

GREEN MICROALGAE AS A RAW MATERIAL FOR BIODIESEL PRODUCTION

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Abstract

The ability of microalgae to adapt to extreme conditions and its efficient production of biomass makes algae a promising source of alternative energy. Some extreme conditions can cause the accumulation of triglycerides, which can be used to make biodiesel. To improve commercial production, research has focused on cultivating algae to produce high lipid content and biomass. This experiment evaluated lipid production in two microalgal strains *Dunaliella salina* and *Dunaliella bardawil* with nitrogen deprivation. The two strains were cultivated in both normal nutrient (+N) and nitrogen-deprived (-N) media for six days. Cell growth and oil accumulation was monitored from 3 to 6 days of culture. While cell growth was slightly inhibited with N-deprivation, oil content in N-deprived cells was higher than cells grown in N+ media. Oil extraction from *D. salina* and *D. bardawil* was performed after 6 days, and the extracted lipids were separated by sucrose gradient. Nutrient deprivation was proven as a promising method to elevating lipid content in algae.

Introduction

One of the major issues facing industrialized nations in the twenty-first century is the limited availability of resources needed to meet energy demands. At the current rate of consumption, world fossil oil reserves will most likely be exhausted in less than fifty years (1). The search for alternatives to fossil fuels has resulted in the development of solar and wind energies, however, these resources are impractical for large scale national or even global production. Biodiesels from crops such as soybean, corn, and oil palm are quickly emerging as viable replacements for petroleum derived transport fuels. While these oil crops are already in development, they utilize valuable arable land, reduce food supply, and require fresh water. Furthermore, production in large quantities is not sustainable as soybean and palm oil produce oils in miniscule amounts of less than 5% of

biomass (1). Microbial photosynthesis offers many advantages over oil crops as microalgae need less water than terrestrial crops, can be grown in seawater or salt-contaminated land, and possess a higher oil content per dry weight of biomass. The fixation of carbon dioxide by algae can remove gases from power plants and create a carbon neutral fuel production system.

Microalgae are photosynthetic organisms which convert sunlight, water, and carbon dioxide to algal biomass. Certain oil-rich microalgae can be cultivated and processed to produce biodiesel on a large scale in variable climates. Furthermore, unlike most oil crops, algae grow extremely rapidly and double biomass within 24 h (2). However, limitations of microalgae cultivation are still being overcome. Microalgae must be selected for productive lipid-rich strains and their ability to be grown outdoors in variable

conditions. The cost-effective extraction of oil from algal biomass also presents another difficulty for commercial production.

Large-scale production of microalgae has focused on unicellular, eukaryotic green algae. Algae from the genus *Dunaliella* has been successfully cultivated in open ponds and thrives in high salt habitats where other crops could not be grown. Only two strains of this genus *D. bardawil* and *D. salina* have been shown to produce large quantities of β -carotene which is located within lipoidal globules in the interthylakoid space (1). In this study, we cultivated these two strains to investigate their potential for biodiesel production.

Current research on biodiesel from algae has included attempts to enhance the oil content of algal biomass. Under stressful conditions, such as nitrogen and phosphorus deprivation, lipid content and productivity is enhanced in certain strains of algae (1). Therefore, the purpose of this experiment was to examine the effects of nitrogen deprivation on oil accumulation in two strains of green algae and purify the oil-bodies from the algae that produced the highest amount of oil.

Experimental Procedures

Algal stock preparation - Two microalgal strains *Dunaliella bardawil* and *Dunaliella salina* were cultivated in 500 mL of appropriate nutrient medium with shaking at 25°C and constant illumination with initial cell concentrations of 4×10^5 cells/mL. Samples were collected on days 0,3,4,5, and 6. For both algal species, a wild-type algae in standard medium (+N) and algae in nitrogen-deficient medium (-N) was grown by centrifugation and reinoculation of stock algae

in N-deficient medium. Cell growth was monitored daily using a hemacytometer after sample treatment with formamide (1 μ M).

