheehan et al., 1998; Chen et al., 2009). Current designs for photobioreactors may not be cost-effective, but availability would likely increase demand and demonstration-phase potential (Vasudevan & Briggs 2008).

Outdoors, in ponds that are 1,000 m^2, 50 $g/m^2/d$ of algal biomass can be achieved (Sheehan et al., 1998). The energy derived from 200,000 ha is equivalent to 10^{15} Btu (one quadrillion Btu) (Sheehan et al., 1998).

Harvesting microalgal cells efficiently can be a challenge. Separating aqueous media from solid cells via centrifugation can be quite effective, but may leave debris in the pellet which could lead to misinformation about dry weight and oil content. Ultrasound has been investigated as a potential harvesting tool that can recover up to 10^8 cells per mL (Bosma et al., 2003). More recently, cationic starch and chitosan have been gaining the attention of researchers as effective flocculants for fresh water microalgae; efficacies of up to 98% have been reported (Vandamme et al., 2009; Gordo et al., 2014; Liu et al., 2014).

The esterification of fatty acids or the transesterification of oils with short-chain alcohols is used to produce biodiesel from vegetable oils (Vasudevan & Briggs 2008). A solvent is used to extract the lipids: methanol, chloroform-methanol mix, hexane, ethanol, and hexane-ethanol mix are the most widely used. In a review of algae to biofuel, Chen et al. (2009) discuss thermochemical conversion, fermentation of cells directly into ethanol, and anaerobic digestion of biomass to methane, as alternative ways to produce bio-oil.

Methods for analyzing cell density and lipid yield are usually conducted with a microscope, hemocytometer, and gravimetric oil extraction (cell lysis and hexane, ethanol, hexane-ethanol, or chloroform-methanol extraction; Chen et al., 2009). Cell densities can also be observed through utilization of a spectrophotometer or in some instances a spectrofluorometer (Liang et al., 2009). Conventional techniques of quantifying biomass and lipid yield are time consuming and involve many steps. Utilizing a flow cytometer may reduce time and complexity. There is a local source of waste glycerol that may serve as a low-cost feedstock for the zero-waste project at DKI-PBARC. The objectives of this research were to: (i) determine if waste glycerol from Big Island Biodiesel is a viable feedstock for *C. prototheco*ides; (ii) determine the most rapid and reliable method to quantify the concentration of algae cells;

and (iii) develop a dual staining technique to determine cell density and lipid yield utilizing flow cytometry.

2. Material & Methods

2.1 UTEX strains and growth conditions

Three strains of *Chlorella protothecoides* were obtained from the Culture Collection of Algae, University of Texas at Austin, USA: UTEX B25, UTEX 249, and UTEX 256. These strains were axenically maintained on glycerol-plant agar plates and transferred to 50.0 mL molecular grade glycerol plus autolysed yeast liquid media in 250 mL Erlenmeyer flasks (see Table 3, Appendix). After one week of growth, counts via hemocytometer and light microscopy were made to obtain cell density and inoculation rate of new media.

The initial cell concentration for all experiments were 10^5 cells/mL (5 x 10^6 cells in 50mL). The culture pH was measured on days 0, 7, and 14 using 2.0 mL aliquots (pH probe, Fisher Scientific). Batch cultures were maintained on a platform shaker set at 200 rpm (MaxQ 4000, Thermo Scientific) in the dark. On Day 14, a 500 µL sample was taken from each flask, pipetted into a pre-weighed 1.5 mL microcentrifuge tube, and dried overnight (to constant mass) in a drying oven at 80 C. The dry pellet was used to calculate grams of oil per gram dry weight (dwt). Experiments were duplicated.

2.2 Media

Waste glycerol was obtained from Big Island Bio Diesel, LLC (Shipman Industrial Park, Keaau, Hawaii, USA). Samples included the following waste glycerol collected on different days: unrefined waste glycerol 04/04/2013 (UG); refined waste glycerol 01/22/2013 (RG1); and refined waste glycerol 04/04/2013 (RG2). Molecular grade glycerol (MG; Fisher Scientific, Waltham, MD, USA) was used as the positive control. Elemental analysis of UG, RG1 and RG2 was conducted by the University of Hawaii-Hilo Analytical Laboratory. Total organic carbon,

total dissolved nitrogen, Al, B, Cr, Cu, Fe, K, Mg, Mn, Na, Pb, S and Zn were analyzed and compared to other studies (Chen & Walker 2011; Sivakaminathan 2012).

Liquid medium was made in 150 mL batches within one week of each experiment. Bristol salts medium (modified Bristol medium; Bold 1949) was made by adding 1.5 ml of each of the following components to 135 mL deionized water in the following order: $NaNO_3$ (2.94 mM), $CaCl_2 \cdot 2H_2O$ (0.17 mM), $MgSO_4 \cdot 7H_2O$ (0.3 mM), K_2HPO_4 (0.43 mM), KH_2PO_4 (1.29 mM), and NaCl (0.43 mM). Autolysed yeast cells extract (0.525 g of Bacto Yeast Extract, Becton, Dickinson and Company (BD), Sparks, MD, USA) and molecular grade glycerol (5.25 mL) was then added and the flask was placed on a stir-plate with a magnetic stir bar. The media was pH adjusted to between 6.5 and 6.7. The pH-adjusted medium was then divided between three 250 mL Erlenmeyer flasks, covered in doubled aluminum foil, and autoclaved.

Agar medium was made within two days of plating. Bristol salts medium was added to 536 mL deionized water in a 1.0 L Erlenmeyer flask containing 2.1 g yeast extract, 4.5 g plant agar (PhytoTechnology Laboratories, Shawnee Mission, KS, USA), and 21 mL glycerol. The pH was adjusted to between 6.5 and 6.7 and autoclaved. Upon cooling, approximately 20 – 25 mL were poured into Fisherbrand 100mm x 15mm sterile polystyrene plates which were stored at room temperature (21 – 25°C) until use. The cooled agar was labeled according to the media composition: UG (unrefined glycerol); RG1 (refined glycerol 1/22/13); RG2 (refined glycerol 4/4/13); MG (molecular grade glycerol). A second control medium was made following the recipe above minus the glycerol (Bristol and yeast, BY).

2.3 Growth experiments

2.3.1 Production on waste glycerol

Erlenmeyer flasks, in triplicate, containing the following five media types were made: Bristol's salts (BY), unrefined glycerol (UG), refined glycerol 1 (RG1, 1/22/13), refined glycerol 2 (RG2, 4/4/13) and molecular grade glycerol (MG). Each 250 mL flask contained 50 mL medium

and 10^5 cells/mL of UTEX B25, UTEX 249, or UTEX 256; there were no duplicate inoculum (e.g. one BY flask with UTEX B25, one with 249, one with 256); however, this was only a preliminary experiment to determine if *C. prototheocoides* could grow on waste glycerol medium. This experiment was duplicated.

Aliquots of inoculated medium were analyzed with a flow cytometer (Accuri C6, BD, North America). BODIPY 505/515 and NucRed Live 647 ReadyProbes Reagent (both acquired via Life Technologies, Fisher Scientific, Waltham, MD, USA) were used to stain lipid and nucleic material, respectively, according to manufacturer's directions. BODIPY 505/515 excites in the green channel, while NucRed Live 647 excites in the far red channel of the flow cytometer. These subsamples were used for preliminary statistical analyses.

2.3.2 Strain and medium selection

Strains UTEX B25, UTEX 249, and UTEX 256 were grown in BY, UG, RG1, RG2, and MG media, following the above recipes (see section 2.2).

UTEX B25, UTEX 249, and UTEX 256 were grown, in triplicate, in RG1 and MG media, following the above medium recipes. A comparison of UTEX B25 produced in five media types (BY, UG, RG1, RG2, MG) was done.

All flasks were produced in triplicate, inoculated with 10^5 cells/mL in 50mL medium and grown for two weeks. All three experiments were duplicated.

2.4 Comparison of cell count techniques

Established methods of Neubauer chambers (Hoff & Snell 2008; LeGresley & McDermott 2010) and colony forming units were correlated. A next-generation method of flow cytometry and the development of a dual staining technique were compared with the established methods.

2.4.1 Cell density comparisons

Colony forming units, hemocytometer counts, and flow cytometer cell counts were compared within experiments. One-mL aliquots of inoculated medium from every flask were serially diluted for ease of quantification. Cultures were dilution plated and grown on waste glycerol agar plates for 7 days to determine colony forming units (cfu) and compared to hemocytometer counts.

Ten microliters of diluted sample from the above cultures were pipetted on to a Neubauer chamber (hemocytometer), placed under a light microscope, and cells were counted at 10x magnification. Cell densities were calculated using equation 1.

$$\frac{cells}{mL} = \left(\frac{count}{4}\right) * (dilution) * 10{,}000 \qquad \text{Eq. 1}$$

The remaining diluted culture samples were used for flow cytometry (fcm). Triplicate aliquots were mixed for an average cell density and to supply the fcm with the required amount of sample. Following pipetting of 500 µL samples into wells of a 48-well plate, the samples were analyzed automatically by the BD Accuri C6. Four wells were used for each UTEX strain grown in each medium. Samples included: no staining, BODIPY, NucRed, and dual staining. Data were recorded, saved in a template file and exported for FloJo software analyses.

