Determination of Algal Cell Lipids Using Nile Red
Using Microplates to Monitor Neutral Lipids in Chlorella Vulgaris

Paul Held Ph. D. and Keri Raymond, Applications Department, BioTek Instruments, Inc., Winooski, VT

Lipid production by algal cultures has become an important area of research in biodiesel and other natural products. The fluorophore Nile red has been shown to be quite useful in detecting neutral lipids in many different microalgae. Here we describe the use of the Synergy™ H4 Multi-Mode Microplate Reader to monitor lipid levels in algal cultures using fluorescence.

Key Words:

Biofuel

Neutral Lipid

Nile Red

Chlorella Vulgaris

Algae

Microplate

Fluorescence

BioTek Instruments, Inc.
P.O. Box 998, Highland Park,
Winooski, Vermont 05404-0998 USA
Tel: 888-451-5171
Outside the USA: 802-655-4740
E-mail: **customercare@biotek.com**
www.biotek.com

Introduction

Microalgal cultures have been used to produce natural products that have been derived into pharmaceuticals [1] and nutraceuticals [2] for a number of years. As a result of global climate change and the ever rising price of transportation fuels, recent interest has focused on the ability of algae to produce lipids that are easily converted to biodiesel. Microalgae are a diverse group of lower plant photosynthetic microorganisms that live in fresh or marine water environments and are able to use solar energy to fix carbon into biomass and synthesize lipids. Like higher plants, microalgae produce both neutral and polar lipids. Neutral lipids are composed primarily of triacylglycerol esters (TAG). Under favorable and unlimited growth conditions microalgae produce primarily polar lipids (e.g. glycolipids and phospholipids), which enrich chloroplast and cellular membranes. However, under unfavorable or restricted growth conditions microalgae accumulate neutral lipids in lipid droplets located in the cytoplasm [4].

Nile red (9-(Diethylamino)-5H benzo [∞] phenoxazin- 5-one) is a red phenoxazone dye present in trace amounts that is responsible for the metachromatic coloring of tissue lipids when staining procedure that use Nile blue are employed [3]. Purified Nile red is a lipid soluble dye that has many characteristics that make it amenable for use as a means to detect neutral lipids. It is relatively photostable, highly fluorescent in non-polar hydrophobic environments, yet very poorly fluorescent in aqueous solutions [5]. This dye has been shown to pass through the cell wall of many different species of algae when dissolved in aqueous/DMSO solutions. Inside algal cells, the dye partitions to cytoplasmic lipid droplets where is becomes fluorescent [7]. The degree to which it fluoresces is an indicator of the amount of neutral lipid present.

Chlorella vulgaris is a single celled green algae species. It is spherical in shape, about 2-10 µM in diameter and lacks flagella. C. vulgaris has chloroplasts containing chlorophyll-a and –b, which provide photosynthetic energy. Under normal growth conditions lipids make up approximately 20% by weight of the dry mass of C. vulgaris [8]. Under certain conditions, such as stress, the oil content can exceed 80% by weight of dry biomass [9].
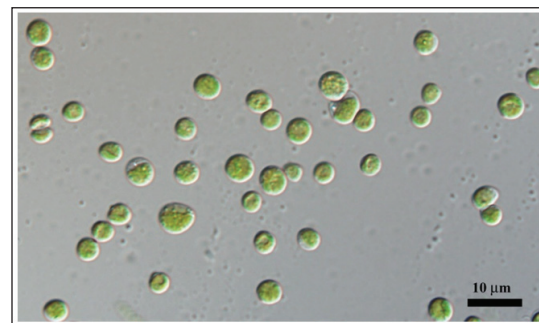

Figure 1. Light Microscopic observation of Chlorella vulgaris algae.

