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# Screening of Florida Native Green Microalgae as a Potential Source of Biodiesel

## Research Article

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**Abstract**

Twenty seven strains of green algae from the Florida aquatic habitats were screened for their ability to accumulate lipids. In addition, *Botryococcus braunii* was used as the reference strain. This preliminary screening for lipid content was performed with laboratory grown algal cultures using the fluorescent dye Nile red. The lipid accumulation was followed during the course of cultivation as well as under conditions of nitrogen and phosphorus deprivation. Of all strains tested, an increased lipid accumulation was found in five strains (*Coelastrum* sp. strain 46-4 and strain 108-5, *Chlorococcum* sp. strain64-12, *Dactylococcus* sp. strain 64-10 and *Stigeoclonium* sp. strain 64-8) and they were used for further studies. A linear relationship between algal biomass yield (culture age) and lipid accumulation was observed in most strains tested (*Coelastrum* sp. strain 46-4 and strain 108-5, *Chlorococcum* sp. strain 64-12, and *Stigeoclonium* sp. strain 64-8). Significantly higher lipid accumulation under nitrogen deprivation was observed in only two strains while five strains had elevated lipid accumulation under phosphorus deprivation. Apparently, nutrient deprivation conditions affect different strains differently. Three strains contained more than 50% of lipid of dry biomass (*Coelastrum* 108-5, *Chlorococcum* 64-12 and *Stigeoclonium* 64-8) and therefore were identified as potential organisms to be used in algae-based biodiesel technology.

**Keywords**: Biofuel; Green algae; Lipids; Nile red

## Introduction

The need for renewable energy sources is increasing rapidly as result of rising global population and industrialization. Diminishing of fossil fuel reserves, and increasing concerns about energy security and climate change have led to an increased interest in biofuel research [1]. One form of biofuels, which is currently receiving much attention, is biodiesel. Current sources of commercial biodiesel include soybean and palm oil, animal fat and cooking oil waste [2]. The advantage of using biodiesel is that it is a renewable fuel, non-toxic and biodegradable [3]. However, despite the benefits that biodiesel commercialization can provide, the economic aspect of its development at large-scale is limited by the high cost of vegetable oil [4], resulting in a search for alternative sources of feedstock.

Microalgae are a promising alternative sources for biodiesel [5,6]. The algae-for-fuel concept started in the 1970s [7] and has recently been re-invigorated with steady increases in energy prices [2]. Using algae for biofuels have several advantages over conventional crops including high growth rates and oil production per area unit [8,9] and they can be grown in regions of non-arable land, thereby avoiding competition with food crops [10]. Algae also use less water than the traditional oilseed crops and contribute to sequestration of the greenhouse gas carbon-dioxide ($CO_2$) [11,12].

Different kind of lipids, hydrocarbons and other oils are produced by different algal species [13]. Some algae can accumulate up to 80% of lipids by weight of dry biomass [14], however the suitability of lipids for fuel conversion depends on species characteristics [1].

In order to develop a technology of producing biodiesel from algae, a selection of a productive organism adapted to local conditions is crucial. Since green algae are ubiquitous and easy to grow they represent the major groups of algae within lipid-producing species [2]. Florida is considered a suitable location for mass cultivation of algae and therefore screening of native green algal strains for their ability to accumulate lipids has great potential. In this work the cell

lipid accumulation was determined in cultures of different strains of green algae under varying culture conditions.

## Materials and Methods

### Organisms and culture conditions

Twenty-seven green algal strains (Chlorophyta) that are part of a culture collection at Florida International University were used in the study (Table 1). Taxonomic identification of the strains to the genus level was based on morphological characteristics [15]. As a reference strain, we used *Botryococcus braunii*, which was purchased from University of Texas, Austin. Algal cultures were grown in BG11 medium [16] which was buffered with MES buffer(Sigma-Aldrich) at pH 7.20.The cultivation was carried out in laboratory conditions on a shaker with agitation speed of 150 rpm under cool white light (30 μE $m^{-2} s^{-1}$) and temperature of 24°C.

### Growth rate

The growth rate determination was based on dry weight and chlorophyll measurements. For the dry weight measurements, five milliliters (three replicas) of samples were filtered onto pre-weighed GF/C (Whatman) filter papers. Filters were dried in an oven at 60°C to a constant weight and dry weight determined. In the time course experiment (Figure 1) the growth rate was determined using changes in chlorophyll content. Five milliliters of culture were centrifuged and the pellet was extracted with methanol and the absorbance measured at 666nm and 653nm [17]. The samples were taken every five days.