2.4.2 Lipid analyses

Gravimetric lipid analysis was analyzed at Day 14 of growth. Cultures were centrifuged for 20 minutes at 4500 rpm (Sorvall ST 40R, ThermoScientific, Waltham, MD, USA) and the supernatant was discarded. Pellets were made from 13.0 – 14.0 mL aliquots in 15 mL Falcon tubes. Following centrifugation, the pellet was re-suspended in 500 – 1000 µL deionized water

and transferred to 2.0 mL screw-cap tubes. These tubes contained 1.0 g of zirconium beads and the pellet was placed in a Fast-prep (MP Biomedics) for one minute to lyse cells. Approximately 1.0 mL hexane was added to the tubes, tubes were Fast-prepped for an additional minute and centrifuged for 10 minutes at maximum speed. This method was conducted once, and subsequent oil extractions were conducted using 15 mL Falcon tubes. For 15 mL tubes, 5 g of zirconium beads were added to the pelleted media, and deionized water was added up to 7.0 – 7.5 mL mark. Tubes were placed in the Fast-prep for one minute to lyse cells; 5 mL hexane was added to the 12 mL mark. After a second cycle in the Fast-prep, the tubes were centrifuged for 20 – 30 minutes at 4500 rpm. The extraction process was performed twice. The oil-hexane layer that formed was decanted using a Pasteur pipette and placed in a pre-weighed micro-centrifuge tube, or glass vial, then air-dried under a fume hood overnight. The tubes or glass vials were subsequently weighed to calculate oil accumulation per volume. The oil calculation was also used to determine grams of oil per gram dry weight (see section 2.1).

Volumetric lipid analysis (relative units) was achieved via flow cytometry. Diluted samples were stained with BODIPY 505/515 (5 µL dye in 500 µL of sample) in a 48-well plate and allowed to sit for five minutes. The plate was placed on a platform and data were collected automatically. Data were recorded and saved in a template file. The file was exported for further analysis by the FloJo software.

2.5 Statistical analysis

Cell densities and lipid yield from triplicate are presented for each experiment. Student's t-test was used to determine alpha ($p = 0.05$) through the TTEST function in Excel. Linear regression in Excel was conducted for the dual staining technique using the flow cytometer. Two-way ANOVA, one-way ANOVA, paired t-test, Box-Cox transformations, and correlation/regression analyses (R^2 and R^2 adj.) were performed in Minitab 16.

3. Results

3.1 Glycerol stock elemental analysis

Total organic carbon (TOC); total dissolved nitrogen (TDN); and the detected elements of the various waste glycerols used can be found in Table 1. The complete analysis, as well as a comparison with Chen and Walker (2011) and Sivakaminathan (2012) appear in the appendix.

Table 1. Elements found via analysis of waste glycerol, expressed as mg/L. A complete comparison of all 12 elements can be found in the appendix.

Element	UG	RG1	RG2
K	30764.6	30798.05	35646.55
Mg	5.74	6.26	13.00
Na	208.21	847.61	810.15
S	3753.92	18291.4	25017.60
TOC	333150	473550	487850
TDN	124.2	339	1120

3.2 Cell density quantification

3.2.1 Production on waste glycerol

In the first experiment, all three strains of *C. protothecoides* achieved average densities of between $5.49 \times 10^6 - 3.76 \times 10^8$ cells/mL, in all media (data not shown). No cells were observed during trial 1 on day 14 for UTEX 256 in RG2 medium, or for UTEX 249 in MG medium. During trial 2, no cells were observed on days 7 and 14 for all three strains in RG2 medium, and on day 14 no cells were observed for UTEX 249 in UG medium, or for UTEX 256 in RG1, RG2, and MG media. On day 7 of the first trial, densities for UTEX B25, UTEX 249, and UTEX 256 were 5.40×10^7, 1.83×10^7, and 3.57×10^6, respectively (averaged across media). Day

14 densities for UTEX B25, UTEX 249 and UTEX 256 were 5.78 x 10^7, 5.49 x 10^6 and 8.48 x 10^6. Trial 2 densities were 4.87 x 10^7, 8.25 x 10^6, and 1.21 x 10^7 and on day 14 they were 3.76 x 10^8, 2.90 x 10^8, and 6.80 x 10^6. Without the RG2 medium, however, cell densities on day 7 for UTEX B25, UTEX 249, and UTEX 256 were 6.08 x 10^7, 1.03 x 10^7, and 1.51 x 10^7 while on day 14 cell densities were 4.70 x 10^8, 3.62 x 10^8, and 8.50 x 10^6.

The decrease in densities from days 7 to 14 may be an indicator of possibly pipetting error upon inoculation or cultural die-off. Since in some cultures no cells were observed on day 7, pipetting error may be the leading cause. Visual observations were able to ascertain that all three UTEX strains are able to grow on waste glycerol medium. Strains grew differently in different media, but all three strains nearly doubled in density in all media except in RG2.

3.2.2 Strain and medium selection

Cell densities for UTEX B25, 249, and 256 at 14 days were between 10^7 to 10^9 cells/mL (Fig. 1). UTEX B25 and 249 grew to significantly higher densities on RG1 and MG media resulting in an approximately 2 - 4 orders of magnitude increase over 14 days ($p<0.05$). Significantly lower cell growth occurred with UTEX 256 indicating this strain was not able to utilize the local source of waste glycerol as a feedstock. UTEX B25 out-performed the others, and was therefore selected as the best-fit for high density and high lipid yield in waste glycerol. For these reasons, UTEX B25 was chosen for all subsequent experiments. Experiments were repeated with similar results.

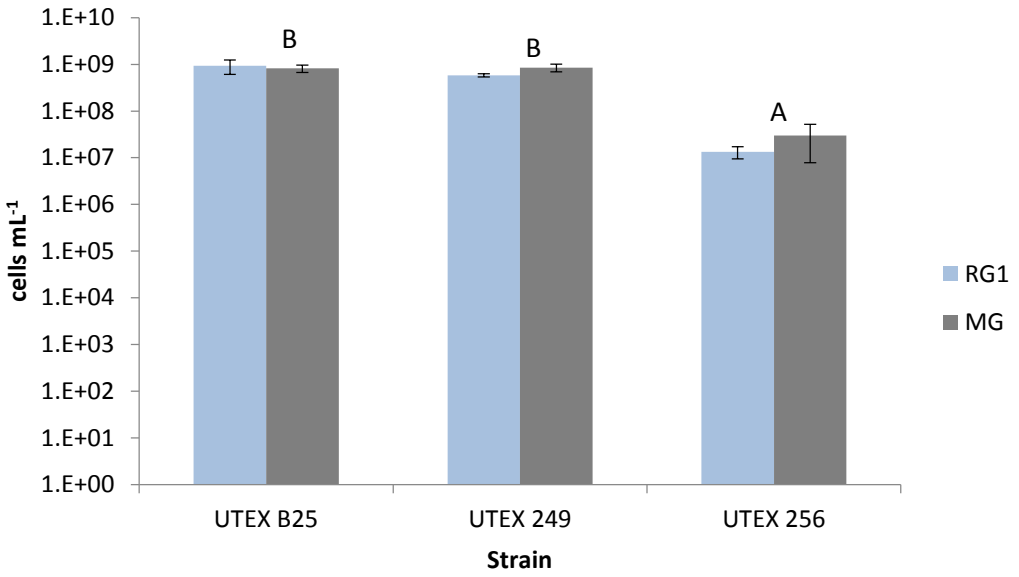


Figure 1. Average cell densities (cells/mL) of *Chlorella protothecoides* on day 14 of growth in RG1 (refined glycerol 1, 1/22/13) and MG (molecular grade glycerol; + control) media. Y-axes log(10) scale; bars represent standard error. One-way ANOVA resulted in no significant difference between medium ($p = 0.87$; $F = 0.03$), while there is a significant difference between UTEX 256 and the other two strains.

Average cell densities for UTEX B25 after 14 days of growth in various glycerol media types are shown in Fig. 2. UTEX B25 grew to significantly higher cell densities in RG1 medium with similar results to the positive control, MG medium. Results indicate that UTEX B25 was also able to utilize the local source of unrefined glycerol as a feedstock. Significantly less growth occurred in RG2. Experiments were repeated with similar results.