Materials and Methods

Several different algal species including Chlorella vulgaris (2714) were obtained from the UTEX Culture Collection of Algae at the University of Texas at Austin. Cells were grown in TAP and TP media as needed. Ultrapure Nile Red dye (catalog # ENZ-52551 was obtained from Enzo Life Sciences (Farmingdale, NY). Nile red powder was dissolved in DMSO (1 mg/mL) and stored at -20°C. Working 2X (1 µg/mL) solutions were prepared in 50% DMSO. Complete TP and TAP growth media, as well as nitrogen and sulfur deficient variants were made from components as described by Deng et al. [4].

Algal cell cultures were grown over a period of days in sterile 250 mL Erlenmeyer flasks. Suspension cultures were maintained at room temperature and agitated using a rotating shaker rotating at 100 RPM with constant light illumination. Aliquots (100 µL) were removed and pipetted into Corning 3603 black-sided clear bottomed plates. Absorbance and fluorescence measurements were made using a Synergy™ H4 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) using the discontinuous kinetics feature of Gen5™ Data Analysis Software (BioTek Instruments, Winooski, VT) to manage the data.

Chlorella vulgaris cell growth from low cell density was carried out by inoculation of experimental cultures with 100 µL of a stock culture. Cell number and lipid production were monitored daily using light scatter optical density and fluorescence respectively. Lipid production were made by first recording cell number growth using light scatter measurements at 600 nm as described previously [6] followed by the addition of 2X working Nile red solution. Fluorescence was measured after 10 minute incubation at ambient temperature. Microplates containing samples were incubated in the dark in the microplate reading chamber with incubation time tightly controlled by the reader. Fluorescence measurements were made from the top using 530 nm excitation and 570 nm emission wavelengths.

Neutral lipid induction was accomplished by media exchange with deficient media. Cell suspensions from log phase stock cultures were centrifuged at 4000xg. The supernatant was discarded and the cell pellets were resuspended in either nitrogen or sulfur deficient TP or TAP media as needed and the cultures placed in 250 mL flasks. Discontinuous kinetic absorbance and fluorescence measurements were started using the initial reading as a baseline.

Nile red measurements were performed by the addition of equal volume amounts of a 2X working dye solution. Final dye and DMSO concentration for lipid measurements were 0.5 µg/mL and 25% respectively. Nile red dye uptake experiments were performed by adding working dye solution using the dispenser module of the Synergy H4 Multi-Mode Microplate Reader to C. vulgaris cultures induced to produce lipids by culture in nitrogen deficient media. Fluorescence measurements were made every 0.5 seconds for 5 minutes. Longer kinetic uptake kinetics measurements were made every minute for 30 minutes.

Fluorescent spectral scans were performed using C. vulgaris cultures grown in nitrogen deficient TAP media. Excitation scans were from 450 nm to 610 nm in increments of 1 nm using an emission wavelength of 620 nm. Emission spectral scans were from 530 nm to 700 nm in 1 nm increments using an excitation wavelength of 510 nm.

Lipid induction experiments were carried out using several different algal species. Each strain was grown in complete TAP media for 72 hours under light illumination. Cultures were centrifuged and resuspended in nitrogen deficient TAP media. Lipid production in each culture was monitored as described previously for Chlorella vulgaris.

Results

Fluorescent spectral scans of Chlorella vulgaris that has been cultured in nitrogen deficient TP media demonstrates excitation and emission peaks at 534 nm and 589 nm respectively after blank subtraction (Figure 2). An excitation wavelength of 530 nm in conjunction with an emission wavelength of 570 nm were chosen for subsequent measurements in order to maximize Nile red fluorescence while minimizing background chlorophyll fluorescence.
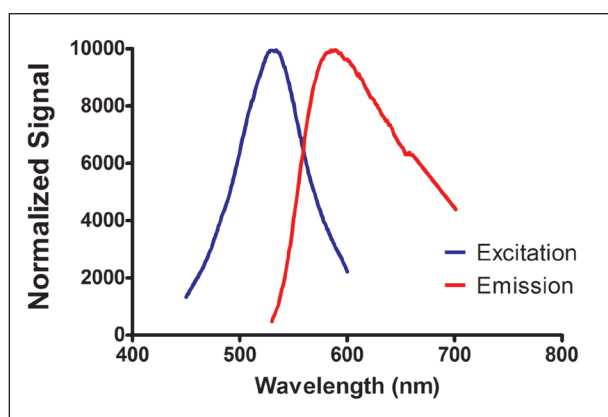

Figure 2. Fluorescent excitation and emission spectrum of C. vulgaris stained with Nile red dye.