### Screening for lipid content

Screening the algal strains for lipid content was based on the Nile red method [18,19]. Algae cultures were grown in 250 ml Erlenmeyer flasks in BG11 medium on a shaker at 25°C under constant light ($30\mu Em^{-2}s^{-1}$). Samples were taken on the 13th and 45th day of cultivation. Sample of every culture was normalized by dilution to an optical density (OD600) of 0.2. One milliliter of a sample (three replicates) was transferred into eppendorf tube, homogenized by vortexing, and then DMSO was added (final concentration 20%) to improve penetration of the dye. To this cell suspension, the Nile red solution (6.5 mg of Nile red in 26 ml acetone) [20] was added to final concentration of 1% (v/v). This mixture was incubated in the dark for 10 min and transferred into 96-well plates. The plates were read by using the excitation wavelength of 530 nm and emission wavelength at 575 nm [21] on a plate reader Synergy 2 (BioTek, Winooski, VT). The relative fluorescence intensity of Nile red in the stained cells was obtained after subtraction of both the autofluorescence of algal cells (cells without Nile red) and the fluorescence of Nile red alone in the medium [20]. These values were normalized by dividing them by the absorbance values measured at a wavelength of 600nm. In the case of the primary screening (Table 1) the intensity of Nile red fluorescence was further translated into lipid content per 100 μl of the culture by using standard curve generated with Triolein as a lipid standard (Fischer Scientific, USA).In other experiments the Nile red fluorescence was translated into lipid amount calculated as a percentage of the dry weight.

Triolein standard curve was used in the determination of lipid content. One milliliter of triolein which weighed 0.9194g was dissolved in 9ml of chloroform and diluted to $10^{-4}$. The concentration of the lipid was brought to 90 μg and further diluted in chloroform in order to obtain a concentration in the range of 5 to 50 μg $ml^{-1}$ with the total working standard volume of 3ml. To 3ml of solution, 300 ml of chloroform and 150 ml of methanol was added. Also, 7 μl of Nile red was added and boiled for 1- 1.5 min and allowed to cool down to room temperature [22]. Fluorescence was read using the plate reader at 530nm and 575 nm respectively. The blank in this case was chloroform, methanol and Nile red. The calibration curve was constructed with the fluorescence intensity against the lipid concentration.

### The effect of nitrogen and phosphorus depletion

Those strains showing the highest lipid content were cultured under varying nitrogen and phosphorous concentrations to provide information on how different strains respond to nitrogen and phosphorus limitation. Three-week old cultures grown in BG11 medium were centrifuged and the biomass washed with the nitrogen-free or phosphorus-free medium. This biomass was used to inoculate media containing 0%, 50% and 100% of a standard nitrogen and phosphorus content in BG11 medium. The lipid content was determined using the Nile red method for samples taken after 5 and 10 days of growth. Nile red fluorescence was translated into percent lipid per unit cell dry weight.

### Statistical analysis

All statistical analysis were conducted using SPSS 15.0 software. In this study, it was hypothesized that the lipid content determined by both gravimetric technique and Nile red method were the same. In order to test our hypothesis, Independent sample T test was used by comparing the means. It was also hypothesized that nitrogen and

**Table 1:** Sites from which the strains were isolated.

| Site | Strain |
|---|---|
| Florida Everglades, Shark Valley – periphyton | *Chlamydomonas*, EV-29; *Chlorella* 2-4; *Chlorococcum;* 5-1; *Scenedesmus* 3-11; *Scenedesmus* 66-1; *Scenedesmus* 79-1; *Scenedesmus* 80-15; *Scenedesmus* 81-5; *Scenedesmus* 103-4; *Selenastrum* 2-7 |
| St Johns River, Florida - water | *Chlorella* 71-4; *Planktospaeria* 56-4; *Chlorococcum* 56-5 |
| Lake Howard, Florida - water | *Chlorococcum* 45-3; *Coelastrum* 46-4; *Coelastrum* 108-5; *Pediastrum* 108-4 |
| Doctor's Lake, Florida - water | *Chlorococcum* 55-2; *Chlorococcum* 55-5; *Chlorococcum* 64-12; *Dactylococcus* 64-10; *Eremosphera* 103-6; *Stigeoclonium* 64-8; *Pediastrum* 81-6; *Planktospaeria* 81-7; *Kirchneriella* 104-7 |
| Canal C-111, South Florida | *Selenastrum* 34-4 |