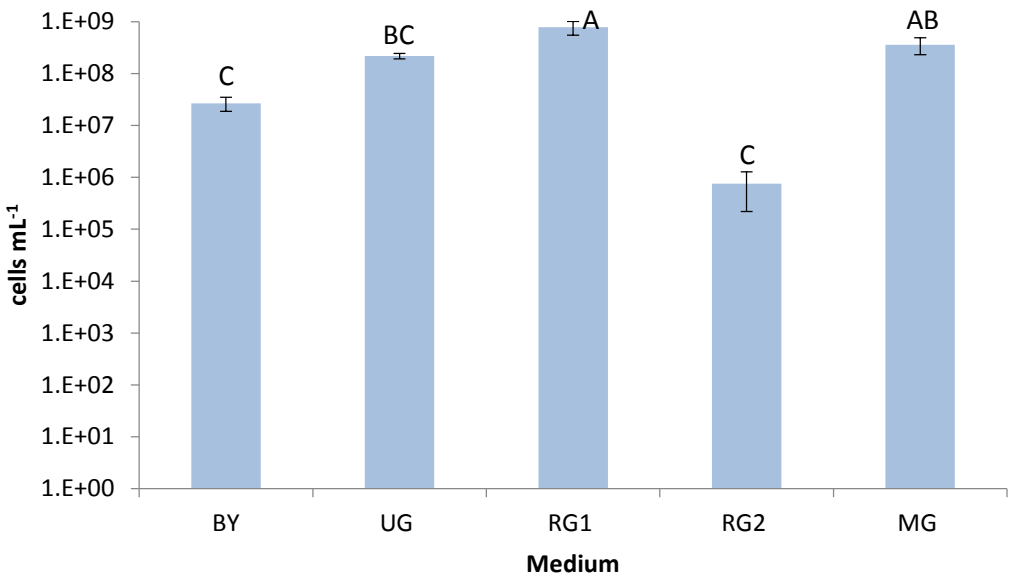


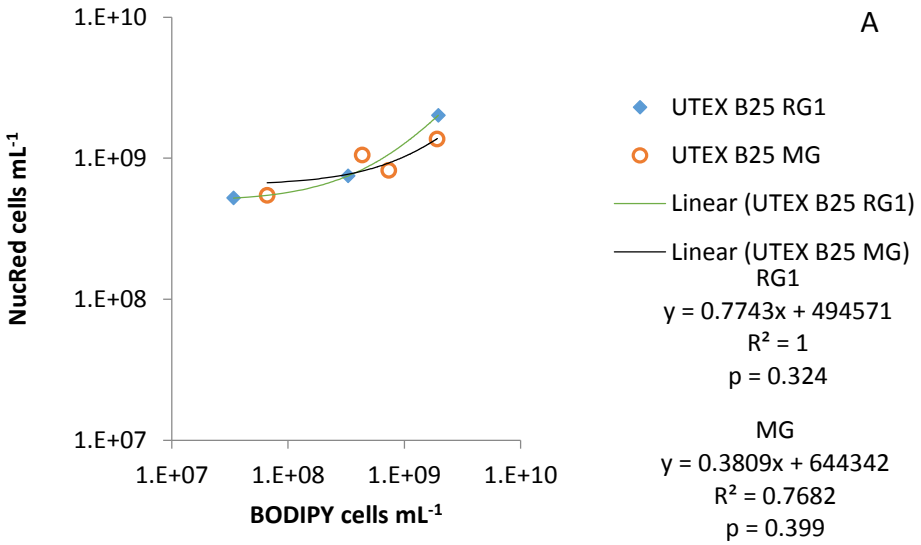
Figure 2. Average cell density per mL with standard error bars for UTEX B25 grown in five media types, on day 14. Letters above bars represent statistical significance of means, as analyzed via one-way ANOVA. BY (Bristol's modified medium; + control), UG (unrefined), RG1 and RG2 (refined glycerol 1, 1/22/13; refined glycerol 2, 4/4/13) and MG (molecular grade; + control) glycerol media types.

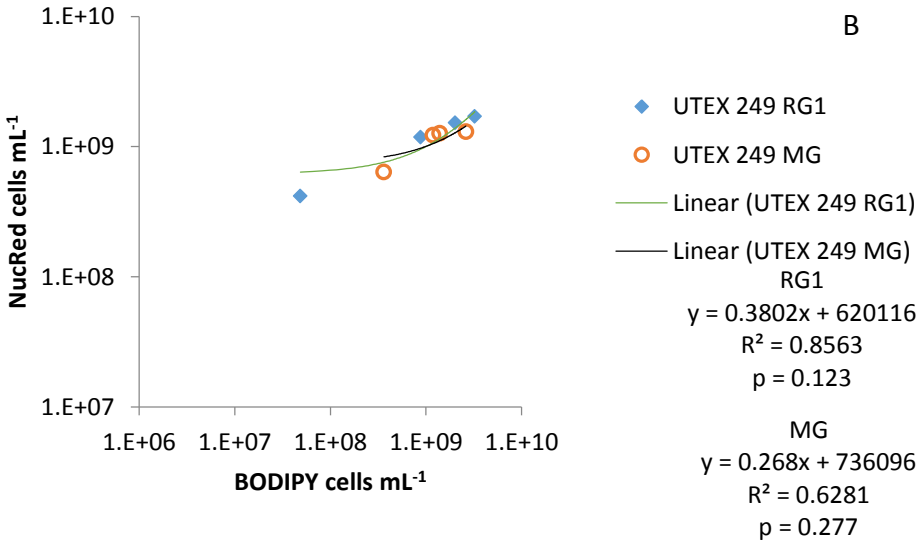
3.4 Comparison of techniques for cell counts and lipid production

After analysis with the flow cytometer, preliminary statistics of the subsamples suggested there was no significant difference between strains across media; however, there was significant difference between media ($p < 0.05$) within the strain. The relationship between NucRed Live 647 and BODIPY 505/515 is shown in Fig. 3. These results are observationally consistent with the hemocytometer counts, as can be seen in Fig. 3D ($p = 0.008$). Cell density determination by BODIPY and NucRed were not significantly different from each other ($p > 0.05$) and not significantly different from dual-staining ($p>0.05$). There was, however, a significant difference between dual staining of UTEX 256 in RG1 medium and NucRed Live 647 ($p = 0.045$). Linear regressions indicate strong correlation (all $R^2 > 0.6$) between NucRed Live 647 and BODIPY 505/515, although the relationships in Fig. 3A and 3B were not significant, likely because of low

sample size. These staining techniques therefore result in a more rapid assessment of cell density than hemocytometer counts, yet are just as accurate.

Results from this preliminary experiment indicate that obtaining cell densities from the flow cytometer, BODIPY 505/515, and NucRed Live 647 is observationally not different from hemocytometer counts. Therefore, further utilization of this method was recommended for the subsequent experiments.





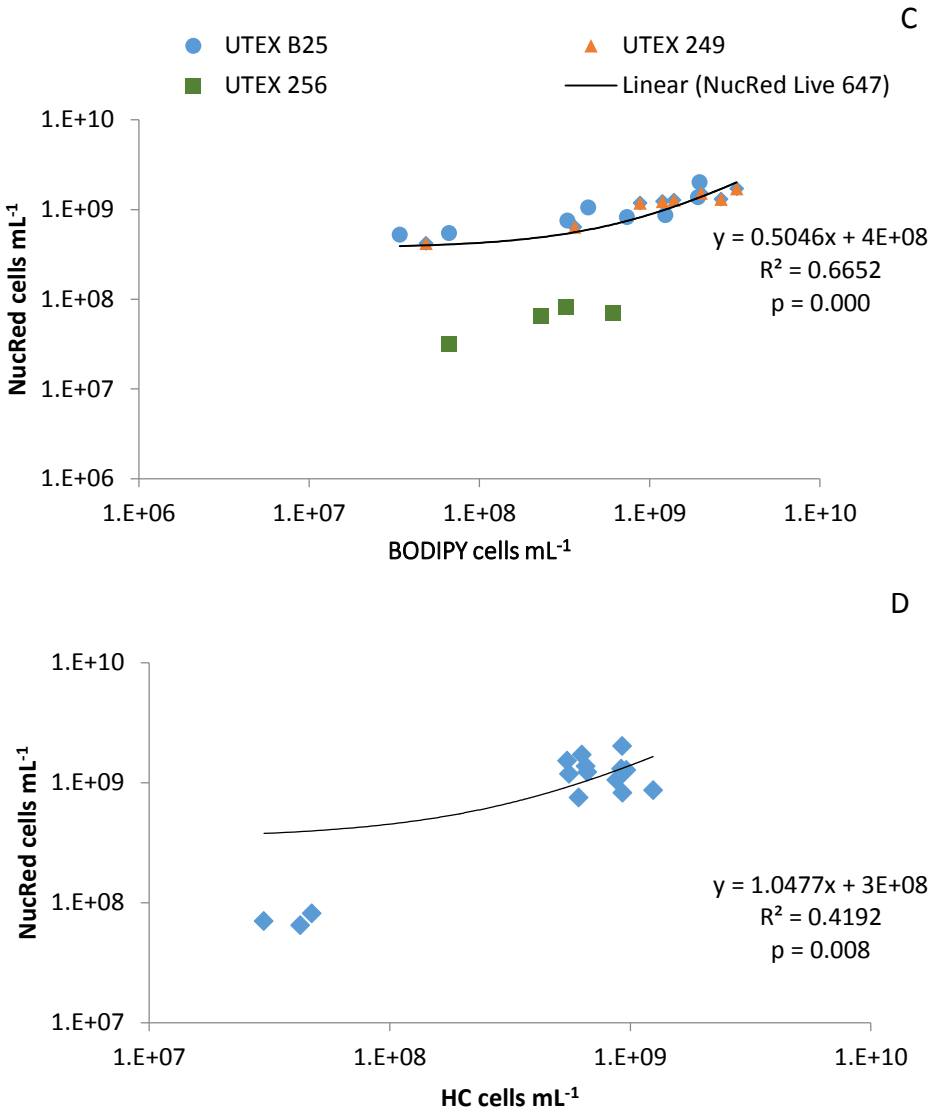
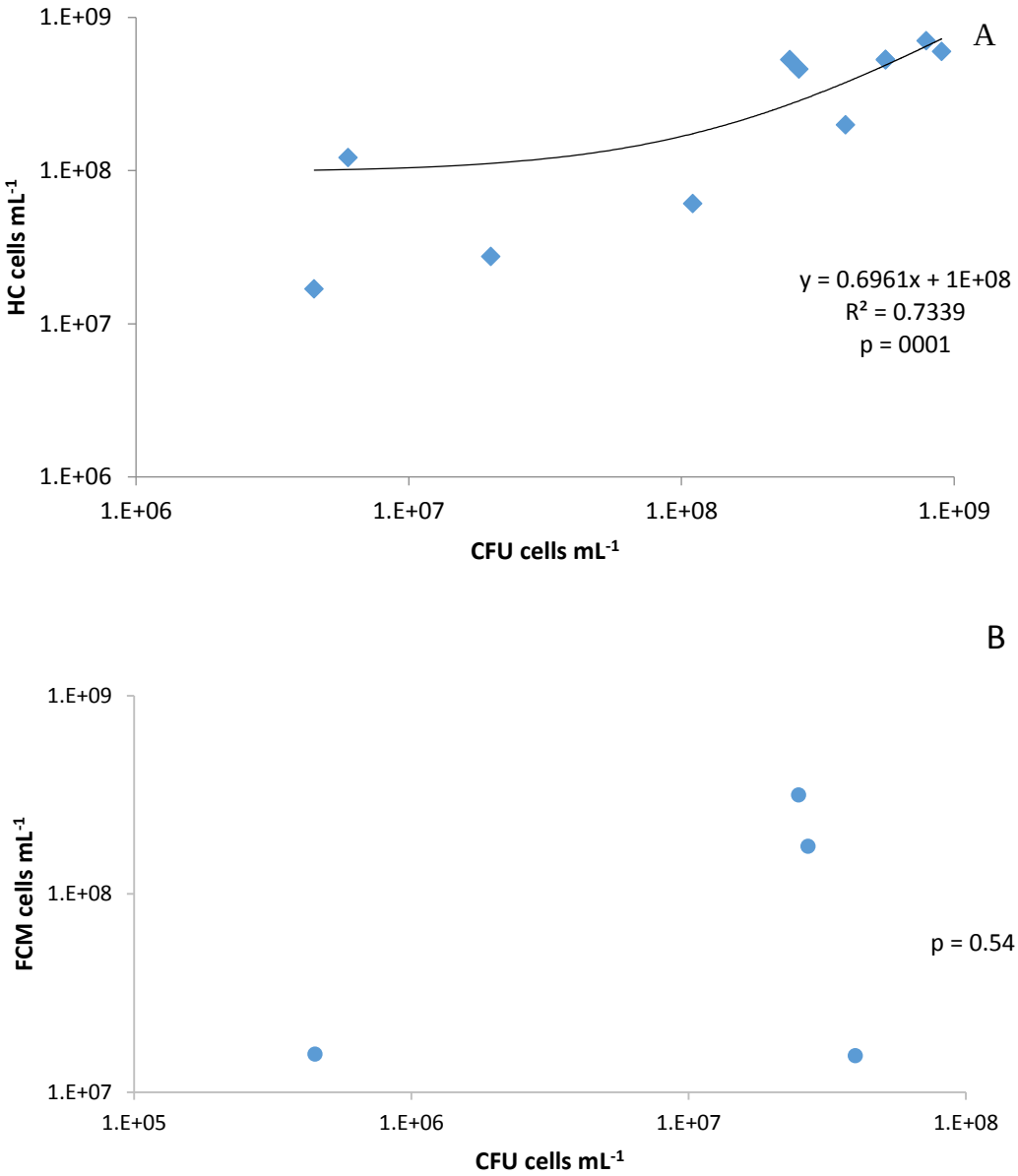