Nile red dye incorporation into C. vulgaris cultures is rapid and stable. Maximal fluorescence is reached within 2 minutes and the signal is stable for at least 30 minutes (Figure 3). In the presence of 25% DMSO Nile red rapidly crosses the microalgae cell wall and partitions to the lipid droplets contained in cells induced to form lipid by nitrogen deprivation stress. A small but often repeatable fluorescence peak was occasionally observed within the first 3 or 4 minutes after the addition of dye. An incubation time of 10 minutes was chosen for subsequent studies to allow for complete dye equilibration.
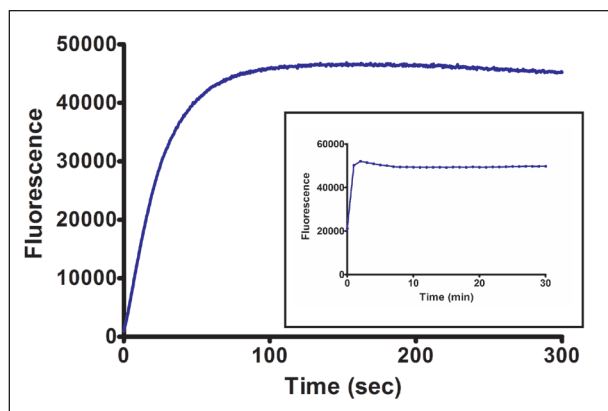

Figure 3. Loading kinetics of Nile Red dye into Chlorella vulgaris algal cultures.

All C. vulgaris cells contain measurable amounts of neutral lipids in the normal growth state (Figure 4). The addition of Nile red dye increasing numbers of C. vulgaris cells results in an increase in measureable fluorescence. The increase in observed fluorescence is not the result of inherent cellular fluorescence (from chlorophyll) as parallel measurements on cultures without Nile red dye had no increase in fluorescence (Figure 4).
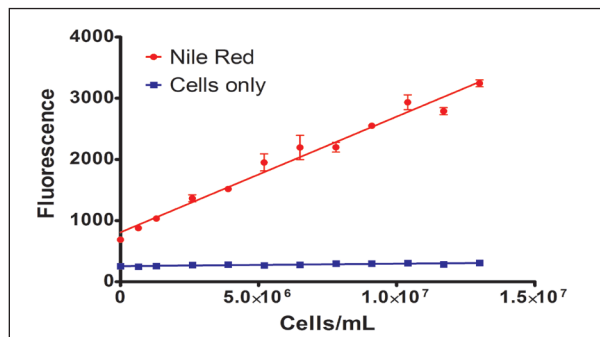

Figure 4. Fluorescence of Nile red stained C. vulgaris cell dilutions.