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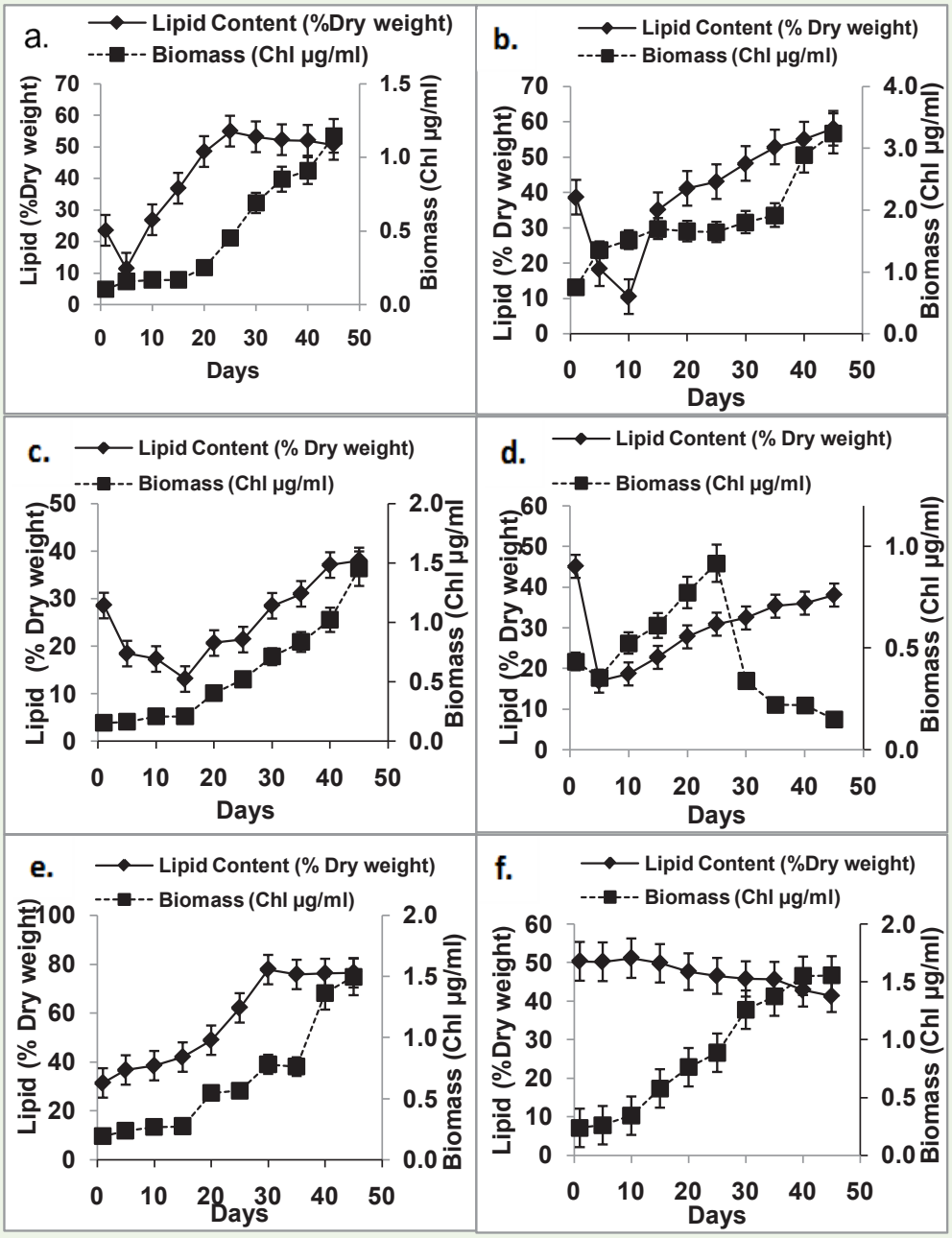


**Figure 1:** Biomass growth and lipid accumulation over time. Lipid content was estimated by Nile red fluorescence and calculated as percent per dry weight. (a. *Chlorococum* sp. 64-12; b. *Coelastrum* sp. 46-4; c. *Stigeoclonium* sp. 64-8; d. *Dactylococcus* sp. 64-10; e.*Coelastrum* sp. 108-5; f. *Botryococcus braunii*).

phosphorus depletion would increase lipid levels in selected strains. The significance of differences were tested using one way ANOVA. Comparisons that show p-values< 0.05 were considered significant.