Figure 3. NucRed Live 647 counts as a function of BODIPY 505/515 for (A) UTEX B25. Open circle – MG; linear regression line – black. Filled square – RG1 (1/22/13); linear regression line – green. (B) UTEX 249. Filled squares – RG1 (refined glycerol 1, 1/22/13); linear regression line - green. Open circles – MG (molecular grade glycerol; + control); linear regression line - black. (C) Pooled data with linear regression for UTEX B25 (circles); UTEX 249 (triangles); and UTEX 256 (squares). (D) Pooled data with linear regression for all strains in RG1 and MG media.

3.4.1 Colony forming units

A scatter plot with linear regression of colony forming units (CFU) and hemocytometer counts (HC) is shown for all three strains in Fig. 4A. An R^2 value of 0.73 indicates significant correlation between the two counting methods ($p = 0.001$), indicating that either method can be used to predict cell density. Colony forming units and NucRed Live 647 counts of UTEX B25 on day 14 of growth in five medium types do not have as strong of a correlation ($R^2 = 0.14$, $p = 0.54$)

yet the slope indicates NucRed counts will increase more than two-fold as CFU also increase with a factor of 5 x 10^7 (Fig. 4B). A strong correlation does exist, however, for flow cytometer counts and HC (R^2 = 0.96; Fig. 4C); but as with CFU and HC for all three strains, there would be NucRed counts of 2 x 10^7 cells/mL even if the HC count were zero for that sample.



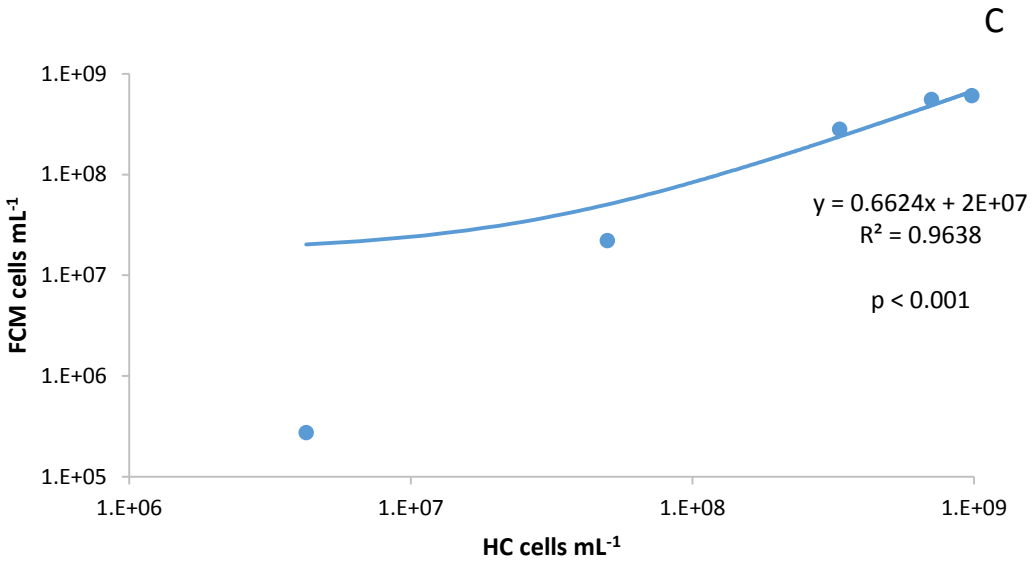


Figure 4. (A) Linear regression of colony forming units and hemocytometer counts for UTEX B25, 249, and 256 in Bristol's (BY, + control), unrefined (UG), refined 1 and 2 (RG1 1/22/13, RG2 4/4/13) and molecular grade (MG, + control) glycerol media. This shows that as cfu counts increase, there is a more likelihood of predicting hc counts (absolute). p = 0.001 indicates statistically significant fit of regression with about 74% of hc variability explained by cfu. (B) UTEX B25 in five media cfu vs flow cytometer (fcm; NucRed Live 647) Four points are shown as cfu for RG2 were zero. No linear regression shown due to poor R^2 (0.14) value and high p-value. (C) UTEX B25 in five media hc vs fcm (NucRed Live 647). All points represent averaged data for each medium at day 14 of culture.

3.4.2 BODIPY 505/515 and NucRed Live 647

There is a very strong correlation between BODIPY 505/515 and NucRed Live 647 cell counts for UTEX B25 ($R^2 > 0.9$; $p > 0.05$; Fig. 5A), suggesting that either can be used for cell density quantification. Cell density counts for UTEX B25 are significantly affected by medium for both dyes ($p < 0.001$), suggesting medium composition influences dye absorbance in these cells. The slope implies that as BODIPY counts increase, NucRed counts will increase at a similar rate.

Similarly, either dye can be used to quantify cell densities for UTEX 249 ($R^2 = 0.76$; Fig 5B). Cell densities for these two strains are not influenced by medium ($p>0.05$), but there is a significant interaction between strains ($p<0.05$). NucRed Live 647 and BODIPY 505/515 therefore appear to be absorbed differently depending on strain, but that does not affect the over-all accuracy of the FCM.