Reports describing an increase in lipid production as a result of nutritional deficiency prompted us to attempt to grow C. vulgaris cultures in nitrogen or sulfur deficient TP and TAP media. Using 600 nm optical density light scatter measurements, cell numbers for cultures were determined using a previously generated calibration curve [6]. As demonstrated in Figure 5, complete TAP media resulted in the greatest amount of cell growth over a period of 140 hours from cultures seeded at low density. Complete TP media, which lacks the carbon source acetate as compared to TAP media, also resulted in significant cell growth, albeit 50% less than complete TAP media (Figure 5). Interestingly, cell numbers for C. vulgaris cultures seemed to grow more rapidly in TP media, but slowed sooner as nutrients (presumably carbon) became scarce. Media deficient in either nitrogen or sulfur grew at much slower rates than either complete media formulations. No significant difference between the corresponding TP and TAP formulations deficient in either nitrogen or sulfur were observed suggesting that the nutritional deficiency is the rate limiter in either case. Also noted was that sulfur deficiency reduced growth to greater extent than did nitrogen.
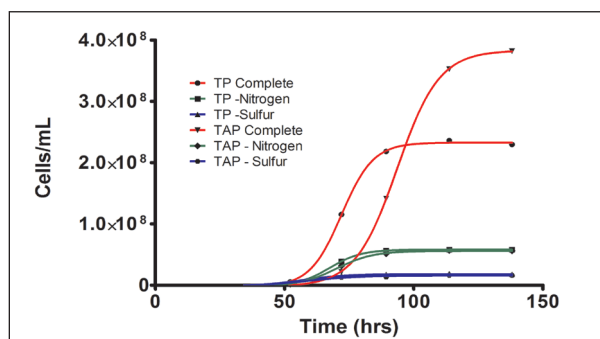

Figure 5. Increase in C. vulgaris cell number over time with complete and deficient media.

When lipid content of the same cultures is examined, neutral lipids are found to be present in all cultures. Nitrogen deficient cultures were found to have over twice the amount of lipid per culture volume as that found in the cultures with complete media, despite the complete media cultures having a 5-8 times greater concentration of cells. Cells grown in sulfur deficient media were also capable of producing significant amounts of lipid despite having substantially fewer cells (Figure 6).
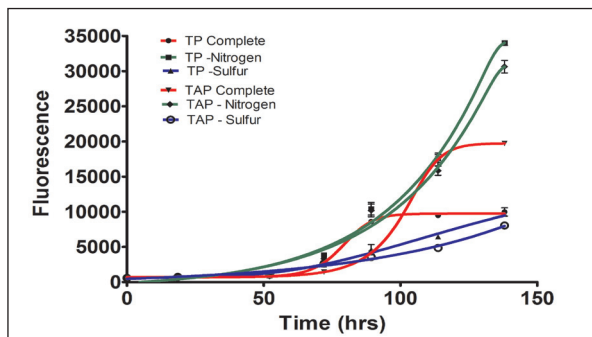

Figure 6. Nile Red fluorescence in C. vulgaris cultures.

When the data is normalized for cell number using 600 nm optical densities, the amount of lipid per cell is consistent between the different media formulations. Growth of cells in nitrogen deficient media results in a nearly 15-fold increase in the amount of lipid per cell, irrespective of the presence of acetate, after 140 hours of exposure. Sulfur deficiency results in a 5-fold increase in cellular lipid content, while complete media does not result in an increase on a per cell basis (Figure 7).
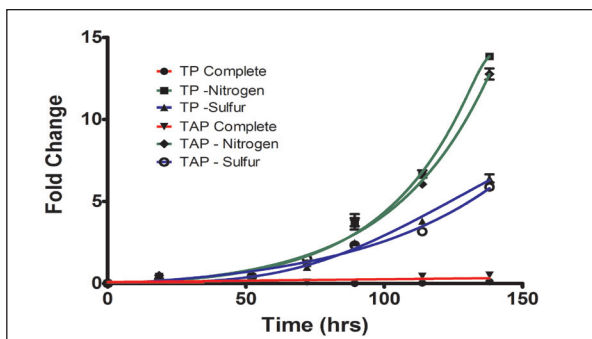

Figure 7. Fold increase in Nile Red fluorescence in C. vulgaris cultures normalized by A600 absorbance.