## Results

### Screening the isolates by Nile red method

The initial screening was performed with 27 algal strains. Lipid content was estimated during the exponential phase of growth (day 13) and at the end of cultivation (day 45). During early exponential phase there was no substantial accumulation of lipidsin any of tested strains except in reference strain *Botryococcus braunii* (Table 2). Lipid content went up in stationary phase for all the strains. Strains that had lipid content higher than 27µg per 100µl of culture,were considered promising strains and used in further studies including*Coelastrum* sp. 46-4; *Chlorococcum* sp. 64-12, *Dactylococcus* sp. 64-10; *Stigeoclonium* sp. 64-8; and *Coelastrum* sp. 108-5, (Table 2). In a separate experiment using the selected strains, lipid content was determined as a percentage of cell dry weight (Figure 2). *Coelastrum* sp. strain 46-4 had the highest lipid content. After 45 days of cultivation, this strain


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**Table 2**: Lipid content in the culture on the 13th and 45th day of cultivation. Lipid content was determined by using Nile red method. The biomass of each strain was normalized by the dilution to an optical density (OD600) of 0.2. Six strains (figures in bold) that showed high lipid content were selected for further analysis. Standard error data were obtained from triplicate measurements.

| | Lipid content (µg/100µl culture) | |
|---|---|---|
| **Strain** | **After 13 days** | **After 45 day** |
| *Chlamydomonas*, EV-29 | 10.53 ± 1.59 | 17.82 ± 0.63 |
| *Chlorella,* 2-4 | 8.17 ± 0.52 | 12.36 ± 0.32 |
| *Chlorella*, 71-4 | 2.11 ± 0.60 | 18.79 ± 0.52 |
| *Chlorella*, 104-1a | 1.02 ± 3.92 | 16.01 ± 0.71 |
| *Chlorococcum*,5-1 | 14.71 ± 0.71 | 26.88 ± 0.22 |
| *Chlorococcum*, 45-3 | 6.69 ± 1.27 | 25.73 ± 0.71 |
| *Chlorococcum*, 55-2 | 3.96 ± 0.69 | 18.99 ± 0.63 |
| *Chlorococcum*, 55-5 | 5.17 ± 1.23 | 27.11 ± 1.01 |
| *Chlorococcum* , 64-12 | 8.17 ± 0.60 | **36.44 ± 1.34** |
| *Chlorococcum*, 56-5 | 0.92 ± 0.14 | 24.02 ± 0.21 |
| *Chlorococcum* 111-4 | 4.656 ± 1.21 | 21.03 ± 0.87 |
| *Coelastrum*, 46-4 | 7.68 ± 0.45 | **38.00 ± 0.62** |
| *Coelastrum,* 108-5 | 12.98 ± 0.65 | **27.98 ± 0.28** |
| *Dactylococcus*, 64-10 | 3.25 ± 1.18 | **29.86 ± 0.43** |
| *Eremosphaera*, 103-6 | 14.10 ± 0.06 | 20.96 ± 1.97 |
| *Scenedesmus*, 66-1 | 12.36 ± 0.79 | 18.11 ± 0.28 |
| *Scenedesmus*, 79-1 | 1.84 ± 0.61 | 26.92 ± 0.16 |
| Scenedesmus, 80-15 | 1.57 ± 1.10 | 16.78 ± 0.45 |
| *Scenedesmus*, 81-5 | 10.32 ± 1.28 | 14.42 ± 0.83 |
| *Scenedesmus*, 103-4 | 1.59 ± 0.87 | 22.65 ± 0.36 |
| *Selanstrum*, 34-4 | 9.40 ± 0.86 | 24.17 ± 0.61 |
| *Stigeoclonium*, 64-8 | 7.70 ± 2.15 | **29.72 ± 0.58** |
| *Pediastrum,* 81-6 | 9.39 ± 1.55 | 25.81 ± 0.17 |
| *Pediastrum,* 108-4 | 16.58 ± 0.36 | 25.03 ± 0.50 |
| *Planktosphaeria*, 81-7 | 0.50 ± 0.11 | 21.07 ± 0.52 |
| *Planktosphaeria*, 56-4 | 6.40 ± 0.60 | 15.88 ± 0.26 |
| *Kircherniella*, 104-7 | 2.84 ± 1.61 | 27.50 ± 1.29 |
| *Botryococcus braunii* | 33.91 ± 0.40 | **27.54 ± 0.64** |

contained 75% lipids, *Coelastrum* sp. strain 108-5 and *Chlorococcum* sp. strain 64-12, contained 73% and 51% of lipids respectively.