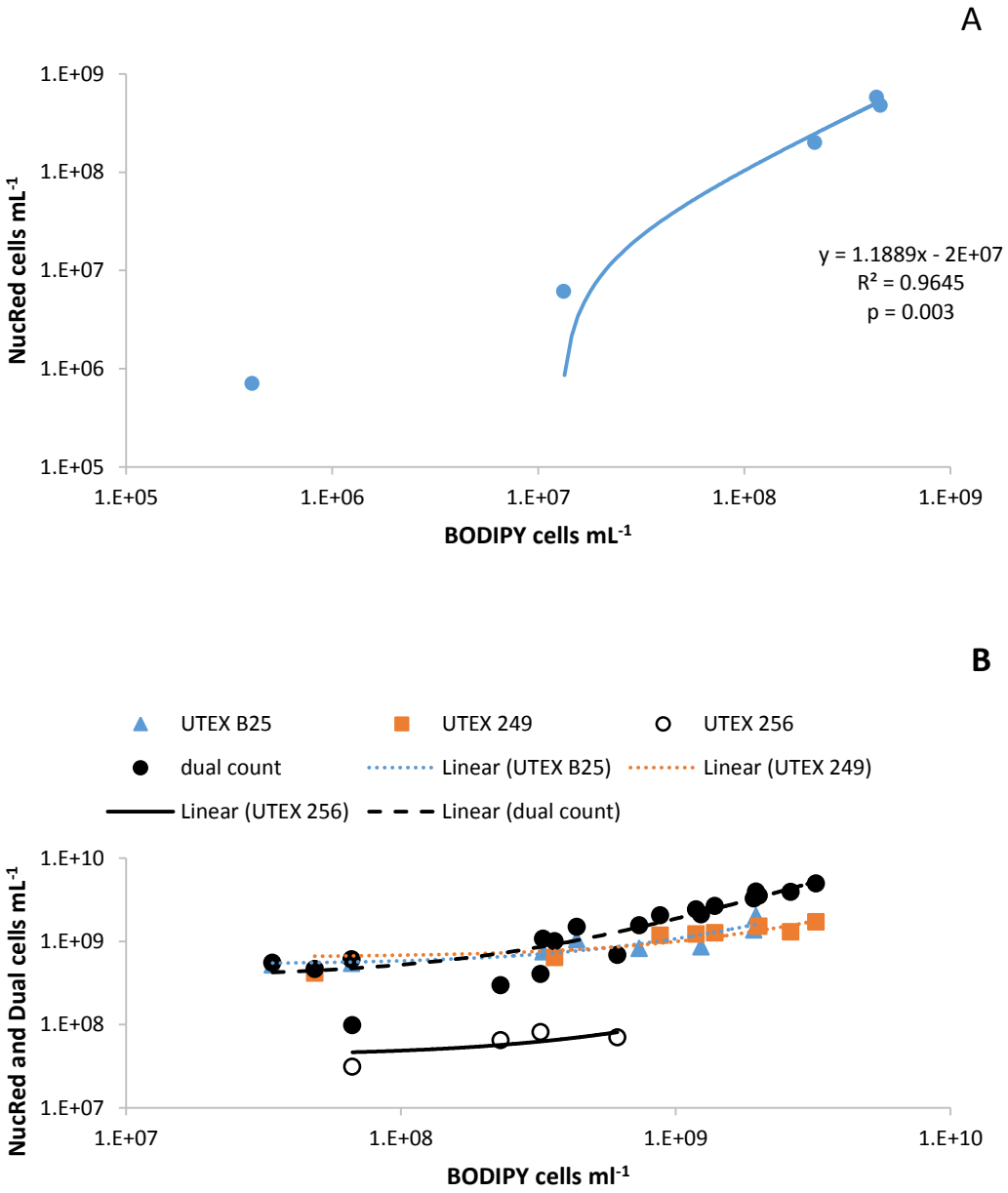
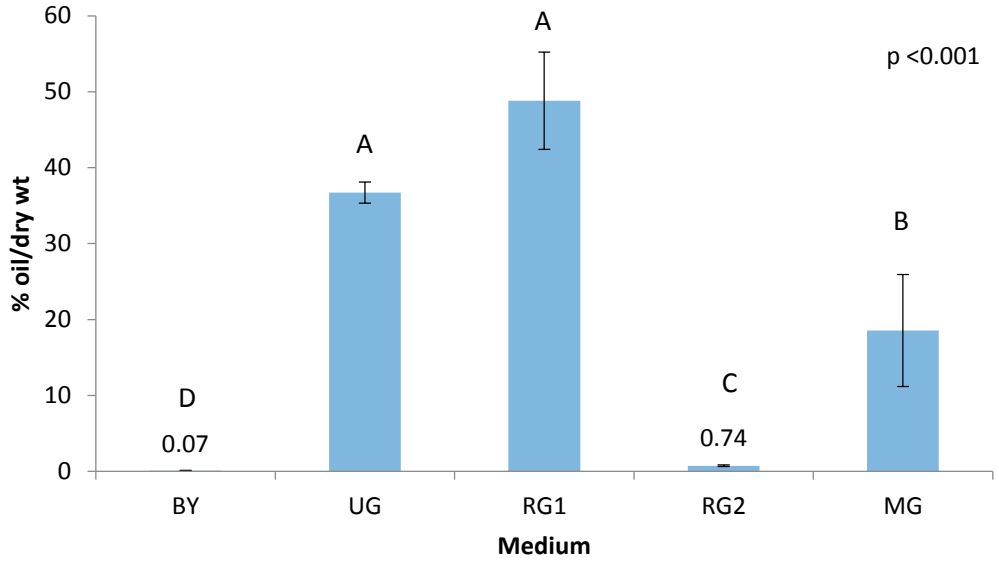


Figure 5. Scatter plot of BODIPY 505/515 cell counts, NucRed Live 647 cell counts, and their summation (dual count/vol) with linear regressions. (A) UTEX B25 on day 14 of culture (B) represents all three UTEX strains on Day 14 of culture. Bristol's (BY, + control), unrefined (UG), refined 1 and 2 (RG1 1/22/13, RG2 4/4/13) and molecular grade (MG, + control). Slope equations and R^2 values are as follows: UTEX B25 (triangles) - $y = 0.5486x + 5 \times 10^8$ and $R^2 = 0.7497$ and $p = .059$; UTEX 249 (squares) $y = 0.3469x + 6 \times 10^8$ and $R^2 = 0.785$ and $p = 0.001$; UTEX 256 (open circles) - $y = 0.0632x + 4 \times 10^7$ and $R^2 = 0.4521$ and $p = 0.955$; dual counts (filled circles) - $y = 1.5046x + 4 \times 10^8$ and $R^2 = 0.9464$ and $p < 0.001$.

3.4.2 Lipid analysis

Per g dry weight, UTEX B25 grown in RG1 medium yielded 0.48 g oil (Fig. 6). UTEX B25 oil extraction yielded 3.0 g oil/L in RG1 medium and 9.3 g oil/L in MG medium (Fig. 7). There are significant differences in oil yield from UTEX B25 utilizing the various glycerol media types. Oil extracted from all three strains is non-significant between RG1 and MG media. These two findings indicate (i) cells grown on RG1 media will have similar oil yields to those produced on positive control medium (99% pure glycerol) and (ii) different media will have different outcomes.



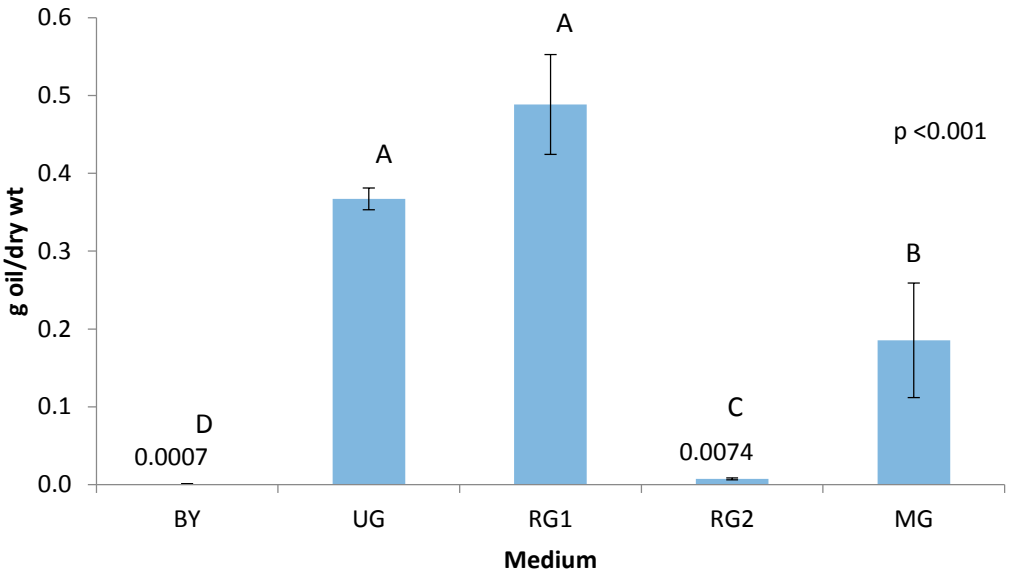


Figure 6. Oil yield expressed as %dry weight and grams of oil per dry weight, with standard error bars for UTEX B25. Letters above bars represent statistical significance of means via one-way ANOVA. Bristol's (BY; positive control), unrefined (UG), refined 1 and 2 (RG1 1/22/13, RG2 4/4/13) and molecular grade (MG; + control).

