Screening for Optimal Algal Cell Growth and Neutral Lipid Production Conditions in Microplates

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Abstract

The increase in the price of fossil fuel based products, such as gasoline and diesel fuel, and the ever present reality of global climate change has brought an increased interest in the use of renewable sources of energy products. While initial efforts have focused on the fermentation of sugars obtained from sugar cane or corn, the use of food based sources has proved untenable for long term production of renewable fuels. While algae based products offer many potential advantages over the food based sources of energy, extensive research is still required in order to make them an economically viable option. Unfortunately, algal research has not widely utilized the tool of high throughput screening using microplates that has been successfully employed in fields such as drug discovery.

Microplate-based screening and experimentation offers the ability to measure large numbers of samples in multiple experimental conditions rapidly and simultaneously. Here we describe the quantitation of algal cell growth under various growth media conditions using a microplate reader to make absorbance and fluorescent determinations. Light scatter absorbance measurements at 600 nm were used to monitor cell growth. Nile Red stain fluorescence monitored neutral lipid production under various nutrient deficient states. Algal cells grow to a 20-fold greater density in complete-nutrient rich media than in either nutrient poor or nitrogen deficient media. However, despite the marked disparity in cell number, cultures grown in nitrogen deficient media exhibit more neutral lipid staining. On a per cell basis, *Chlorella vulgaris* cultures grown in nitrogen deficient media have 15-fold more lipid than cells grown in complete media. When nutrient deprived cells are placed in complete media, lipid droplets are quickly reabsorbed and Nile red staining returns to basal levels. The use of microplates enables the rapid measurement of multiple samples and experimental conditions at the same time.

Introduction

Growth of single celled organisms in suspension culture can be monitored using turbidity or light scatter measurements. As the number of cells increases, the solution becomes increasingly cloudy or turbid because light passing through it is scattered by the microorganisms present [1]. While not obeying Beer's law, as light scatter increases, the percentage of the total light beam reaching the detector diminishes and is recorded as absorbance.

Neutral lipids are composed primarily of triacylglycerol esters (TAG). Under favorable and unlimited growth conditions, microalgae produce primarily polar lipids, which enrich chloroplast and cellular membranes. However, under unfavorable or restricted growth conditions, microalgae accumulate neutral lipids in lipid droplets located in the cytoplasm [2]. Nile red is a red lipid soluble phenoxazone dye that is relatively photostable, highly fluorescent in non-polar hydrophobic environments, yet very poorly fluorescent in aqueous solutions [3]. 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 [4]. The degree to which it fluoresces is an indicator of the amount of neutral lipid present.

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. The most common biodiesel constituent used today is fatty acid methyl ester, which can be obtained from the triglyceride lipid (TAG) droplets by glycerolysis and transesterification. *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* [5]. Under certain conditions, such as stress, the oil content can exceed 80% by weight of dry biomass [6].

Synergy H4 Hybrid Multi-Mode Microplate Reader

Figure 1 – *All Turbidimetric and fluorescent measurements were made on the Synergy H4 Multi-Mode Microplate Reader.*

Light Scatter

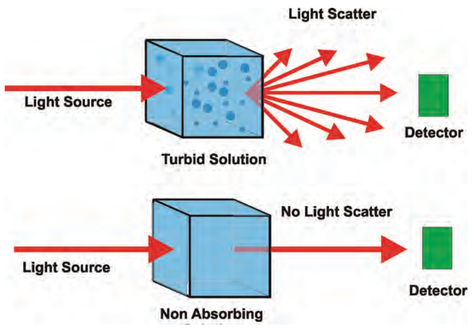

Figure 2 – Basis of 600 nm Turbidimetry Measurements. *Unlike true absorbance, the light is not absorbed, but rather the molecules within the cell diffract incident light as a result of changes in refractive index and density as light passes from the buffer to the cells. Some of the diffracted light will be deflected away from the optical path to the detector and be recorded as optical density by the reader. The degree of light loss due to light scatter is influenced by both the suspended particle, as well as the configuration of the instrument optics.*