Optimal algal-cell lipid production is a function of both cell number as well as stress induction. Total lipid content is in part a function of cell number. Complete media cultures, which demonstrate significant amounts of lipid production do so through cell density. Small amounts of lipid in each cell multiplied by large numbers of cells can and does result in measurable amounts of lipids. The differences in total lipid content observed between the TP and TAP complete cultures (Figure 6) is solely explained by differences in cell number.
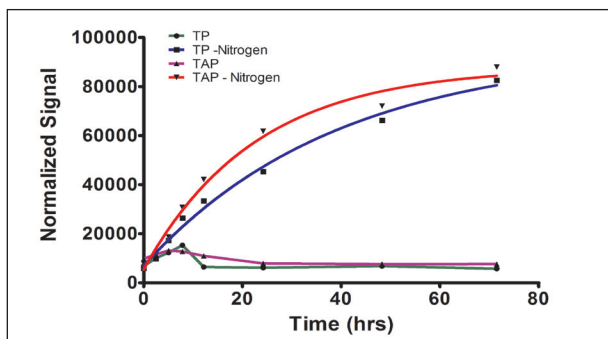
Figure 8. Total lipid fluorescence in C. vulgaris cultures with and without stress induction.

Because growth rates of C. vulgaris cultures in deficient media is very low relative to that observed with complete media, lipid production is inefficient. In order to improve lipid production efficiencies, we sought to grow C. vulgaris algal cultures to high density in complete media then induce lipid production by switching the culture to be nitrogen deficient. As observed in Figure 8, switching large numbers of algal cells to deficient media results in the rapid production of lipids. Interestingly TAP media (complete and nitrogen deficient) offers an advantage towards lipid production as compared to the TP counterpart through an increase in cell numbers. When fluorescence is normalized for optical density at 600 nm both (an indicator of cell number) cells grown in either TP and TAP media deficient for nitrogen show the same amount of fluorescence (Data not shown). Regardless of whether the starting media was TP or the acetate rich TAP; nitrogen deficiency induces 10-fold increase in lipid relative to complete media. This increase is quite rapid, with significant increases in lipids within a few hours (Figure 8).
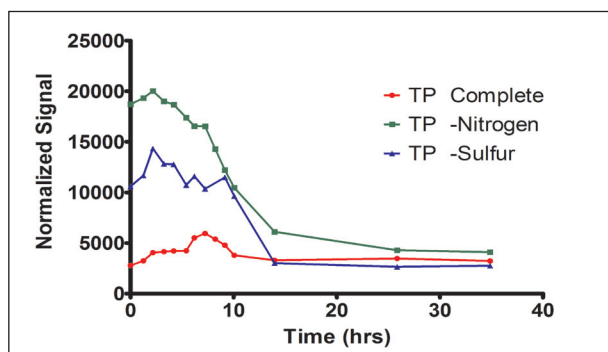

Figure 9. Stress Recovery of C. vulgaris cultures returned to complete medium.

Stress induced lipid production is rapidly reversed when the stress is removed. When C. vulgaris cultures are grown to high density in TP complete media, then induced to produce lipids by either nitrogen or sulfur deprivation are returned back to complete media, cellular lipid levels quickly return to normal. As observed in Figure 9. Nitrogen and sulfur deprived cells rapidly lost Nile red fluorescence after the return to complete media. Interestingly, complete media demonstrates a transient increase in lipid content as a result of the media switch. Removal of cultures from one media type to another involves centrifugation of the cells, removal of the supernatant and the replacement of the media with new. The physical act of centrifugation can be construed as a cellular stress, resulting in an short increase in cellular lipids. The stress seems to be additive as the already induced cultures (nitrogen and sulfur deprived) demonstrate the same phenomenon.
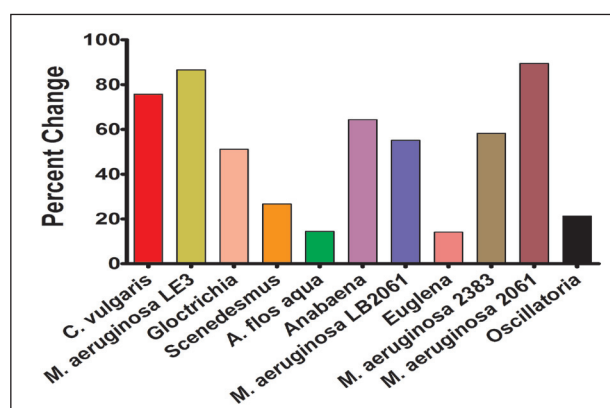

Figure 10. Effect of nitrogen deprivation on various microalgal strains. Data is expressed as the percent change of the A600 normalized Nile red fluorescent signal after 70 hours.