## Lipid accumulation depending on the culture age

In order to determine effect of culture age on lipid accumulation, lipid levelwasdetermined in the biomass of selected six algal strains every five days over a period of 45 days. In most tested strains, biomass continuously increased during the entire cultivation period (Figure 2). The exception was *Dactylococcus* sp. strain 64-10 (Figure 2d) that reached the maximal biomass yield at day 25 followed by a sharp biomass decline. The pattern of lipid accumulation differed from one strain to another. In four stains (*Chlorococcum* sp. strain 64-12; *Stigeoclonium* sp. strain 64-8; *Coelastrum* sp. strain 46-4; *Dactylococcus* sp. strain 64-10) there was an initial evident decline in lipid content. After that initial phase, several different patterns of lipid accumulation were observed. There was (1) a continuous increase of lipid content during the entire cultivation period (*Coelastrum* sp. strain 46-4; *Stigeoclonium* sp. strain 64-8; *Dactylococcus* sp. strain 64-10) (Figure2b, 2c and 2d); (2) the lipid content reached a plateau after 25 days of cultivation (*Chlorococcum* sp. strain 64-12; *Coelastrum* sp. strain 108-5) (Figure2a and 2e); (3) and in the case of *Botryococcus braunii*, there was no initial change of lipid content with the slow decline towards the end of cultivation period (Figure 2f).

A linear regression test showed that only in four strains (*Chlorococcum* sp. strain 64-12, *Coelastrum* sp. strain 108-5, *Stigeoclonium* sp. strain 64-8 and *Coelastrum* sp. strain46-4) there was a linear relationship between algal biomass yield and lipid accumulation with the following correlation coefficients ($R^2$) of 0.834, 0.703, 0.632 and 0.612 respectively.

## Effect of nitrogen concentration on lipid accumulation

In order to assess effect of nitrogen depletion on lipidlevel, biomass of a 30-day-old culture was washed with nitrogen-free medium and transferred into nitrogen-free medium and medium containing 50% and 100% of nitrogen concentration normally found in BG11 medium. The lipid level of the inoculum (day 0) was determined and used as control (Figure 3). Of six strains tested, a significant increase ($P > 0.05$)in lipid levelin nitrogen-free medium was observed in only two strains. The lipid level increased in *Chlorococcum* sp. strain 64-12 (Figure 3a); and *Stigeoclonium* sp. strain 64-8 (Figure 3c) 105% and 57% respectively. Nitrogen depletion did not significantly affect lipid levelin other tested strains (*Coelastrum* sp. strain 108-5; *Dactylococcus* sp. strain 64-10; *Coelastrum* sp. strain46-4; *Botyrococcus braunii*).

## Effect of phosphorous concentrationon lipid accumulation

Of the six strains tested, five showed significant lipid accumulation in phosphorus-free medium after 10 days of cultivation. The highest increase of lipid level of 116% was observed in *Chlorococcum* sp. 64-12, followed by *Stigeoclonium* sp. 64-8(83%); *Coelastrum* sp. 46-4 (58%); *Coelastrum* sp. 108-5 (30%); and; *Botryococcus braunii*(26%) (Figure 4). The only strain that showed decrease of lipid content in phosphorus-depleted medium was *Dactylococcus* sp. strain 64-10

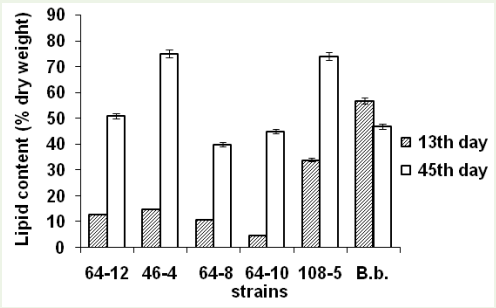


**Figure 2:** Lipid content determined by Nile red method as a percentage of dry biomass of six selected strains after 13 and 45 days of cultivation. Error bars represent standard error (n=3). Strains tested: *Chlorococcum* 64-12; *Coelastrum* 46-4; *Stigeoclonium* 64-8; *Dactylococcus* 64-10; *Coelastrum* 108-5; *Botryococcusbraunii* (B.b.).