Measurement of oil accumulation - 4 mL samples of *D. bardawil* and *D. salina* were collected at 5×10^5 cells/mL and 1×10^6 cells/mL, respectively, starting on day 3 through day 6. The fluorescent dye Nile red was added to the samples at a concentration of 1 μ M per sample. Nile red was applied for 5 min for the two *D. bardawil* samples and 2.5 min for the two *D. salina* samples. Fluorescent intensity was read using an Eclipse fluorescence spectrophotometer ranging from 520-750 nm.

Thin Layer Chromatography - Samples were collected with cell counts of 10^7 cells per a sample on days 3,4,5, and 6. After centrifugation at 3,000 rpm for 7 min, dimethylsulfoxide was added to penetrate the cell membrane. Samples were then incubated for 5 min at 70°C. 3 mL of MeOH were vortexed with each sample followed by centrifugation to extract the supernatant. 3 mL of diethylether, N-hexane, and double distilled water were mixed with the supernatant and spun for 5 minutes at 1,000 rpm. The upper phase was extracted with evaporation by vacuum. The remaining sample was dissolved in 200 μ l of chloroform and stored at -4°C until testing. 5 μ l of each sample was placed on the TLC plate followed by iodine staining and run with 2 μ l of triolein standard (0.5 μ g/ μ l). Analysis of plates was performed with ImageQuant TL.

Triglycerides globules extraction - Cells were harvested on the sixth day of growth and filtered to remove dead cells and debris. After harvestation by centrifugation at 2,500 rpm for 10 minutes, cells were washed and centrifuged with first NaCl solution and then glycerol buffer. For cell bursting, pellet was suspended in glycerol buffer to 2×10^8 cells/mL and cold bursting buffer was added (1:4 for *D. salina* and 1:2.5 for *D. bardawil*). Protease inhibitors were added (1:100 dilution) to prevent degradation of cell components. Subsequent centrifugation for 15 min at 3,200 rpm separated chloroplasts and cytoplasm. 4mM of EDTA was added to the supernatant and chilled for 30 min. The pellet was resuspended in 5 mL of chloroplast buffer and chloroplasts were burst by sonication in three cycles of 10 sec each. The pellet was centrifuged for 10 min at 10,000 g and the resultant pellet discarded.

Oil-body separation and purification - Carotene globules were separated using a sucrose gradient of 30%, 15%, 5%, and 0% sucrose. 85% sucrose was used to dilute samples to 30% sucrose. Subsequent layers of sucrose with 20 mM tricine buffer of pH 8 were gradually added. Samples were ultracentrifuged in vacuum at 30,000 rpm for 2 hrs at 4°C. After centrifugation, layers of oil globules were extracted using a needle syringe. To precipitate the proteins, trichloric acid (1v:2v) and acetone (4v:1v) were added to the samples. Samples were then chilled at -20°C.

Results

Cell Growth – The two strains *D. salina* and *D. bardawil* were cultivated and tested for their

lipid production potential by evaluating cell growth over 6 days (Fig. 1). Cell growth leveled out after the fourth day, and nitrogen-deficient algae exhibited production of β -carotene by color change. The best growth productivity strain was *D. salina*; however, deprivation of nitrogen reduced cell growth in both *D. salina* and *D. bardawil* strains.

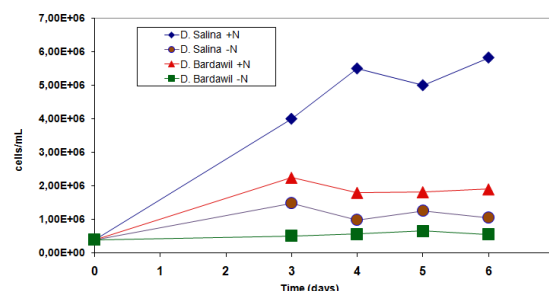


Fig. 1 Cell growth over six days. Counting was performed using a hemocytometer and cells treated with formamide.