The cellular increase in lipid content is not exclusively unique to Chlorella vulgaris cultures. When several other microalgae strains were tested for lipid production as a result of nitrogen deprivation many different species increased cellular lipids (Figure 10). Not all species tested responded with increases in lipid content. The species Scenedesmus, Oscillatoria, and Aphanizomenon flos aqua had little to no induction of lipid production. Other strains, such as Euglena did not sustain high lipid levels past 48 hours.

Discussion

The production of lipids that can be converted to biofuels has become an important area of research. A number of different pilot studies have shown the capability of manufacturing biodiesel from algal cultures. Biodiesel is the monoalkyl esters of long chain fatty acids, an alternative for fossil fuel. The most common biodiesel constituent used today is fatty acid methyl ester, which can be obtained from the triglyceride lipid droplets by glycerolysis and transesterification.

The conditions where lipid is optimally produced is a key element in the process of biodiesel can be produced. These data presented demonstrate the utility of microplate based measurements in regards to algal lipid testing. The ability to quickly and easily measure small amounts of large numbers of experimental cultures is an important advantage that microplates have over tube or cuvette based instrumentation.

In regards to algal lipid production as a means to produce an alternative fuel source, the total amount of neutral lipid per culture volume is the most important parameter. Achieving large amounts of lipid can be accomplished in a number of different ways. Uninduced high cell density cultures can provide significant amounts of lipid, as demonstrated by Figure 5. The growth of algal cells from low density using complete media results in significant amounts of total lipid due to the high density of cells. The advantage of this method is that cultures need not be manipulated in any fashion; they need only to be grown to high density and then harvested. However, extracting the entire amount of cellular lipid may be difficult. Growth of cells in nitrogen deficient media results in significantly more neutral lipid on a per cell basis and despite significantly fewer cells, more total lipid per unit of culture volume than growth in complete media. This method, like growth in complete media does not require any manipulation, but cell growth rates are significantly slower.

Regulation of lipid content as a result of nutrient deficiency is tightly controlled. Upon induction, increased amount of neutral lipid are observed within a few hours of the cultures being placed into deficient media. Likewise when the stress has been removed lipids levels quickly return to normal. Induction of lipid production through stress of an algal culture grown in complete media enables the rapid growth of algal cultures to high density prior to the induction of lipid production. Stress induced lipid production occurs quite rapidly (24-48 hours) and results in significantly more total lipid than growth from low density in deficient media.

The choice of algal species can have a tremendous impact in regards to lipid production. We have demonstrated that not all algal species can be induced to produce significant amounts of neutral lipid by nitrogen deficiency stress. Some species that can be induced to produce lipid will not retain their lipid for significant periods of time. In addition, many microalgal cultures do not grow as discrete single cells in suspension, but rather as flocculated masses of cells.

The Synergy™ H4 Multi-Mode Microplate Reader has the ability to measure both fluorescence and absorbance during the same read step. This allows the researcher to ascertain algal cell numbers prior to determining lipid content on the same sample at the same time.

The dual measurement of cell number and total lipid content enables a lipid per cell calculation. The use of single-mode readers would require separate determinations on different measurement devices. Researchers with only a fluorometer at their disposal have used fluorescent measurements at 530 excitation and 570 emission wavelengths prior to the addition of Nile red dye as an estimator of cell number. In our hands the signal response of this method was found to be poor. Use of light scatter optical density measurements provides much better resolution in regards to cell number quantitation.

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