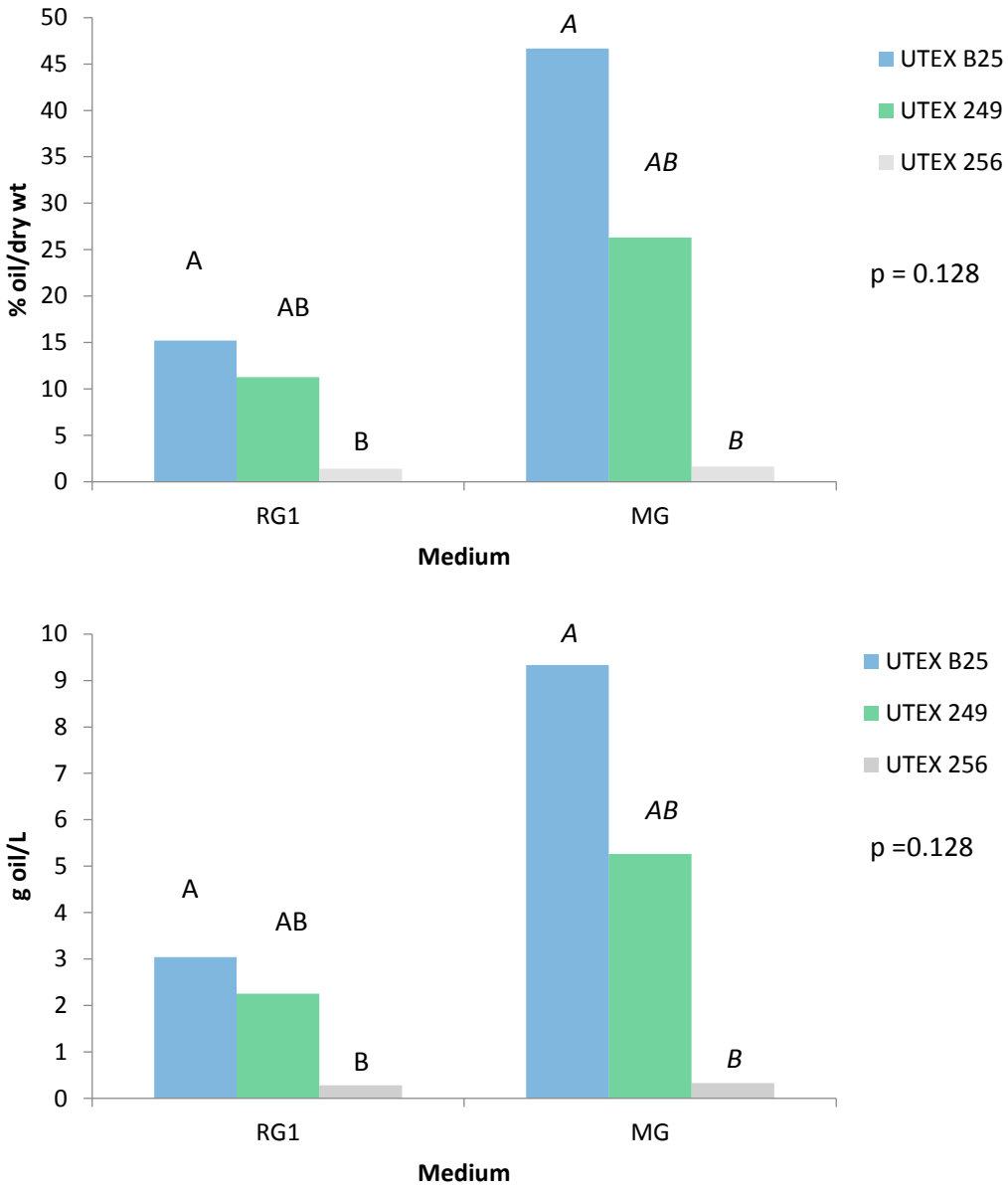


Figure 7. Grams of oil expressed as %dry weight and per L, with for UTEX B25, 249, and 256. Letters above bars represent statistical significance of means between strains (RG1, *MG*), via one-way ANOVA. Refined glycerol 1 (RG1 1/22/13) and molecular grade (MG; + control).

Regression analysis for lipid yield indicates a mild correlation between gravimetric and volumetric lipid yield for UTEX B25 (Fig. 8), but not for the other strains (data not shown). Volumetric lipid yield is a relative unit (RU); as seen by the slope equation, for every increase in RU, grams oil per L will increase at a fraction of RU with only 70% accuracy ($R^2 = 0.70$). Lipid

yields reveal similar trends for both trials of the UTEX B25 experiment (Fig. 6) and for both trials of experiment 3 (Fig. 7). For UTEX B25 experiment trial 1, RG1 and UG media are most likely to affect lipid yield than the other media ($p < 0.001$, $F = 205.75$). Similarly, lipid yields from trial 2 are also more likely to be affected by RG1 and UG media ($p = 0.001$, $F = 11.43$). The RG1 and UG media contain lower concentrations of K, Mg, and S than RG2 medium, and could therefore positively affect lipid yields compared to other media.

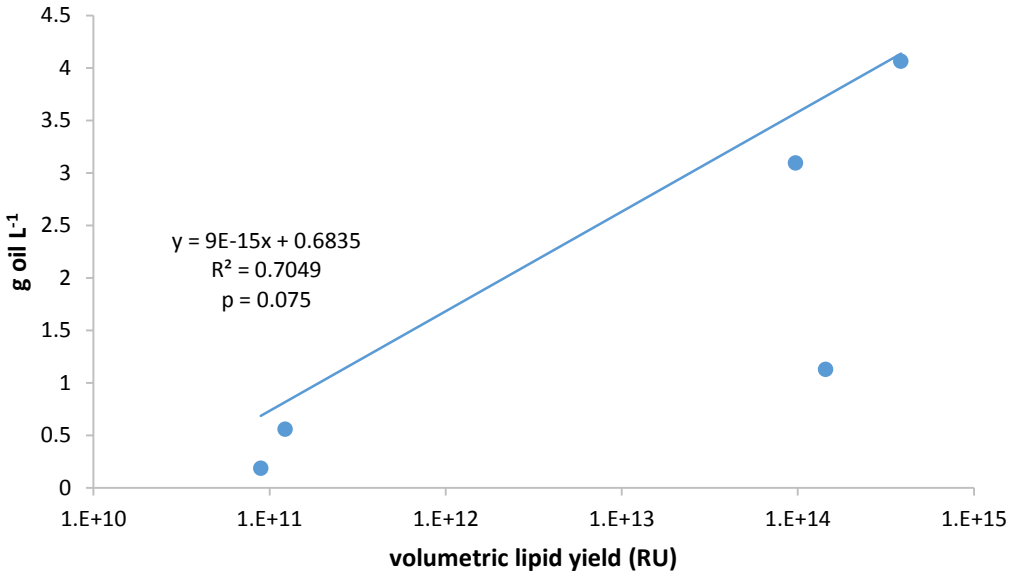


Figure 8. Volumetric lipid yield (relative units; RU) and grams of oil per liter with linear regression for UTEX B25 produced on five media, after 14 days of growth. Trial 2. Bristol's (BY, + control), unrefined (UG), refined 1 and 2 (RG1 1/22/13, RG2 4/4/13) and molecular grade (MG, + control).

One-way ANOVA (Minitab 16) results indicate significant influence of medium composition on flow cytometer counts under no staining, BODIPY 505/515 staining, or NucRed Live 647 staining, with BY and RG2 media being statistically different from the other media (these media also had significantly less cells/mL). One-way ANOVA of volumetric lipid yield also gave similar results; UG, BY, and RG2 are statistically different from RG1 and MG media.

Regression analysis of BODIPY and NucRed for UTEX B25 yielded significant correlation ($p < 0.001$; $R^2 = 0.97$; $F = 475.8$). Oil yield, expressed as g oil/dry wt, for trial 1 of UTEX B25 is significantly influenced by medium ($p<0.001$; $F = 205.75$; CI = 99.18%); RG1 and UG are isolated from MG, RG2, and BY which are statistically different from each other, respectively. For trial 2, RG1 medium is statistically isolated while the other media are grouped together ($p = 0.001$; $F= 11.43$). One-way ANOVA for all three strains reveals no significant influence of medium for trial one ($p = 0.128$; $F = 2.58$), and for trial two ($p = 0.868$; $F= 0.03$).

3.5 Imaging

To visualize the BODIPY 505/515 and NucRed Live 647 stains, aliquots from two cultures in stationary phase were sampled for epifluorescent microscopy and photographed. An image taken with normal light was layered over an image under green excitation to create Fig. 9. The images below were taken with an Olympus epifluorescent microscope and camera. Images A and D were taken under normal light (bright field) conditions; image D was taken after cells had been stained with both dyes. Images B and E were taken under green fluorescence after cells were stained with BODIPY 505/515 to emphasize lipid vacuoles. Image C and inset were taken under red fluorescence after cells had been stained with NucRed Live 647 to emphasize nuclei.

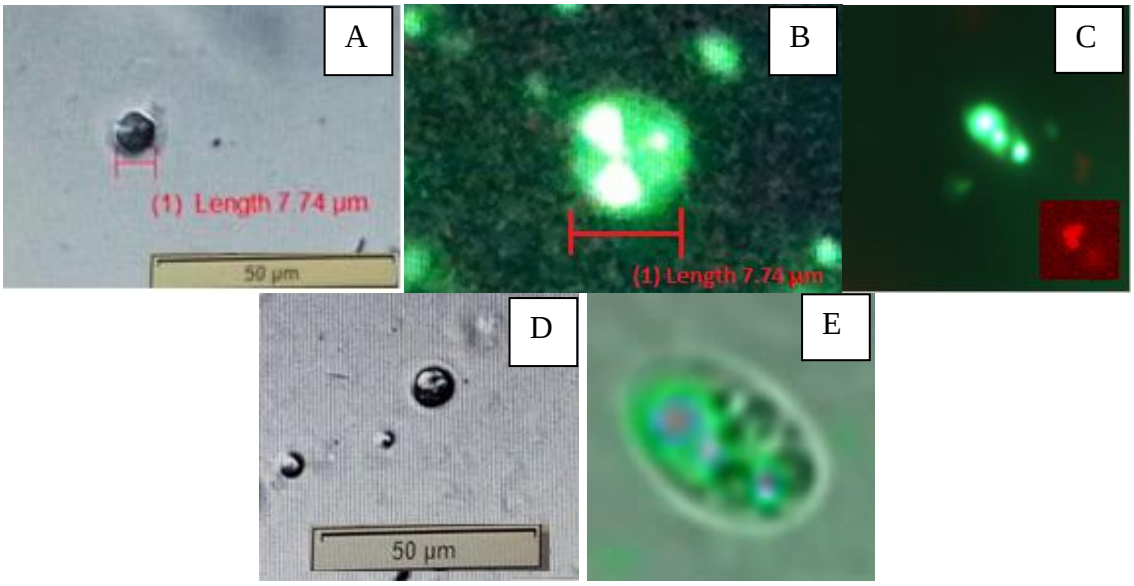


Figure 9. (A) Bright field (normal light); (B) Green channel fluorescence; (C) Red channel fluorescence dual stain (inset – NucRed Live 647 only stained nucleus; not from the same cell); (D) Dual stain under bright field; (E) Green and red fluorescence and normal light overlay images of *Chlorella protothecoides* under an epifluorescent microscope. Cells are approximately 8 microns across (no scale available for C and E).