Oil Accumulation - The lipophilic fluorescent marker Nile red (emission spectrum 400-600 nm) was used to determine oil accumulation in algal samples. Peak intensities were noted between 550-600 nm and 650-700 nm (Fig. 2). The 550-600 nm peak was indicative of the presence of Nile red in oil globules and hence oil accumulation in the samples. The second peak intensity of 650-700 nm correlated with the emission wavelength of chlorophyll. After four days of growth, chlorophyll content in *D. bardawil* +N and *D. salina* +N was higher than the nitrogen deficient samples. However, Nile red fluorescence indicative of oil accumulation was elevated in the nitrogen deficient samples. The oil content of *D. salina* -N and *D. bardawil* -N was higher than the *D. salina* +N and *D. bardawil* +N, respectively (Fig. 3). Cell oil accumulation gradually increased over the course of the experiment.

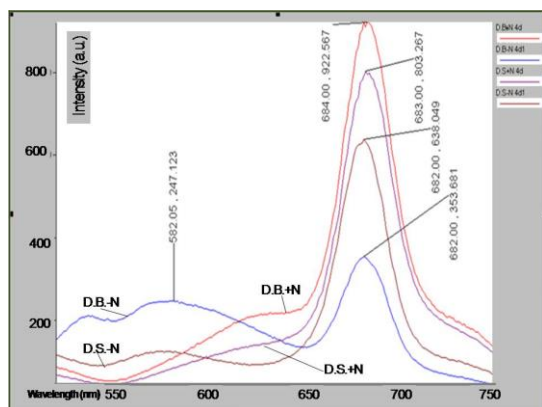


Fig. 2 Nile red fluorescent intensity at 4 days. (D.B. = *D. bardawil*, D.S. = *D. Salina*)

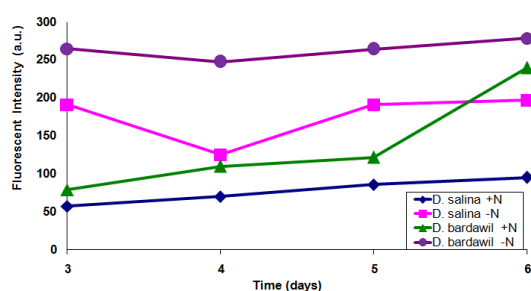


Fig. 3 The Nile red fluorescent peak intensities were measured over 3 to 6 days.

*Day 6 of *D. bardawil* +N had an abnormally high reading.

Thin Layer Chromatography - Thin layer chromatography (TLC) analysis was performed on previously collected samples. Using a standard curve, oil content in samples was quantified and nutrient deprivation compared. For both *D. salina* and *D. bardawil* samples, the nitrogen-deficient algae were richer in lipid content than the wild-type algae. Over days 3 to 6, oil content gradually increased in *D. salina* -N samples.

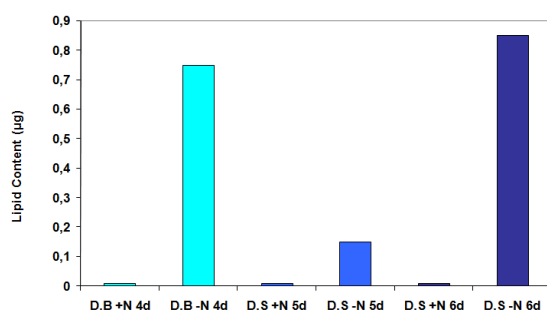


Fig. 4 Thin layer chromatography (TLC) oil accumulation on days 4,5, and 6.

Oil-body separation - Extraction of oil globules from cytoplasmic and chloroplastic fractions was performed. Oil globules were separated after extraction using a sucrose gradient. In both *D. salina* -N pellet and supernatant samples, two oil fractions were noted at the 0% and 30% sucrose layers (Fig. 5). In the *D. bardawil* -N samples, four oil layers were present at the 30%, 15%, 5%, and 0% sucrose layers.