Calibration Curve

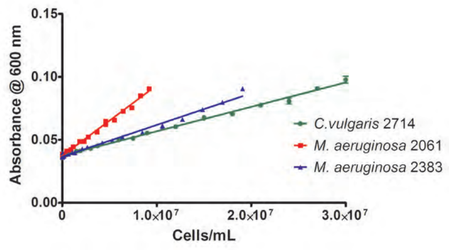

Figure 3 – Calibration curve for algae strains grown in BG11 media. *Absorbance measurements, reflecting the extent of turbidity of the cells, when plotted against cell number demonstrate a linear relationship for all three algal cell lines tested. Calibration curves such as these can then be used to determine cell number based on the absorbance reading. We did note a marked difference in the absorbance response on a per-cell basis with absorbance between different strains and organisms.*

Comparison of Algal Strains

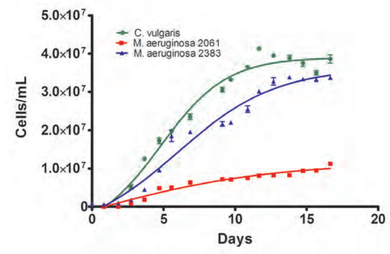

Figure 4 – Comparison of cell number from different algal strains grown in BG11 media.

All three strains exhibit classical sigmoidal shaped growth patterns. An initial lag phase after inoculation where cells are adapting to the new environment and growing slowly, which is followed by a log-phase where cells are rapidly growing and dividing and as nutrients become scarce and metabolic by-products increase the cells enter a stationary phase. Aliquots of all three strains increased in 600 nm absorbance until reaching stationary or plateau phase at approximately 10 days. *C. vulgaris* and *M. aeruginosa* 2383 resulted in very similar absorbance values, while *M. aeruginosa* 2061 plateaus at cell densities about one third of the other two strains of algae (Figure 3).

Nutrient Dependency for Cell Growth

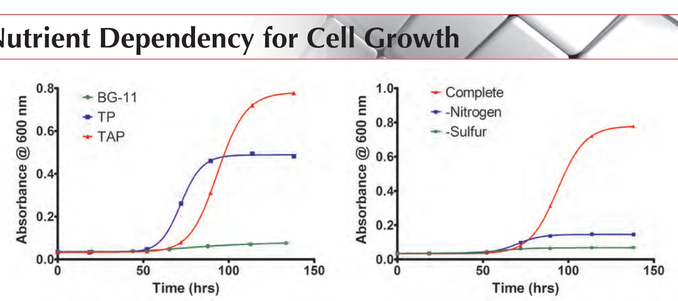

Figure 5 – Nutrient Dependency for Algal Cell Growth. (A) *Comparison of A_{600} growth curves of Chlorella vulgaris grown in different complete media formulations.* ***(B)*** *Increase in Chlorella vulgaris cell number over time with complete and deficient TAP and TP media.*

The nutritional effects of different media formulations can markedly influence the growth rate and final cell density of the algal cultures. *C. vulgaris* cultures grown in different media formulations, with varying amounts of total carbon, have markedly different final cell densities as measured by absorbance at 600 nm (Figure 4A). Cultures grown in TAP, which has high amounts of carbon, grow to an absorbance density approximately 10 fold greater than cultures grown in BG11, which is carbon poor. Cultures grown in TP media, which has less carbon than TAP, have an intermediate final density. TAP media resulted in the greatest amount of cell growth over a period of 140 hours from cultures seeded at low density. 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 4B).

Nile Red Assay Process

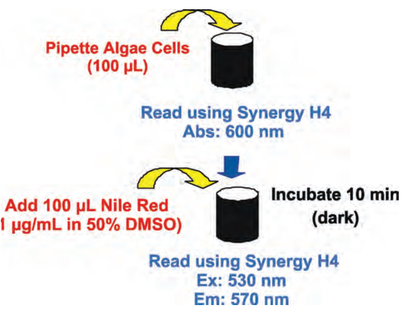

Figure 6 – Assay Process for Lipid Production by Algal Cultures. *Lipid production measurements were made by first recording cell number growth using light scatter measurements at 600 nm followed by the addition of 2X working Nile red solution. Final dye and DMSO concentration for lipid measurements were 0.5 µg/mL and 25% respectively. 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 using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader. Measurements were made from the top using 530 nm excitation and 570 nm emission wavelengths.*