4. Discussion

4.1 Waste glycerol feedstock

Results presented here indicate that the local supply of waste glycerol is a potentially viable feedstock for producing algae oil which could be converted to biofuels in Hawaii. A local source of waste glycerol is invaluable, as it provides a sustainable feedstock that does not require importation. At least three strains of *C. protothecoides* from the UTEX collection can grow with varying success on waste glycerol from Big Island Biodiesel. It should be noted that because not all waste glycerol contains the same source material (vegetable oil, poultry or animal fat), heterotrophic algae will react differently from batch to batch. Utilizing the three strains of *C. protothecoides* on various batches may be the best solution to providing the most effective oil yield. However, there may also be potential for other heterotrophic or mixotrophic organisms [particularly those capable of high lipid yields such as the cyanobacterium *Botryococcus braunii*

(Shen et al. 2008)] to be utilized for biofuel production on Hawaii Island due to the high carbon and nitrogen content of the BIB waste glycerol.

Other research utilizing *C. protothecoides* produced on waste glycerol have yielded similar conclusions (Chen & Walker, 2011; Sivakaminithan, 2012). Although the waste glycerol origins were different (poultry, enzymatic and vegetable; current study – vegetable), there is evidence that multiple types of waste glycerol can be utilized as feedstock for biofuel production. Compared to Sivakaminathan (2012) and Chen and Walker (2011), the results of the elemental analysis for the current study were very different. Al, B, Cr, Cu, Fe, Mn, Pb, and Zn were not detected in this analysis, but were detected in the other two studies. There were more K and S than in the other two studies, and less Na than in Chen and Walker (2011), but more than in Sivakaminathan (2012). Mg was less in UG and RG1 but more in RG2 than found in Sivakaminathan (2012) and Chen and Walker (2011) for crude glycerol, and much less than in Sivakaminathan for enzymatically derived glycerol. Although Fe may retard algal growth (Hoff & Snell 2008) while Mn increases growth, neither were detected in the Big Island Biodiesel samples. N, P, Mg, Fe, Cu, Mn, Zn, and Mb are required by all algae. Essential to many species are N, P, K, Mg, Ca, S, Fe, Cu, Mn, Zn, Mb, Na, Co, V, Si, Cl, B and I (Hoff & Snell 2008). Of these, P, Ca, Mb, Co, Si, Cl, I, and V were not tested, and B, Zn, Fe, Cu, Mn, and Zn were not detected. Nitrogen was as high as 1120 ppm in RG2, and as low as 124.2 ppm in UG.

UG may be influencing the oil yield as it is unrefined and was the only medium that appeared to contain extraneous material which pelleted when centrifuged. The refined waste glycerol collected on 04/04/2013 performed poorly across the board, while the refined waste glycerol collected on 01/22/2013 performed quite well. The differences are most likely a result of their different compositions.

4.2 Cell density quantification

A striking result from the current research is that there is a significant interaction ($p = 0.032$) of medium and cell density, within a strain. However, medium alone does not cause a significant effect between strains ($p>0.05$). Hemocytometer counts and colony forming units have a very strong correlation with flow cytometer counts; therefore, a flow cytometer can be used for quantifying cell density and be as accurate as the established methods. Counting cells with a hemocytometer or waiting a week or more for colonies to grow on agar is time consuming and may involve human error. Therefore, utilizing a flow cytometer can reduce the time spent and minimize human error while providing accurate results.

The relationship between colony forming units and hemocytometer counts is strong: CFU explain 73% of the variation in HC despite the differences in their orders of magnitude (Fig. 4), indicating either method is reliable for quantifying cell density. Historically, bacteria counted using these two methods gave a similar trend as the results presented here, indicating direct counts as being more accurate for enumerating absolute cell numbers (Pickup 1991). Contamination, improper identification, incubation temperature, and media composition can also be factors in obtaining accurate counts from CFU. It has been suggested, however, that direct counts using a haemocytometer cannot distinguish between dead, viable, and viable but non- culturable cells (Pickup 1991). The use of viability stains such as Trypan blue may aid the user in distinguishing viable from non-viable cells.

4.3 Lipid yield quantification

With an inoculation rate of 10^5 cells per mL, a saturation rate of 10^8 cells can be achieved within seven days. Optical optimum density for culture of cells is near 10^6 cells per mL at between 600 – 700 nm in a laboratory setting (e.g., see Heredia-Arroyo et al., 2010; Phukn et al., 2011). Staining cells with NucRed Live 647 throughout the culture cycle – lag, exponential, and stationary growth phases – can quickly quantify cell densities. BODIPY 505/515 stains lipid

vacuoles; staining cells throughout the culture cycle can aid in distinguishing the point at which cells begin producing lipids in higher quantities (g oil/L, g oil/dry wt, %oil/vol). The BD Accuri C6 flow cytometer data collection software can be set to a user-programmed template so that only the data the user needs is collected.

Volumetric lipid yield is calculated by multiplying lipid per cell (a relative unit expressed as green channel fluorescence) and cells/mL as analyzed via flow cytometer. This is a relative unit (RU) so the capability of deriving a similar result for an absolute value (of grams of oil for example) through regression analysis should be taken conservatively. RU can be misleading when plotted against an absolute value, as is the case with Fig. 8 (see Sec. 3.4.2). Data is needed to provide for a better understanding of this correlation.

Toxicity of certain metals can affect algal growth and reproduction; however, the analysis of the waste glycerol for this study did not detect any of the potential toxic metals. Of the 12 elements analyzed, four were detected (Appendix Table 3). Franklin et al. (2002) studied the combined effects of various doses of Cu, Cd and Zn on a *Chlorella* sp. and used a flow cytometer to detect cell size and fluorescence. Intracellular and extracellular uptake of Cd and Zn were reduced in the presence of Cu; Cu was most inhibiting to cell growth. Cell division was negatively affected by the Cd + Cu interaction. An increase in cell size by 51% occurred with the addition of Cu in a single-metal experiment and by more than 50% in any of the binary combinations of metals (Franklin et al. 2002). Cu and Zn were not detected in the current study; therefore growth inhibition, especially in RG2 medium, was most likely not from Cu toxicity. RG2 medium differed from RG1 medium by date of refining (04/04/2013 and 01/22/2013, respectively) and in elemental composition. Potassium was found to be nearly 5000 ppm more than that found in UG and RG1 media and nearly 10,000 ppm more S.

4.4 Imaging

The relatively large cell sizes of approximately 8.0 μm in diameter may be a result of heterotrophic growth. *Chlorella* produced photosynthetically obtains diameters from 2 – 10 μm, depending on species and growth conditions (Hoff & Snell 2008).

The stains used in the images taken for this study (sec. 3.5, Fig. 9) fluoresce at different wavelengths, yet there may be enough of an overlap at the tail-ends of green and red excitations for a cancellation effect. As seen in Fig. 9C: the cell was dual stained, the image was taken with a red fluorescence filter, yet the BODIPY 505/515 lipid stain appears to excite more than the nucleic acid staining of NucRed Live 647. Time-series data of cell densities, lipid yields, and epifluorescent imaging would lead to a better understanding of the interaction of the two stains.

4.5 Future directions

Biofuels are an important energy source for today's global demands, and third-generation fuels (e.g. derived from microalgae) can meet those demands. Alternative carbon sources such as waste glycerol are becoming cheaper and more widely available for production of third-generation fuels (Chen et al., 2009; Chisti 2007; da Silva 2009). Utilizing these carbon sources has the added benefit of mitigating the rise of atmospheric CO_2, recycling and sequestering carbon which conventional fuels do not provide (Liu et al., 2014).

Further investigation is needed to determine optimal production conditions for *C. protothecoides* high lipid yield and high biomass. Differences in lipid yield and cell density may be attributed to different strains of microalgae, and different waste glycerol compositions. High through-put assays of *C. protothecoides* (and UTEX B25 in particular) lipid yield via flow cytometry rather than gravimetric lipid analysis is beneficial. The novelty of the research presented here is in the comparison of techniques to quantify cell density and lipid yield.

To become independent of conventional fuels, the State of Hawai'i has implemented several laws (H.R.S. §§ 196, 206M, 269, 296, 304A; HRS 2015). The need for renewable, cost-

effective alternative energies plays a large role in the quest for sustainability. The zero-waste program at the DKI-PBARC in Hilo is one that addresses this need by redirecting waste streams to high value added products such as biofuel, animal feed, utilizing low-cost feedstock. The research presented in this study represents one of the many steps taken towards embracing second- and third-generation fuels and encouraging self-sustainability.

References

1. Al-Haj, L.A. 2012. Biofuels from microorganisms. Asia-Pacific Biotech News 16: 30-33.

2. Bold, H.C. 1949. The morphology of *Chlamydomonas chlamydogama*, sp. nov. Bulletin of the Torrey Botanical Club 76:101-8.

3. Chen, P. et al. 2009. Review of the biological and engineering aspects of algae to fuels approach. International Journal of Agricultural and Biological Engineering 2: 1-30.

4. Chen, Y.H. and T.H. Walker. 2011. Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. Biotechnology Letters 33:1973-1983.

5. Chisti, Y. 2007. Biodiesel from microalgae. Biotechnology Advances 25: 294-306.

6. da Silva, G.P., M. Mack, J. Contiero. 2009. Glycerol: a promising and abundant carbon source for industrial microbiology. Biotechnology Advances 27: 30-39.