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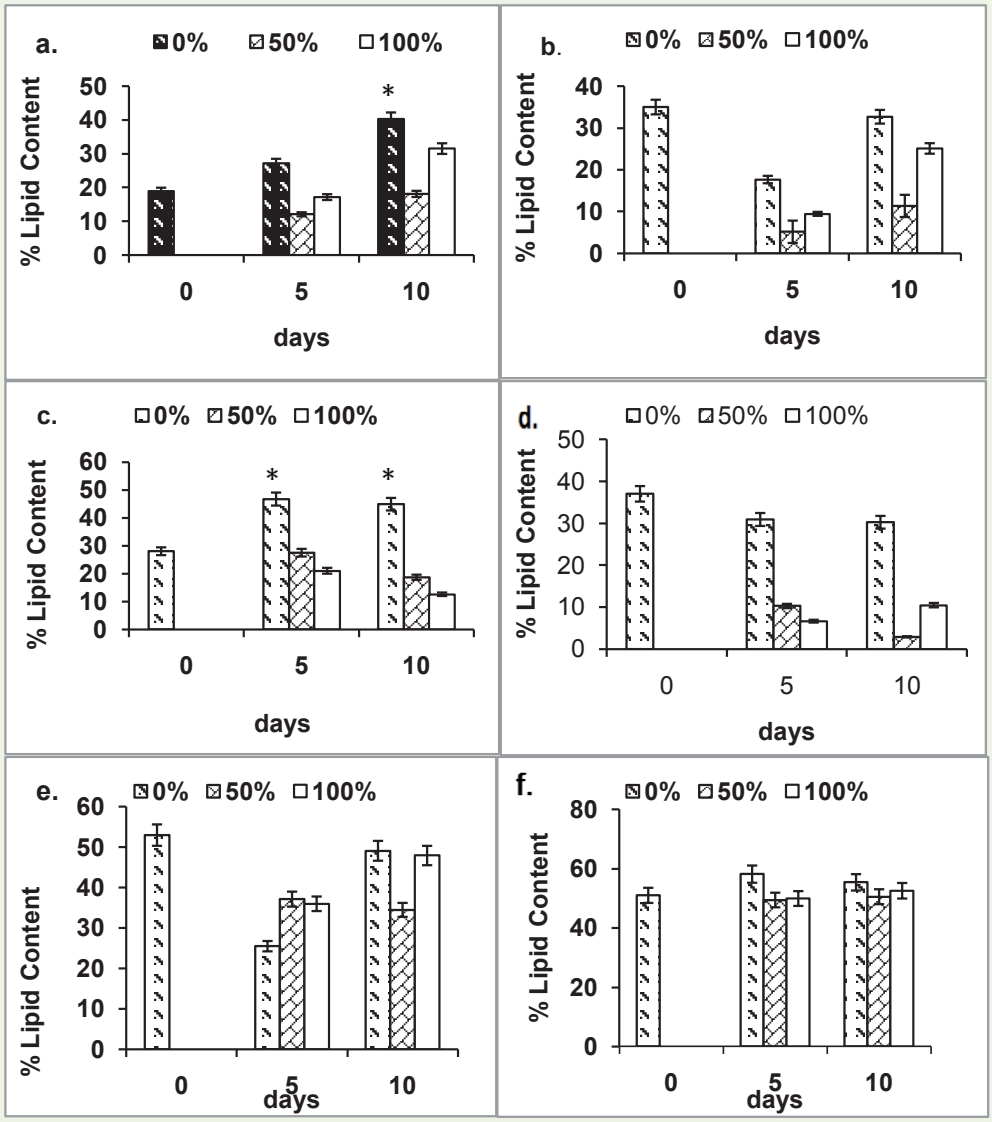


**Figure 3:** Lipid accumulation in nitrogen depleted medium after 5 and 10 days of cultivation. The cultures were grown in nitrogen-free medium (0%); in 50% nitrogen strength of normal BG11 medium and in a full strength medium (100%). Error bars represent standard error (n=3). * indicates significant increase in lipid content compared to control - day 0. (a. *Chlorococum* sp. 64-12; b. *Coelastrum* sp. 46-4; c. *Stigeoclonium* sp. 64-8; d. *Dactylococcus* sp. 64-10; e. *Coelastrum* sp. 108-5; f. *Botryococcus braunii*).

(Figure 4d). In the strains affected by phosphorus depletion, the significant accumulation of lipids occurred after 10 but not after 5 days of cultivation. Reduction of phosphorus concentration in the medium by 50% did not affect lipid levels significantly.