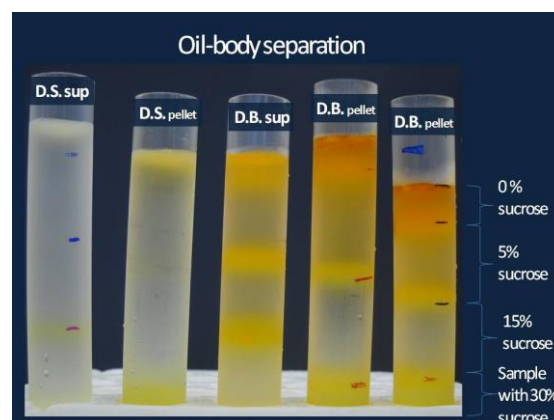


Fig. 5 Oil globule fractions after extraction and separation of chlorophyll and cytoplasmic globules followed by separation with sucrose gradient.

Discussion

Improving lipid yield per area of microalgae may prove essential towards the viability of algae as a source for biodiesel. One of the major limiting factors of biodiesel from microalgae is the high cost associated with oil production and purification in comparison to petroleum. Microalgal cultures must withstand outdoor environments while maintaining a high constitutional lipid content in order to compete with cost effectively for large scale production. Furthermore, harvestation of oil must be taken into account as small cell size and other limitations can reduce commercial viability.

Previous studies have focused on enhancing oil production in algae and

selecting for strains that can be easily grown in high salt environments (1). In this study, two strains of the genus *Dunaliella* were studied based on their halotolerant properties and established successful culture in outdoor vats (3). The high salinity of these cultures prevent the contamination of farms with predatory or invasive species. Furthermore, *Dunaliella* lacks cell walls, lowering the time and costs associated with oil extraction. *D. salina* and *D. bardawil* have been widely cultured for commercial production of β -carotene and were selected for our experiments.

Nutrient deprivation, in particular nitrogen starvation, has been associated with enhanced oil yield. Within a short time span of six days, both strains exhibited signs of oil accumulation with nitrogen deprivation. Measurement of lipid storage with the Nile red marker demonstrated lipid accumulation in the *D.salina* -N and *D.bardawil* -N samples over several days. These results were further confirmed with the thin layer chromatography tests where -N samples also showed greater oil accumulation with a gradual increase over time. We identified in both lipid content assays *D. bardawil* as the more lipid-rich strain in comparison to *D. salina* and therefore a more promising candidate for production.

While both algae strains responded to nitrogen deprivation with increased oil accumulation, cell growth was restricted compared to the wild-type algae. Our fluorescent intensity results indicated that levels of chlorophyll production were similar to lipid accumulation in the nutrient deficient samples. The similar levels may point towards a compensation of cellular processes for oil production. Consequently, further study would include examining the relationship between

biomass and oil accumulation to improve economics of production.

In our work with *D. salina* and *D. bardawil*, for the first time, extracted algal lipids were successfully separated by density gradient. Two separate fractions were observed in the *D. salina* samples, and four fractions were observed in the *D. bardawil* samples. This development allows the possibility of identifying the types of oil in each fraction and eventually promoting the synthesis of desired oils. Future plans may involve identification and examination of the proteins such as oleosins in the oil globules to better understand lipid accumulation in cells.

While current technology and research has not yet developed an economical way to produce biodiesel, enhancing lipid production of algal cultures could establish competitiveness with petroleum-derived fuel. The coupling of production of biodiesel from algae and a carbon neutral facility where biomass could be used to produce methane and fertilizers is a more cost-effective strategy without jeopardizing food supply and carbon output. Unlike other renewable biodiesels, oleaginous algae can be cultivated on a scale large enough to provide a sustainable alternative to many of the world's energy demands.

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