7. European Biodiesel Board (EBB). 2011. 2010-2011: EU biodiesel industry production forecasts show first decrease in 2011 since data is gathered. Press Release 664/COM/11: 4pp.

8. Franklin, N.M., J.L. Stauber, R.P. Lim, P. Petocz. 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environmental Toxicology and Chemistry 21: 2412-2422.

9. Gordo, C.O.L., S.L. Holdt, D. De Francisci, D.B. Katakashev, I. Angelidaki. 2014. Effective harvesting of the microalgae *Chlorella protothecoides* via bioflocculation with cationic starch. Bioresource Technology 167: 214 – 218.

10. Greenspan, P., E.P. Mayer, S.D. Fowler. 1985. Nile Red: A selective fluorescent stain for intracellular lipid droplets. Journal of Cell Biology 100: 965 – 973.

11. Hawaii Clean Energy Initiative (HCEI). 2015. Available from http://www.hawaiicleanenergyinitiative.org (Accessed July 2015).

12. Hawaii Revised Statutes (H.R.S.). 2014. Title 12 Ch 196. Available from http://www.capitol.hawaii.gov/hrscurrent/Vol03_Ch0121-0200D/HRS0196/ (Accessed March 2014).

13. Hawaii Revised Statutes (H.R.S.). 2015. Hawaii State Legislature homepage. http://www.capitol.hawaii.gov/ (Accessed July 2015).

14. Heller, W.P., K.R. Kissinger, T.K. Matsumoto, L.M. Keith. 2015. Utilization of papaya waste and oil production by *Chlorella protothecoides*. Algal Research 12: 156 – 160.

15. Heredia-Arroyo, T., W. Wei, B. Hu. 2010. Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978-1995.

16. Hoff F.H., T.W. Snell. 2008. Plankton Culture Manual. Sixth edition. Florida Aqua Farms, Inc. Florida, USA.

17. Hsieh, C-H, W-T Wu. 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology 100: 3921 – 3926.

18. Kun, L.Y. 2012. Carbon dioxide bio-mitigation and third generation biofuel: the way forward. Asia-Pacific Biotech News 16: 30-33.

19. LeGresly, M., G. McDermott. 2010. Ch 4 Counting chamber methods for quantitative phytoplankton analysis - haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell. In Microscopic and molecular methods for quantitative phytoplankton analysis. B. Karlson, C. Cusack, E. Bresnan (eds). Intergovernmental Oceanographic Commission of UNESCO. Paris, UNESCO. (IOC Manuals and Guides, no. 55.) (IOC/2010/MG/55) 110 pages.

20. Liang, Y., N. Sarkany, Y. Cui. 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnology Letters 31: 1043-1049.

21. Liu, J., Y. Tao, J. Wu, Y. Zhu, B. Gao, Y. Tang, A. Li, C. Zhang, Y. Zhang. 2014. Effective flocculation of target microalgae with –self-flocculating microalgae induced by pH decrease. Bioresource Technology 167: 367 – 375.

22. Lu, Y., Y. Zhan, M. Liu, Q. Wu. 2010. Biodiesel production from algal oil using cassava (*Manihot esculenta* Crantz) as feedstock. Journal of Applied Phycology 22: 573-578.

23. Miao, X. and Q. Wu. 2004. High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. Journal of Biotechnology 110: 85-93.

24. Minty, J.J., et al. 2011. Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*. Microbial Cell Factories 10: 38pp.

25. Minty, J.J., M.E. Singer, S.A. Scholz, C. Bae, J. Ahn, C.E. Foster, J.C. Liao, X.N. Lin. 2013. Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. PNaS 110: 14592-14597.

26. Pacific Biodiesel. 2013. Where to buy biodiesel. Hawaii. Available from http://www.biodiesel.com/index.php/fuel_sales/where_to_buy_biodiesel (Accessed October 2013).

27. Pacific Biodiesel. 2015. What is biodiesel? Hawaii. Available from http://www.biodiesel.com/biodiesel/what-is-biodiesel/ (Accessed May 2015).

28. Perez-Garcia, O., F.M.E. Escalante, L.E. de-Bashan, Y. Bashan. 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water Research 45: 11-36.

29. Phukn, M.M., R.S. Chutia, B.K. Konwar, R. Kataki. 2011. Microalgae *Chlorella* as a potential bio-energy feedstock. Applied Energy. doi:10.1016/j.apenergy.2010.11.026

30. Pickup, R.W. 1991. Development of molecular methods for the detection of specific bacteria in the environment. Journal of General Microbiology 137: 1009 – 1019.

31. Sheehan, J., T. Dunahay, J. Benemann, P. Roessler. 1998. A look back at the U.S. Department of Energy's Aquatic Species Program – biodiesel from algae. National Renewable Energy Laboratory, Golden, Colorado.

32. Shekh, A.Y., Shrivastava P., Krishnamurthi K., Mudliar S.N., Devi S.S., Kanade G.S., Lokhande S.K., Chakrabarti T. 2013. Stress-induced lipids are unsuitable as a direct biodiesel feedstock: A case study with *Chlorella pyrenoidosa*. Bioresource Technology 138: 382 – 386.

33. Shen, Y., W. Yuan, Z. Pei, E. Mao. 2008. Culture of microalga *Botryococcus* in livestock wastewater. Transactions of the American Society of Agriculture and Biological Engineers 5: 1395-1400.

34. Sivakaminathan, S. 2012. Biomass and lipid production from heterotrophic and mixotrophic fed-batch cultivations of microalgae *Chlorella protothecoides* using glycerol. Master of Science, Biosystems Engineering, Clemson University, USA. 136 pp.

35. USDA-NASS. 2013. http://www.nass.usda.gov

36. Vandamme, D., I. Foubert, B. Meesschaert, K. Muylaert. 2009. Flocculation of microalgae using cationic starch. Journal of Applied Phycology 22: 525-530.

37. Vasudevan, P.T. and M. Briggs. 2008. Biodiesel production – current state of the art and challenges. Journal of Industrial Microbiology and Biotechnology 35: 421-430.

38. Zaslavskaia, L.A., J.C. Lippmeir, C. Shih, D. Ehrhardt, A.R. Grossman, K.E. Apt. 2001. Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. Science 292: 2073-2075.

Appendix

Table 2. Bold's modified Bristol medium (Bold 1949), UTEX proteose medium, and glycerol medium recipes.

#	Component	Amount	Stock Solution Concentration	Final Concentration
	Bold's modified Bristol medium (B)			
1	$NaNO_3$ (Fisher BP360-500)	10 mL/L	10 g/400mL dH2O	2.94 mM
2	$CaCl_2 \cdot 2H_2O$ (Sigma C-3881)	10 mL/L	1 g/400mL dH2O	0.17 mM
3	$MgSO_4 \cdot 7H_2O$ (Sigma 230391)	10 mL/L	3 g/400mL dH2O	0.3 mM
4	K_2HPO_4 (Sigma P 3786)	10 mL/L	3 g/400mL dH2O	0.43 mM
5	KH_2PO_4 (Sigma P 0662)	10 mL/L	7 g/400mL dH2O	1.29 mM
6	NaCl (Fisher S271-500)	10 mL/L	1 g/400mL dH2O	0.43 mM
	UTEX proteose medium			
1	Bristol Medium	1 L		
2	Proteose Peptone (BD 211684)	1 g/L		
	Glycerol medium			
1	B medium			
2	Glycerol	35 g/L		
3	Yeast extract	3.5g/L		

Table 3. Comparison of 12 elements (ppm) found in Big Island Biodiesel (UG, RG1, RG2), Chen and Walker (2011; C&W), and Sivakaminathan (2012; S).

Element	Crude glycerol	Enzymatically derived glycerol (S)	UG 4/4/13	RG1 1/22/13	RG2 4/4/13
Al	14.7 ± 8.81 (C&W) 28.9 ± 2.64 (S)	17 ± 4.4	ND	ND	ND
B	6.4 ± 1.64 (C&W) 12.1 ± 0.14 (S)	10.8 ± 0.27	ND	ND	ND
Cr	0.22 ± 0.11(C&W) 1.2 ± 0.21 (S)	1.1 ± 0.08	ND	ND	ND
Cu	0.74 ± 0.2 (C&W) 1.2 ± 0.1 (S)	1 ± 0.05	ND	ND	ND
Fe	13 ± 3.1(C&W) 17.7 ± 2.93 (S)	20.4 ± 2.41	ND	ND	ND
K	607 ± 72.8(C&W) 15532 ± 49.95 (S)	524.2 ± 33.26	30764.6	30798.05	35646.55
Mg	10.4 ± 0.92(C&W) 6.6 ± 0.09 (S)	40.2 ± 0.13	5.74	6.26	13.00

Table 3 cont'd

Element	Crude glycerol	Enzymatically derived glycerol (S)	UG 4/4/13	RG1 1/22/13	RG2 4/4/13
Mn	0.12 ± 0.03(C&W) 0.5 ± 0.02 (S)	0.5 ± 0.03	ND	ND	ND
Na	8573 ± 960(C&W) 121.9 ± 71.27(S)	111.4 ± 38.76	208.21	847.61	810.15
Pb	1.2 ± 0.66(C&W) ND (S)	ND	ND	ND	ND
S	217 ± 29.4(C&W) 23.6 ± 0.25 (S)	42.2 ± 0.41	3753.92	18291.4	25017.60
Zn	1.8 ± 0.21(C&W) 6.6 ± 0.16 (S)	3 ± 0.19	ND	ND	ND

Elements analyzed by the UH Analytical Laboratory; ND: not detected or below detection limits.