## Discussion

An earlier study conducted by the Aquatic Species Program [23] emphasized the importance of collecting native algal strains and analyzing their potential for lipid accretion. Using strains that are adapted to a climate conditions in which they are expected to be mass-cultured is of crucial significance. In this study, 27 Florida-native green algal (Chlorophyta) strains and their potential to accumulate lipids for the purpose of biodiesel production is investigated. In this initial screening using Nile redmethod, lipid content was determined in the cultures after 13 and 45 days. The first-time sampling (day 13) was selected based on the study of Kalacheva [24] who reported that neutral lipids start accumulating after 13 days of cultivation. Five strains thatproduced 27 mg of neutral lipids or more per 100 ml of culture after 45 days of cultivation were used for further analysis (Table 2). In the strains selected for further study, the pattern of both growth and lipid accumulation differed from one strain to another. During 45 days of cultivation, two strains (*Coelastrum* sp. strain 46-4; *Botryococcus braunii*) reached stationary phase, three strains (*Chlorococcum* sp. strain 64-12; *Coelastrum* sp. strain 108-5; *Stigeoclonium* sp. strain 64-8) continued growing till the end of experiment and *Dactylococcus* sp. strain 64-10 showed sharp growth decline after 30 days.


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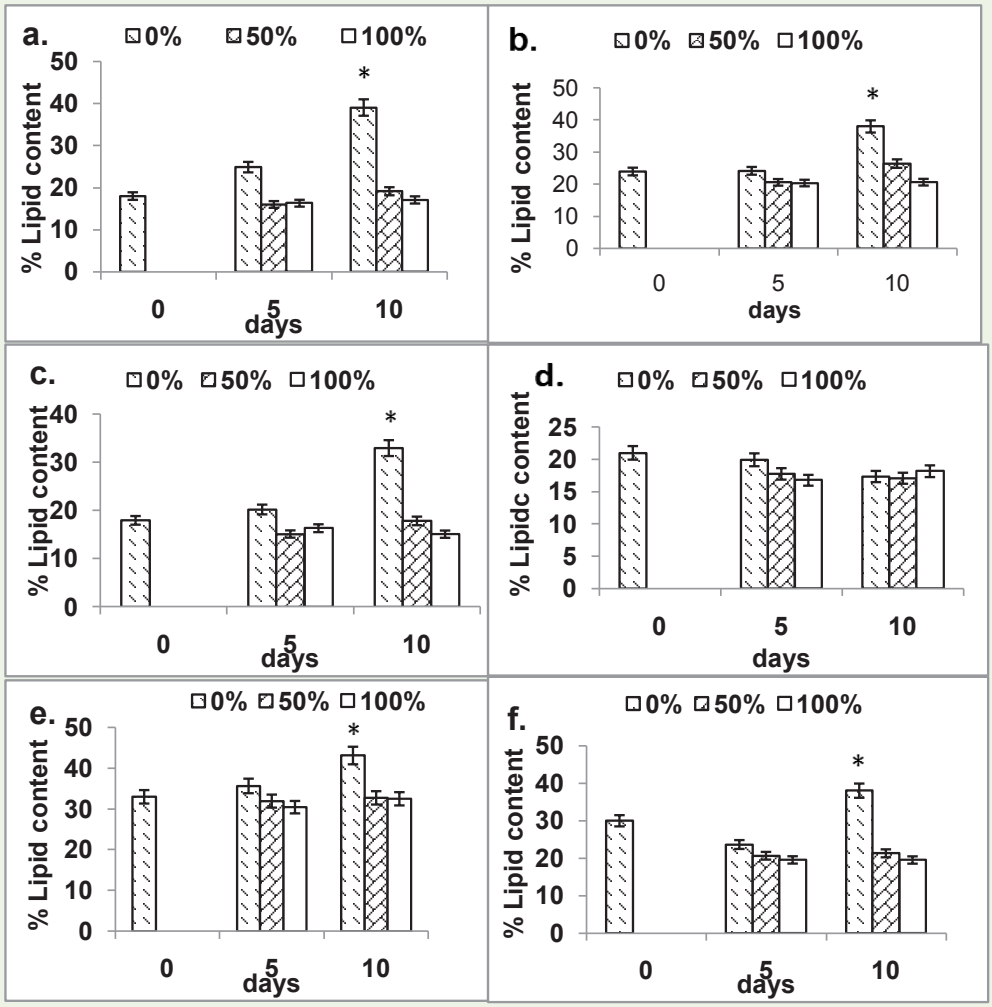


**Figure 4:** Lipid accumulation in phosphorus depleted medium after 5 and 10 days of cultivation. The cultures were grown in phosphorus-free medium (0%); in 50% phosphorus strength of normal BG11 medium and in a full strength medium (100%). Error bars represent standard error (n=3). * indicates significant increase in lipid content compared to control – day 0. (a. *Chlorococum* sp. 64-12; b. *Coelastrum* sp. 46-4; c. *Stigeoclonium* sp. 64-8; d. *Dactylococcus* sp. 64-10; e. *Coelastrum* sp. 108-5; f.*Botryococcus braunii*).