Lipid Production

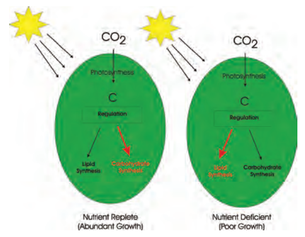

Figure 7 – Nutrient induced shift of Algal cell Energy Storage. *Nutrient deficiency results in a change from carbohydrate and polar lipid production to non-polar triglyceride lipids.*

Nile Red Staining

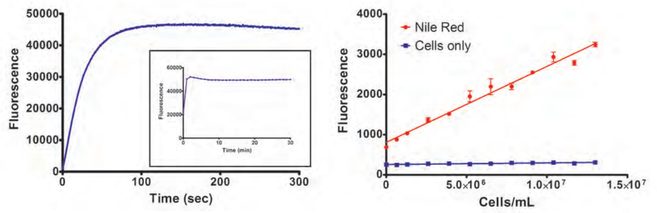

Figure 8 – (A) *Loading kinetics of Nile Red dye into Chlorella vulgaris algal cultures.* ***(B)*** *Fluorescence of cell titration with and without Nile Red stain.*

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 7A). 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. All *C. vulgaris* cells contain measurable amounts of neutral lipids in the normal growth state (Figure 7B). 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.

Stress Response

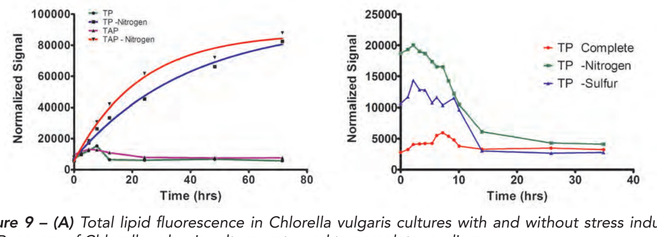

Figure 9 – (A) *Total lipid fluorescence in Chlorella vulgaris cultures with and without stress induction.* ***(B)*** *Recovery of Chlorella vulgaris cultures returned to complete medium.*

Switching large numbers of algal cells to deficient media results in the rapid production of lipids. 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. Stress induced lipid production is also rapidly reversed when the stress is removed. When lipid induced *C. vulgaris* cultures are returned back to complete media, cellular lipid levels return to normal within 20 hours of stress removal.

Algal Cell Lipid Production

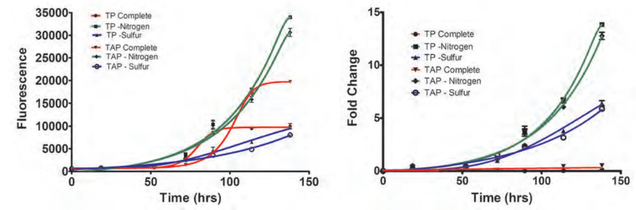

Figure 10 – (A) *Nile Red fluorescence in Chlorella vulgaris cultures.* ***(B)*** *Fold increase in Nile Red fluorescence in Chlorella vulgaris cultures normalized by A_{600} absorbance.*

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 10A). 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. 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 10B).

Conclusions

- Multi-mode microplate readers are an ideal platform to investigate algal cell growth and lipid production
- Light scatter can be used for algal cell quantitation in microplates
- Different algal strains exhibit different growth rates
- Different media formulations result in different growth rates from the same algal species. Nutrient rich media with an added carbon source results in higher growth rates than minimal media.
- Depletion of nitrogen or sulfur from nutrient rich media results in poor cell growth rates
- Nile Red is a suitable dye for monitoring lipid production in algal cultures in microplates
- Nitrogen or sulfur depletion results in neutral lipid production in *Chlorella vulgaris*
- Lipid production is a stress response that is rapidly modulated on and off in response to nutrient conditions
- Cells grown in nitrogen or sulfur deficient media contain more lipid per cell than cells grown in complete media

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[3]Fowler, S.D., W.J. Brown, J. Warfel, and P. Greenspan (1979) Use of Nile Red for the Rapid in situ Quantitation of Lipids on Thin-layer Chromatograms. J. Lipid Research 28:1225-1232. • [4]Chen W. C. Zhang, L. Song, M. Sommerfield, and Q. Hu (2009) A high throughput Nile red Method for Quantitative Measurement of Neutral Lipids in Microalgae. J. Microbial Methods 77:41-47.
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