In most strains tested, the initial lipid content decreased after inoculation (Figure 2) a consequence of using inoculum in the late stationary phase. This phenomenon of decreasing lipid content upon transferring the cells into new medium has been described before and reflects metabolic activity where the lipids are decomposed to supply energy for cell division and growth [25]. The lipid content in all strains, except control strain *Botryococcus braunii*, continued to increase until the end of experiment (45 days). The increase in lipid level in aged cultures is in accordance with the report of Chen [18] who showed that the lipid content in the cells of *Chlorella vulgaris* increased from 30% to 56% from day 1 to day 12. It is known that the old cultures undergo stressful conditions and the majority of the lipids in cells in stationary phase are neutral lipids in the form of triacylglycerides (TAGs). This is apparently due to the shift in lipid metabolism from synthesis of membrane to storage lipids either through *de novo* biosynthesis or through conversion of membrane polar lipids into TAGs [2].

*Botryococcus braunii*showed no increase in lipid level in aging cultures, instead, a decrease of lipid content was observed (Figure 2). Similarly, Alonso [21] reported that culture age had no influence in accumulation of TAGs in the diatom *Phaeodactylum tricornutum*. Possible explanation for reduced lipid synthesis in a stationary phase of*Botryococcus braunii* is that there is an increased rate of polysaccharide accumulation [24]. Additionally, *Botryococcus braunii* has a slower growth rate than other tested strains. According to Wolf [26], the unusual morphology of this alga could possibly be the factor for imposing constraints on growth.

The phenomenon of increased lipid level in older cultures is well documented [27] and could have important implications on the economics of algal biofuels [28]. Selecting strains that have a high growth rate and simultaneously capable of accumulating high lipid levels in a short period of time would be desirable features that could significantly reduce the production costs of biodiesel.


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Once a promising strain is selected, ability of the cell to accumulate lipids can be further improved by manipulating the metabolic pathways through changes in environmental conditions [2]. It is known that under sufficient nutritive conditions, proteins are synthesized, while during nutrient deprivation, the cell division is suppressed and a greater amount of carbon remains available for lipid synthesis [29].

Increased accumulation of lipids, particularly TAGs, has been observed in response to nitrogen deficiency in various strains of microalgae [30,31,32]. With nitrogen and phosphorus deprivation, *Scenedesmus* sp. showed a substantial increase in lipid as high as 30% and 53% respectively [33]. This indicates different algal strains respond differently to nitrogen deficiency. For example, diatoms do not seem to respond to nitrogen depletion by increasing their lipid content [34], while green algae (Chlorophyceae) show different responses to nitrogen depletionranging from several fold increases (e.g., *Chlorella pyrenoidosa*) to no change (*Dunaliella* species) [35].

Phosphorus deprivation has been reported as another factor that contributes to increased lipid accumulation [36,37]. In this study, five out of six strains tested responded to phosphorous depletion with an increased accumulation of lipids after 10 days of culturing. Even though it is widely accepted that a nitrogen-limitation is the most important factor that induces lipid accumulation [38,39,40], in this case, phosphorus deprivation affected most of the strains tested. In phosphorus-depleted medium, phospholipids of cell membranes are broken down into neutral lipids [41]. This implies that when using nutrient deficiency as a method for increasing the lipid synthesis and storage, those conditions should be determined for each strain individually.

The goal of manipulating algal cell metabolism is to increase lipid content without loss of biomass productivity; however, this appears to be a challenging task. Under nutrient shortage, lipid levels will increase but cellular growth will decline, resulting in a decreased lipid production. Therefore, in order to make microalgae-based biofuel technology feasible, it is essential to achieve a balance between lipid production and biomass yield.

In conclusion, of twenty-seven native Florida algal strains screened, three strains contained more than 50% of lipid of dry biomass (*Coelastrum* 108-5, *Chlorococcum* 64-12 and *Stigeoclonium* 64-8) and therefore identified as potential organisms to be used in algae-based biodiesel technology. Deprivation of nutrients such as nitrogen and phosphorus affected various strains differently; most strains showed better response to phosphorus rather than to nitrogen deprivation. According to this preliminary screening, biodiversity of naturally occurring algae in Florida aquatic environments can provide a good feedstock in algae-based biodiesel technology.

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